Metabolic Brain Disease (2019) 34:1281–1298
https://doi.org/10.1007/s11011-019-00437-w

ORIGINAL ARTICLE

Bipolar limbic expression of auto-immune thyroid targets: thyroglobulin and thyroid-stimulating hormone receptor

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Received: 28 September 2018 / Accepted: 20 May 2019 / Published online: 13 June 2019

Abstract

The associations between thyroid auto-immunity and neuro-psychiatric disorders are well-documented. However, there exists limited literature specifically linking auto-immune thyroid disease (AITD) to bipolar disorder (BD). Thus, we investigated the likely association between Hashimoto's disease and BD through the extra-thyroidal localisation of thyroid-stimulating hormone receptor (TSH-R) and thyroglobulin (TG) in limbic regions of normal and bipolar human adult brain. Further, we hypothesised that changes in thyroid expression in bipolar limbic cortex may contribute to mood dysregulation associated with BD. Immuno-chemistry and in-situ PCR were used to localise TSH-R/TG within the amygdala, cingulate gyrus and frontal cortex of normal ($n = 5$) and bipolar ($n = 5$) brains. Reverse-transcriptase qPCR provided fold-change differences in TSH-R gene expression. The results demonstrated reduced thyroid protein expression in bipolar limbic regions; these novel results correlate with other neuro-imaging reports that describe reduced cortico-limbic tissue volumes and neuro-physiological activity during BD. We also demonstrated TG-like proteins exclusive to bipolar amygdala neurons, and which relates to previous neuro-imaging studies of amygdala hyperactivity and enhanced emotional sensitivity in BD. Indeed, reduced TSH-R/TG in limbic regions may predispose to, or bear relevance in the pathophysiology of mood dysregulation and symptoms of BD. Further, we attribute mood dysregulation in BD to limbic-derived TSH-R, which probably provides potential targets for thyroid auto-immune factors during Hashimoto's disease. Consequently, this may lead to inactivated and/or damaged neurons. The neuro-pathology of diminished neuronal functioning or neuronal atrophy suggests a novel neuro-degeneration mechanism in BD.

Keywords Thyroid-stimulating hormone receptor · Thyroglobulin · Limbic regions · Immuno-histochemistry · In-situ RT-PCR · Real-time quantitative PCR

Abbreviations

TH	thyroid hormones
TSH-R	thyroid-stimulating hormone receptor
TRH	thyrotropin-releasing hormone
TG	thyroglobulin
AITD	auto-immune thyroid disease
FFPE	formalin-fixed paraffin-embedded
BD	bipolar disorder
DAB	diaminobenzidine

Introduction

The neuro-immunological association between auto-immune thyroid disease (AITD) and psychiatric illness has been widely reported but not conclusively defined (Heinrich and Grahm 2003; Larsen et al. 2004; Whybrow and Bauer 2005a; Whybrow and Bauer 2005b). Hyper-thyroidism may be accompanied by neuro-psychiatric symptoms that include irritability, anxiety, dysphoria, emotional lability, impaired concentration, intellectual dysfunction and manic-like behaviour

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(Whybrow and Bauer 2005b; Hage and Azar 2012). Similarly, hypo-thyroidism may present depression-like symptoms that include psychomotor retardation, irritability, anger, fatigue and neuro-cognitive dysfunction (Whybrow and Bauer 2005a).

There are some studies that report an association specifically between thyroid auto-immunity and bipolar disorder (BD), (Cowdry et al. 1983; Bauer et al. 1990; Haggerty et al. 1997; Heinrich and Grahm 2003; Larsen et al. 2004; Amann et al. 2017). Bipolar disorder has been reported to affect an estimated 2.4% of the general population and is also linked with increased rates of relapse (Merikangas et al. 2011). Interestingly, thyroid diseases, particularly hypothyroidism, has been associated with an increased risk of manic episode relapse in BD, with a prevalence rate of 18.8% (Amann et al. 2017). Thus, these study findings suggests long-term negative implications of AITD in BD. Other previous studies have also supported the association between thyroid auto-immunity and BD, however the neuro-biological mechanism by which this occurs remains unclear. 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 et al. (1983) and Bauer et al. (1990) 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. 1990). Similarly, Heinrich and Grahm (2003) reported hypo-thyroidism as a probable risk factor for rapid-cycling BD, where significant anti-thyroid antibody titres were reported in over 50% of patients (Heinrich and Grahm 2003. Despite convincing supporting evidence, the prevailing school of thought implicating AITD in BD is neither well-understood nor universally-accepted. Thus, this lack of recent plausible data associating the two disorders provides the rationale for the present study and lends novelty to our results.

In an earlier pilot study, our group demonstrated the presence of thyroid-specific proteins, TSH-R and TG within various human non-limbic brain regions, by standard immuno-histochemical methods (Moodley et al. 2011). The findings from that study suggested an alternate neuro-physiological role for the thyroid system. Our choice of study target proteins, TSH-R and TG, is prompted from previous demonstrations of the distribution of classical thyroid hormones (TH), receptors (T_3 and T_4) within various human neural cell types (Carlson et al. 1994; Carlson et al. 1996; Strait et al. 1997; Carre et al. 1998; Ahmed et al. 2008). TSH-R and TG are key players in TH synthesis (Guyton and Hall 2000). However, the neural distribution of these thyroid-related proteins is limited. Further, there exists no previous evidence, until now, of any quantitative or qualitative comparisons of TSH-R and TG in limbic regions of human brain. Thus, more recently, we extended our investigation of thyroid-specific proteins to major limbic areas of normal adult human brain (Naicker and Naidoo 2017). In that study, we immuno-localised TSH-R to limbic neurons, whilst TG was detected within limbic vasculature and some neurons. The limbic brain represents the control centres for human emotional and behavioural responses (Guyton and Hall 2000); thus, our findings of thyroid-specific proteins within limbic structures contributes significantly to the growing body of knowledge that suggests an association between thyroid disorders and neuro-psychiatric illness.

In order to fully explore this association, in the present study, we extended our investigation into the distribution of TSH-R and TG in limbic regions of bipolar human brain at protein and molecular levels. We postulated that changes in thyroid expression in bipolar limbic regions may contribute to mood dysregulation associated with BD, thereby implying a significant role for the thyroid system in the pathophysiology of BD. Thus, we chose to compare the distribution of thyroid-specific mRNA and proteins in normal and bipolar limbic regions in order to determine whether any regulation of limbic thyroid expression could be related to the pathophysiology of BD.

Materials and methods

Samples

Ethical approval for the study was granted by the University of KwaZulu-Natal's Biomedical Research Ethics Committee (Reference EXPO42/06). Normal human brains were obtained at post-mortem, from 5 subjects ($n = 5$), (3 males with mean age of 36.7 years; 2 females with mean age of 28.0 years) whom had succumbed to causes that were unrelated to head injuries/trauma. Further, there was no evidence of histopathological brain disease. Written consent for collection of post-mortem normal brain was obtained from the next of kin of the deceased. Sections were obtained from the amygdala, cingulate gyrus and frontal cortex, from each of 5 brains. Previously collected, normal human thyroid gland tissue (Raidoo et al. 1996) provided tissue controls for thyroid protein in these experiments.

Bipolar brain tissue sections were obtained from the Netherlands Brain Bank (NBB), (Netherlands Institute for Neuroscience, Amsterdam, Netherlands) which provides post-mortem samples from clinically well-documented and neuro-pathological confirmed cases. Written consent was obtained from either the donor or their next of kin. Sections were obtained from the amygdala, cingulate gyrus and frontal cortex of 5 bipolar subjects ($n = 5$).

Antibodies, primers 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 immuno-staining kit, (LSAB®, Dako) along

with chromogen, 3, 3′-diaminobenzidine (DAB), (Dako, UK). All PCR primers were purchased from Whitehead Scientific (SA). Bio-reagents for these experiments were purchased from Roche Molecular (USA). All chemicals were purchased from Merck (SA).

Immuno-localisation of TSH-R and TG

Immuno-histochemistry

A conventional avidin-biotin immuno-complexation technique that uses polyclonal rabbit anti-human TG and TSH-R antibodies was used to localise thyroid proteins in normal and bipolar limbic tissue as previously described (Naicker and Naidoo 2017).

Image analysis

Immuno-stained tissue sections were captured as digital TIFF images with a Leica digital camera (DCF300X, Leica, Heidelberg, Germany) attached to a Leica microscope (DMLB) and interfaced with Leica IM50 software. We obtained regions of interest (ROI) using the following considerations: tissue integrity; least amount of white matter; minimal background staining and the presence of neurons. Immunolocalisation of thyroid proteins in control and bipolar limbic regions were quantified by image analysis (AnalySIS v5, Soft Imaging Systems, Germany) as previously described (Naicker and Naidoo 2017).

Statistical methods

We obtained all statistical analyses using STATA Software, v 13.1 (StatCorp, USA). It appeared that some of the data analyses for the categories, TSH-R in neurons and TG in vasculature, did not meet the statistical assumption of normality; therefore, these categories were transformed using the natural log (Ln) ± SEM for mean (Ln) intensity. Parametric assessments were obtained for both categories.

Firstly, inter-group statistical comparisons for TSH-R/TG protein intensity levels were determined for all normal limbic regions (amygdala, cingulate gyrus, frontal cortex). A similar assessment was performed for the bipolar limbic groups. Statistical methods for the above assessments included an *F-test* and *Tukey's multiple comparisons for significance test* whereby significance was tested at $p < 0.05$. Thereafter, intra-group statistical 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 $p < 0.05$.

Statistical analyses for the category, TG in neurons, did not appear to follow normal distribution. Non-parametric assessments were therefore employed for comparisons within this category. We obtained median values that were transformed using the natural log (Ln) with *inter-quartile range error* calculations. A *two-sample Wilcoxon rank-sum (Mann-Whitney) test* along with *Bonferonni's multiple comparisons for significance test* corrected for distribution in the normal group. Thus, inter-group statistical comparisons for normal groups was tested at $p < 0.003$. Inter-group 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 determined 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 groups. Significance was tested at $p < 0.05$.

In-Situ reverse-transcriptase polymerase chain reaction (In-Situ RT-PCR) for the detection of TSH-R mRNA

Pre-treatment of tissue sections

All steps were carried out under sterile, RNase-free conditions. Formalin-fixed paraffin-embedded (FFPE) tissue sections (5 μM) were adhered onto 2%, aminopropyltriethoxysilane solution treated slides. Tissue sections were deparaffinised in xylene followed by rehydration through a graded-series of ethanols (100%, 90%, 70% and 50%) at RT for 5 min each. Sections were fixed in 4% paraformaldehyde in 1x phosphate buffered saline at pH 7.4, and digested in 200 mM HCl for 10 min. A 65 μl Gene Frame incubation chamber (Advanced Biotechnologies Ltd., UK) was inserted around each section, followed by optimal proteinase K digestion for 30 min at 37 °C in a thermocycler (Peltier Thermal Cycler-200, MJ Research). Inactivation of the enzyme was achieved through extensive rinsing of sections in cold TBS. Sections were dehydrated through a graded-series of ethanol at RT for 5 min each and treated with chloroform for 1 min to eliminate traces of ethanol.

cDNA synthesis

Complementary DNA (cDNA) synthesis of TSH-R was obtained using the First-strand cDNA synthesis kit (Amersham, UK), according to the manufacturers' instructions. A 60 μl volume of reverse-transcriptase (RT) reaction mix containing 2 μl of TSH-R reverse primer (Table 1), 2 μl of 200 mM DTT, 8 μl 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 μl of RNase-free water was transferred into each incubation chamber and sealed with a plastic coverslip. The slides were incubated at 37 °C for 2 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Table 1 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

provided the endogenous reference gene for these experiments. Negative controls were prepared by omitting primers from the reaction mix.

In-situ PCR

Following incubation, the RT mix was replaced with a 50 μl volume of PCR mix that was prepared using the Kapa Hifi reaction kit (Kapa Biosystems, Inqaba Biotech). The PCR mix contained 10 μl of 5x Kapa Hifi Fidelity buffer, 2 μl of 10 μM forward and reverse primers (Table 1), 5 μl of 10x PCR digoxigenin (DIG) labelling mix (Roche), 1 μl Kapa Hifi DNA polymerase (1 U/μl) and 30 μl of nuclease-free water. The following cycling conditions were used: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation of 98 °C for 30 s, annealing at 60 °C (GAPDH and TSH-R) for 30 s and extension at 72 °C for 30 s, with final extension at 72 °C for 5 min. Negative control reactions were included. Following thermal cycling, tissue sections were washed (Hybaid Wash Module) in 2x sodium citrate (SSC) at 37 °C for 30 min, 1x SSC at 55 °C for 20 min, 0.5x SSC at 55 °C for 20 min and 0.1x SSC at 55 °C for 20 min.

Immuno-detection of DIG-labelled amplicons

Following SSC washing, the sections were rinsed in TBS and treated with 1x blocking solution (DIG wash and block buffer set, Roche) for 15 min at RT, followed by polyclonal sheep anti-DIG (Fab fragments, Roche) conjugated to alkaline phosphatase (1:500 in 10% blocking solution) for 1 h at RT. Thereafter, the sections were treated with a colour substrate solution comprising a 1:50 dilution of NBT/BCIP (Roche) in 1 x detection buffer (1 mM Tris, 1 mM NaCl, 0.5 mM $MgCl_2$, pH 9.5) and incubated in the dark at RT until the development of a red-purple precipitate. The sections were counter-stained with Mayer's haematoxylin (Sigma), dehydrated through a graded-series of ethanols, immersed in xylene, all at 5 min each at RT, and mounted with DPX (Merck).

Documentation of 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 Pro™ image analysis software (Soft Imaging Systems, Germany). Images were digitised and recorded as 24-bit tagged image format files (TIFF). Images were scaled accordingly and archived. Positive labelling for in-situ PCR experiments were identified as blue-black colour indicating precipitation of the chromogen, NBT/BCIP.

Semi-quantitative grading system for the distribution of TSH-R mRNA by In-Situ PCR

A grading system for the in-situ PCR mRNA labelling studies was performed that comprised a two-step approach. For every field of view observed under light microscopy, the cellular labelling of TSH-R was quantified according to (i) visual intensity and (ii) the extent of labelling. A "0" score was assigned when there was an absence of labelling within cells, "1" indicated low labelling, "2" for moderate labelling and "3" for intense labelling. These scores were further 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 evident for labelling, "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 all normal and bipolar limbic regions. Determinations were performed by two independent observers and the mean scores collated.

Reverse-transcriptase, quantitative polymerase chain reaction (RTqPCR) of TSH-R mRNA

RNA isolation from FFPE tissue sections

Total RNA was isolated from FFPE tissue sections (10 μM), using the ReliaPrep FFPE Total RNA MiniPrep System (Promega, USA) according to the manufacturers' instructions.

The concentration of recovered total RNA was determined using the Nanodrop spectrophotometer (ThermoScientific).

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

Following isolation, the RNA samples (undiluted) were subjected to reverse-transcription using the SuperScript IV First-Strand cDNA Synthesis System (ThermoFisher, SA) according to the manufacturers' instructions. A 7 μl volume of undiluted RNA along with random hexamer primers (provided with kit) were used for the cDNA synthesis reaction.

Non-template controls (NTC) were prepared by substituting RNA template with DEPC-treated H_2O. Resulting cDNA concentration was determined spectrophotometrically. The cDNA concentrations occurred in the range 2458.7–3319.2 with accompanying A_{260}/A_{280} ratios in the range 1.7–2.0.

Quantitative PCR (qPCR) assay for cDNA amplification

Quantitative PCR (qPCR) was performed on the Applied Biosystems 7500 Real-time PCR Instrument (Applied Biosystems, Life Technologies) in 96 well microtitre reaction plates. The fluorescent chemistry that was employed to monitor amplification of the target sequence was the DNA-binding dye, SYBR Green 1. GAPDH provided an endogenous reference gene for the normalisation of gene expression in human limbic tissue. Studies have shown GAPDH to be a suitable and stable reference gene in RTqPCR analysis of human brain samples (Grunblatt et al. 2004; Coulson et al. 2008; Kreth et al. 2010). Primer sequences and PCR amplicon size for the target (TSH-R) and reference (GAPDH) genes are presented in Table 1.

Each reaction was performed in a final volume of 20 μl comprising, 6 μl nuclease-free H_2O, 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. Normal limbic samples were selected as the reference samples (calibrator) for these experiments. Non-template control reactions were included. Amplification was performed at 95 °C for 10 min for enzyme activation, followed by 40 cycles of 95 °C for 15 s (denaturation), 60 °C (GAPDH and TSH-R) for 30 s (annealing) and 72 °C for 30 s (extension). All reactions were performed in duplicate and resulting mean values were obtained. To assess the specificity of amplification of the target gene (TSH-R), a *melt curve analysis* comprising a single cycle at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 s, was included per reaction.

Comparative C_T ($2^{-\Delta\Delta CT}$) method

The Comparative C_T method was used to quantify results obtained by RTqPCR. The threshold cycle (C_T) was automatically generated by the 7500 ABI real time PCR system software. Raw fluorescent data, including C_T means, ΔC_T mean, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$, was exported to an MS Excel spreadsheet. Normal amygdala, cingulate gyrus and frontal cortex were selected as calibrator samples for these experiments. The Comparative C_T method ($2^{-\Delta\Delta CT}$) was used to calculate fold-changes in gene expression differences between normal and bipolar experimental groups. The data is presented as the fold-change in TSH-R gene expression normalised to the reference gene, GAPDH, and relative to normal limbic areas.

Statistical methods

We obtained all statistical analyses using STATA Software, v 13.1 (StatCorp, USA) in order to determine the fold-change of TSH-R gene expression, in the bipolar limbic group, relative to normal controls. However, the data did not appear to follow normal distribution; 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 used to determine intra-group comparisons between normal and bipolar groups. Significance was tested at $p < 0.05$.

Bipolar inter-group comparisons for the fold-change of TSH-R expression across the 3 bipolar limbic areas were established using the *Kruskal-Wallis rank test* followed by *Dunn's Pairwise comparison with Sidák adjustment*. Significance was tested at $p < 0.05$.

Results

Localisation of TSH-R and TG by immuno-histochemistry

Thyroid-stimulating hormone receptor (TSH-R)

Immuno-labelling of normal human thyroid tissue demonstrated TSH-R within the thyroid follicular cells (Fig. 1a). The colloid, colloidal vesicles, colloidal spaces and thyroid vasculature were devoid of TSH-R label. Method controls for thyroid tissue was devoid of stain when the TSH-R (Fig. 1a, inset) antibody was substituted with dilution buffer.

In bipolar human brain tissue, immuno-reactive TSH-R was evident in all 3 limbic regions, in all 5 brains examined. Positive labelling for TSH-R was demonstrated within large

neuronal cell bodies in the amygdala (Fig. 1b), cingulate gyrus (Fig. 1c, d) and frontal cortex (Fig. 1e, f). In contrast, labelling was not observed in limbic vasculature nor any neuronal supporting cell type in all bipolar regions.

Labelling intensities of TSH-R in bipolar limbic regions were quantified using image analysis. The results provided a narrow arithmetic mean intensity range of $14.15–19.15 \times 10^2$ pixels.μm^{-2} (Table 2A). Specifically, the cingulate gyrus (19.15×10^2 pixels.μm^{-2}) displayed the greatest intensity within this category, whilst the amygdala (14.15×10^2 pixels.μm^{-2}) stained the least. Table 2A 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 2A, Fig. 2). Hence, there appeared to be a fairly consistent level of TSH-R in diseased limbic regions.

In addition, we obtained statistical comparisons for TSH-R labelled neurons in normal versus bipolar groups that indicated significant differences in all matched regions examined, i.e. amygdala ($p < 0.0001$), cingulate gyrus ($p < 0.0001$) and frontal cortex ($p < 0.0001$), (Fig. 2). All bipolar limbic regions demonstrated appreciable decreases in TSH-R levels.

Thyroglobulin (TG)

TG in cerebral vasculature Immuno-labelling of normal human thyroid tissue demonstrated TG within the cytoplasm of thyroid follicular cells (Fig. 3a). In addition, intense TG labelling was observed within the colloidal space/vesicles and within smooth muscle cells of the tunica media and tunica adventitia of thyroidal vasculature. Method controls for thyroid tissue was devoid of stain when the TG (Fig. 3a, inset) antibody was substituted with dilution buffer.

In bipolar human brain tissue, immuno-reactive TG was identified within cerebral vasculature in all limbic areas, in all 5 brains examined. However, the degree of staining distribution compared to normal limbic vasculature (Naicker and Naidoo 2017), 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 vasculature in the frontal cortex (Fig. 3b), amygdala (Fig. 3c), and cingulate gyrus (Fig. 3d).

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 2B). The cingulate gyrus (98.96×10^2 pixels.μm^{-2}) indicated the greatest intensity within this category, whilst the frontal cortex (75.48×10^2 pixels.μm^{-2}) stained the least. *Log mean (Ln) intensity* values were also obtained in the range of 2.99–3.96 (Table 2B). Inter-group statistical comparisons among all bipolar groups 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 2B, Fig. 4).

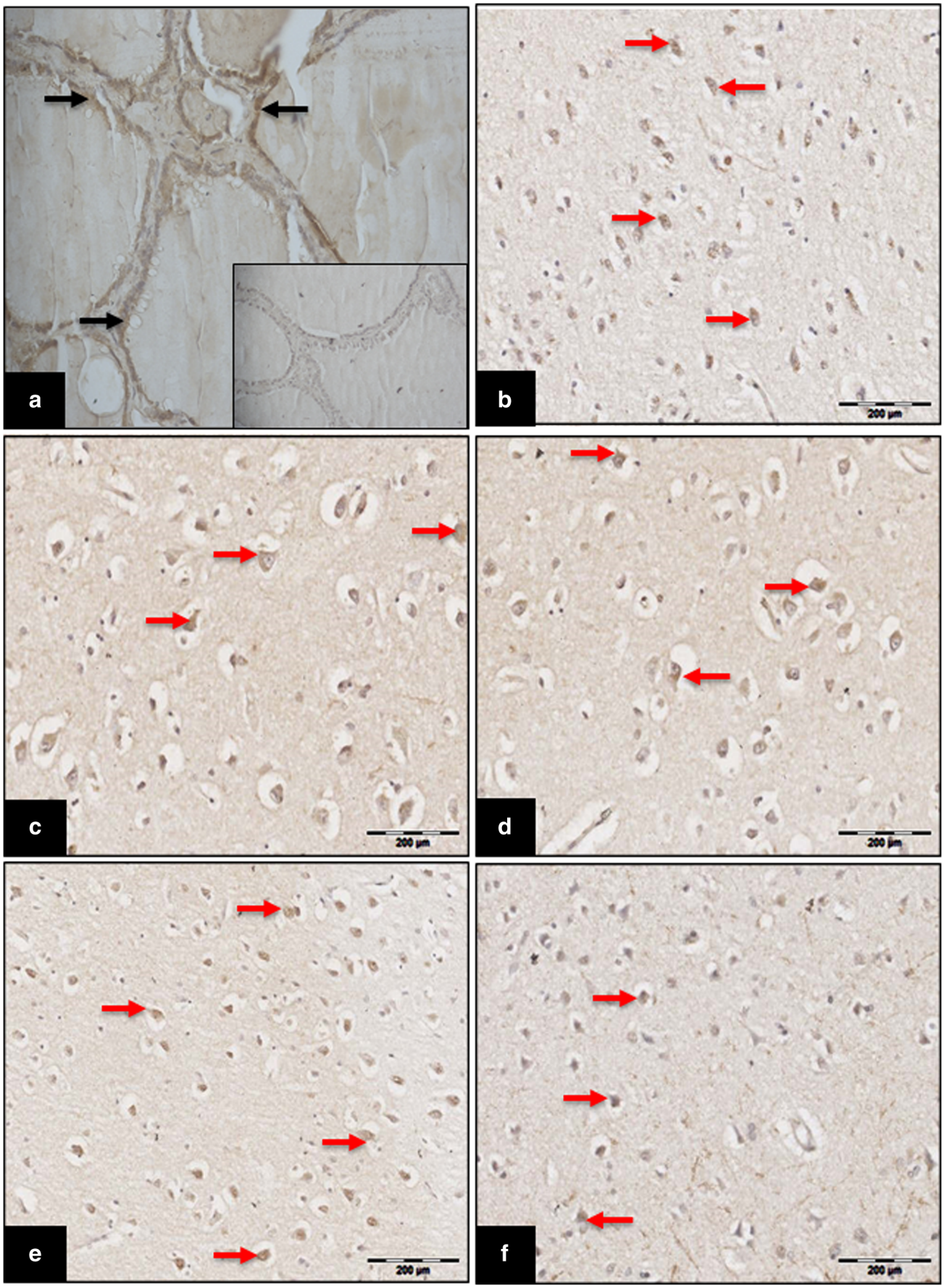
Intra-group statistical comparisons determined for TG in cerebral vasculature in matched normal versus bipolar limbic regions demonstrated significant differences in all areas, amygdala ($p = 0.0007$), cingulate gyrus ($p < 0.0001$) and frontal cortex ($p < 0.0001$), (Fig. 4). Thus, there appeared to be appreciable decreases in the TG levels in bipolar limbic regions.

TG in cerebral neuronal cells Immuno-reactive TG was evident within neuronal cell bodies of all bipolar limbic regions examined, viz. amygdala (Fig. 3e, f), cingulate gyrus (Fig. 3g) and frontal cortex (Fig. 3h). The present findings of TG labelling in bipolar amygdala is in contrast to our observations for normal amygdala, which we had reported to be devoid of stain (Naicker and Naidoo 2017). In contrast to normal brain, bipolar 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.

Quantification of the staining intensities in the bipolar limbic groups, 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 2C). Further, *Log median (Ln) intensity* values obtained for this category had a range of 0.43–0.89 (Table 2C). 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 ($p = 0.0876$).

Intra-group statistical comparisons of neuronal-labelled TG in matched normal versus bipolar limbic regions, indicates significant differences in the amygdala groups ($p < 0.0001$) and frontal cortex groups ($p < 0.001$). In contrast, there was

Fig. 1 Immuno-localisation of TSH-R in bipolar human adult 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**, ×400). Antibody method control for TSH-R in thyroid tissue was devoid of stain (**a**, inset, ×400). In bipolar limbic regions, immuno-reactive TSH-R was evident within neuronal cell bodies (red arrows) in the amygdala (**b**, × 400), cingulate gyrus (**c** and **d**, ×400) and the frontal cortex (**e** and **f**, ×400). Positive labelling for TSH-R in thyroid and brain sections were identified as brown colour. Scale bars = 200 μm


a
b
200 µm
c
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e
200 µm
f
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Table 2 Quantification of the labelling intensities of TSH-R and TG in bipolar limbic structures

A					
TSH-R in bipolar limbic neurons					
Limbic Regions (Bipolar)	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity		
	Mean *(pixels/μm²)*	±SEM	Log Mean (Ln)	±Log SEM	
Amygdala	14.15	2.53	1.97	0.16	
Cingulate gyrus	19.15	3.65	2.3	0.17	
Frontal cortex	15.01	2.42	2.09	0.16	
B					
TG in bipolar 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	
C					
TG in bipolar limbic neurons					
Limbic Regions (Bipolar)	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity		
	Mean (*pixels/μm²*)	±SEM	Log Mean (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)

no appreciable difference in TG immuno-localisation between normal and bipolar cingulate gyrus.

Cellular distribution of TSH-R mRNA by In-Situ RT-PCR

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

For the optimisation of the in-situ RT-PCR experiments, expression of the endogenous reference gene GAPDH, in various normal human tissue sections was determined. GAPDH mRNA labelling was positively identified in all normal human tissue examined, viz. kidney, thyroid and brain. Method controls were devoid of GAPDH mRNA labelling.

For the target gene, normal human thyroid tissue was used to demonstrate TSH-R mRNA labelling in thyroid follicular cells only. There was no evidence of TSH-R mRNA in other thyroidal structures. Method controls for thyroid tissue, showed an absence of TSH-R mRNA labelling in thyroid follicular cells.

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

Positive labelling for TSH-R mRNA expression in normal cingulate was evident in neuronal cell bodies, as well as some axons and dendrites (Fig. 5e). Similarly, normal frontal cortex demonstrated TSH-R mRNA in neuronal cell bodies and axons; however, there was no labelling within dendrites (Fig. 5a). In normal amygdala, TSH-R mRNA labelling was confined to neuronal cell bodies only (Fig. 5c).

In bipolar limbic areas, TSH-R mRNA expression was confined exclusively to neuronal cell bodies in the amygdala (Fig. 5d), frontal cortex (Fig. 5b) and cingulate gyrus (Fig. 5f). In contrast to normal observations, our findings in bipolar limbic regions do not include TSH-R mRNA labelling in neuronal axons.

Further, there was no evidence of TSH-R mRNA in limbic vasculature nor in any neuronal support cell in both normal and bipolar limbic areas. Method controls for brain tissue demonstrated an absence of TSH-R mRNA label in limbic structures (Fig. 5b, d, f, insets).

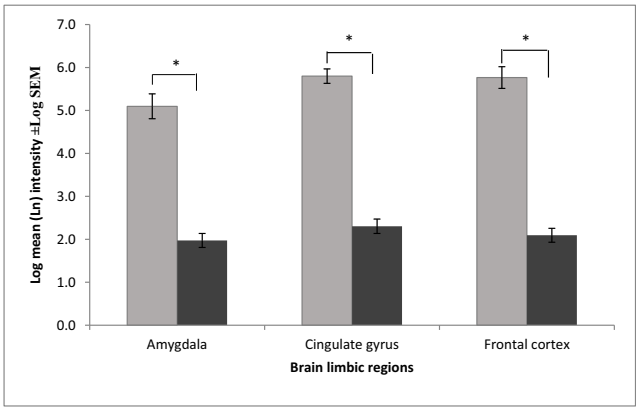


Fig. 2 *Log mean (Ln) intensity* determinations for TSH-R immuno-labelling in cortical neurons of matched normal versus bipolar brain limbic regions**.** Intra-group statistical comparisons demonstrates significant differences between matched normal and bipolar limbic areas examined, amygdala ($p < 0.0001$), 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

Semi-quantitative analysis of 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). The extent of TSH-R mRNA distribution within these regions ranged from 25 to 75% in the cingulate gyrus and frontal cortex and > 75% in the amygdala. In contrast, visual labelling intensity of TSH-R mRNA distribution in the bipolar 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. 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. In-situ RT-PCR semi-quantitative comparisons between matched normal and bipolar groups therefore indicated an under-expression of TSH-R mRNA in all bipolar limbic areas.

Real-time quantitative PCR of TSH-R gene expression

TSH-R gene expression in normal versus bipolar limbic groups

Statistical analyses for the fold-change in gene expression of the target gene, TSH-R, in the bipolar group, relative to the normal control limbic group was determined. Table 4 presents data where the target (TSH-R) and reference (GAPDH) genes were amplified in separate wells of a 96-well microtitre plate. Figure 6 displays the fold-change of TSH-R gene expression in normal and bipolar groups. When compared to normal controls, the expression levels of TSH-R in all bipolar limbic groups appeared to be down-regulated (Fig. 6). Further, statistical comparisons by the *Wilcoxon rank test* between normal and bipolar categories, confirmed significant down-regulation ($p < 0.05$).

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

Table 5 provides *Log median (Ln)* quantification values for TSH-R expression fold-change differences among all matched bipolar limbic areas examined. *Log median (Ln)* values that were 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 (Fig. 6). 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.

Discussion

This study provides the first evidence for the expression and cellular distribution of thyroid-specific proteins in limbic regions of normal and bipolar human adult brain. The study aim was to determine whether our previous findings of TSH-R and TG in normal human adult limbic brain (Naicker and Naidoo 2017) could be reflected in the bipolar state at the protein and molecular levels. Further, we sought to determine whether changes in thyroid-specific protein expression in limbic brain is specific for BD.

The significant finding was the reduction of both TSH-R and TG in limbic regions of bipolar subjects when compared to normal controls. These results were confirmed by both in-situ RT-PCR and RTqPCR which demonstrated down-regulation of TSH-R gene expression in BD. Similar to our previous demonstration of TSH-R in large motor neurons of normal human limbic brain (Naicker and Naidoo 2017), in the present study we demonstrated immuno-reactive TSH-R mRNA and protein in large neurons of bipolar limbic regions, albeit at significantly reduced levels. Our findings of TSH-R in human brain tissue is consistent with the work of Prummel et al. (2000), who demonstrated TSH-R proteins in folliculo-stellate cells of human anterior pituitary tissue (Prummel et al. 2000). Similarly, other authors report the presence of TSH-R in non-human brain tissue (Bockmann et al. 1997; Crisanti et al. 2001; Bolborea et al. 2015). Crisanti et al. (2001) identified TSH-R in rat hypothalamic neurons, whilst Bolborea et al. (2015) reported TSH-R expression in tanycytes of rat hypothalamus. Bockmann et al. (1997) 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).

We detected TG-like proteins in neurons of human bipolar cingulate gyrus, frontal cortex and amygdala groups; these observations were not reflected in the amygdala neurons of normal brain in our previous reports (Naicker and Naidoo 2017). Interestingly, reports from structural and functional neuro-imaging studies associates BD 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 volume when compared to normal amygdala. Thus, based on our present findings along with previous neuro-imaging reports, we may speculate that the presence of TG-like proteins in bipolar amygdala neurons may alter neuronal transmission and functioning, thereby contributing to a hyperactive amygdala in bipolar patients. This is particularly relevant to the suggestion by Miklowitz and Johnson (2006) that elevated amygdala activity may contribute to emotional sensitivity which is a clinical feature of BD (Miklowitz and Johnson 2006).

Another novel finding in the present study demonstrates TG-like proteins in the limbic vasculature of all bipolar groups. However, quantification of TG-staining intensity in the smooth muscle cells of the tunica media/adventitia indicated a significant reduction when compared with normal groups. Our observations of TG proteins in the smooth muscle of cerebral vasculature is supported by Ojamaa and colleagues (Ojamaa et al. 1996) who described the effects of TH on vascular smooth muscle cells of rat aorta (Ojamaa et al. 1996). Specifically, those authors described the vasodilatory effects of T_3 when acting directly on vascular smooth muscle cells in vitro to cause rapid relaxation of coronary arteries. Similarly, Mizuma and colleagues (Mizuma et al. 2001) demonstrated local T_3 production by DII activation in human vascular smooth muscle cells (Mizuma et al. 2001). Further, the significant role of mammalian TH in the development, homeostasis and regeneration of skeletal muscle has been well-documented (Yu et al. 2000; Clement et al. 2002; Udayakumar et al. 2005; Brent 2012).

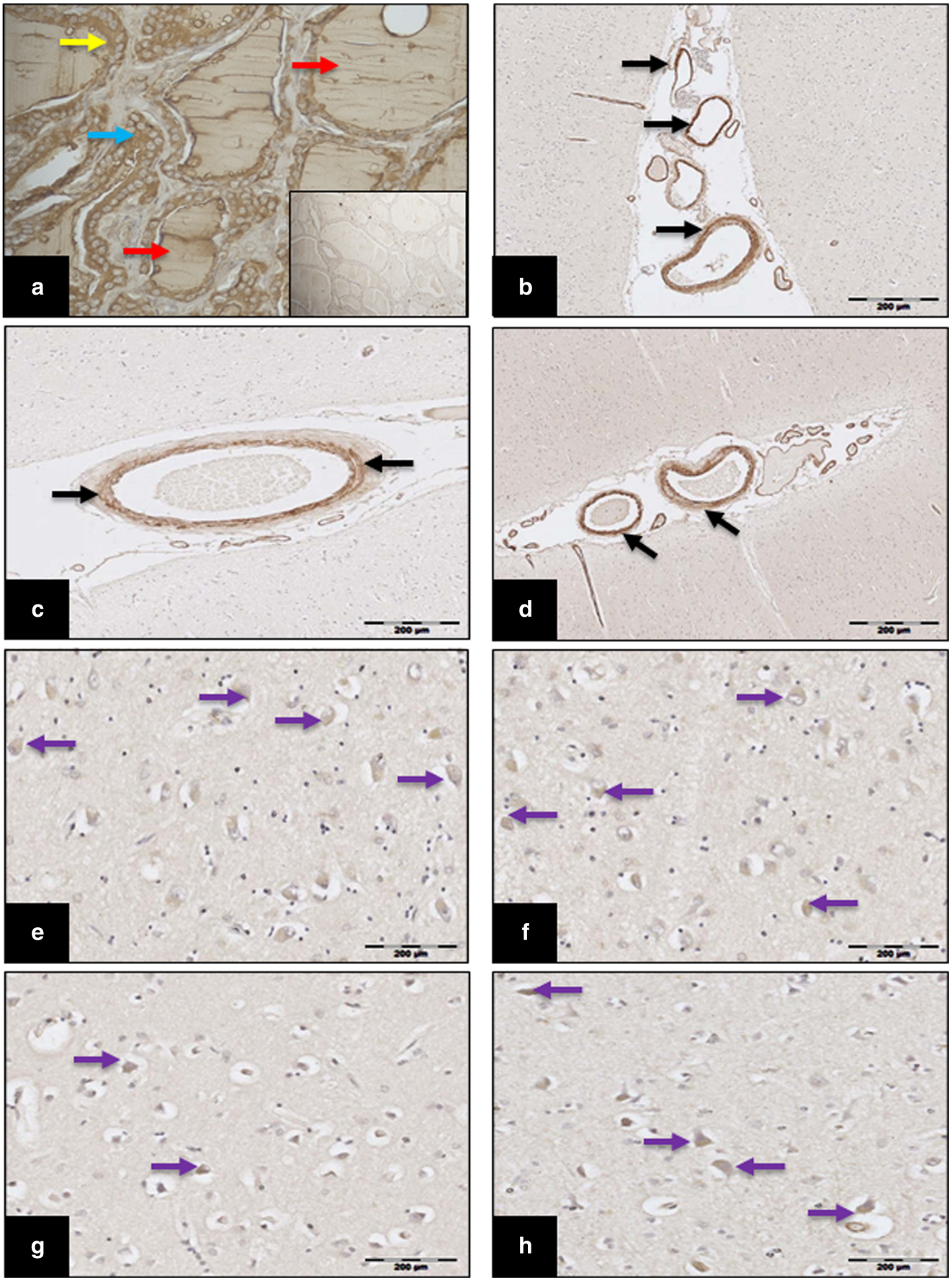
Our findings of TG-like proteins in human cerebral vasculature together with our postulate that circulating anti-thyroid antibodies agonise limbic targets during AITD, suggests a migratory potential for these proteins across the seemingly-impervious blood-brain barrier (BBB). In the recent past, there has been much controversy regarding non-CNS proteins and enzymes breaching the seemingly-impervious BBB. Indeed, there are numerous reports that provide evidence for translocation of non-CNS agents via the protective BBB: Multiple modes of egress have been described, especially when the integrity of the BBB may be altered during neuro-

Fig. 3 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**, ×400). Antibody method control for TG in thyroid tissue demonstrated the absence of stain within thyroidal structures (**a**, inset, ×400). Immuno-reactive TG was highly concentrated within the smooth muscle of the tunica media/adventitia of cerebral vasculature in the frontal cortex (**b**, ×200), amygdala (**c**, ×200) and cingulate gyrus (**d**, ×200) of bipolar brain. Immuno-reactive TG was also evident within neuronal cell bodies (purple arrows) of the amygdala (**e** and **f**, ×400), cingulate gyrus (**g**, ×400) and frontal cortex (**h**, ×400) of bipolar brain. Neuronal axons and other cerebral structures were devoid of stain. Positive labelling for TG in thyroid and brain tissues were identified as brown colour. Scale bars = 200 μm

pathological diseases and these include Alzheimer's disease, HIV-associated dementia, multiple sclerosis, as well as BD (Lou et al. 1997; Minagar et al. 2002; Patel and Frey 2015). Thus, these interactions bear relevance to the present study and lends support to our study postulate that circulating anti-thyroid antibodies and TG-like proteins breach the BBB to bind limbic targets.

The importance of TH in mammalian CNS during various life stages is well-established, especially in foetal and post-natal brain development as well as maintenance of adult brain functioning including mental and psychological stability (Morreale de Escobar et al. 2004; Bernal 2007; Wallis et al. 2010). Here, T_3 and T_4 bind TH receptors that are widely distributed within neurons, astrocytes and oligodendrocytes (Carlson et al. 1994; Carlson et al. 1996; Strait et al. 1997; Carre et al. 1998; Ahmed et al. 2008). Similarly, in our previous study, we demonstrated thyroid-related protein receptors, specifically, TSH-R and TG, in limbic structures of normal human brain (Naicker and Naidoo 2017). Thus, considering the significant roles of TH in mammalian CNS, it is reasonable to suggest that TSH-R and TG in normal limbic components may also exhibit an important alternate neuro-physiological role, specific to mood control. Interestingly, in the present study, we demonstrate down-regulated TSH-R and TG protein expression levels in matched bipolar limbic regions. Thus, we may indeed speculate that a reduced expression of TSH-R and TG in limbic structures will result in reduced thyroid function in the adult brain, which may then predispose or exhibit some involvement in the pathophysiology of mood dysregulation.

Further, our findings of TSH-R expression in limbic structures is suggestive of a neuro-functional role of TSH in limbic brain. We speculate that TSH may act directly on limbic structures by binding to TSH-R [we were the first to report TSH-R in limbic neurons, (Naicker and Naidoo 2017)] and this interaction may stimulate intra-cellular processes (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, provides an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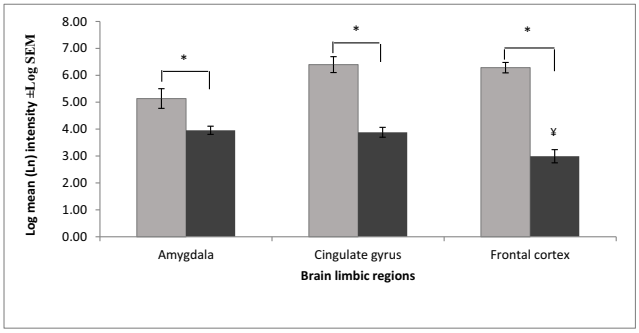

* $p < 0.0001$
¥ vs amygdala, cingulate gyrus, $p < 0.05$

Fig. 4 *Log mean (Ln) intensity* determinations for TG immuno-labelling in cerebral vasculature of matched normal versus bipolar brain limbic regions. 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

Fig. 5 The distribution of TSH-R mRNA in normal and bipolar human limbic areas by in-situ RT-PCR. In normal limbic areas, intense labelling for TSH-R mRNA was detected in neuronal cell bodies (red arrows) in the frontal cortex (**a**, ×400), amygdala (**c**, ×400) and the cingulate gyrus (**e**, ×400). The frontal cortex (**a**, ×400) and cingulate gyrus (**e**, ×400) also demonstrated TSH-R mRNA label in neuronal axons (yellow arrows). Further, TSH-R mRNA was also evident in neuronal dendrites (light blue arrows) of the cingulate gyrus (**e**, ×400). Examination of bipolar limbic areas demonstrated a sparse distribution of labelling for TSH-R mRNA in neuronal cell bodies (red arrows) of the amygdala (**d**, ×400). Moderate distribution of TSH-R mRNA labelling was detected in neuronal cell bodies (red arrows) of the frontal cortex (**b**, ×400) and cingulate gyrus (**f**, ×400). Method controls for brain tissue showed absence of TSH-R mRNA label in limbic structures (**b**, **d** and **f**, insets, ×400). Positive labelling for TSH-R mRNA in brain tissues were identified as blue-black colour. Scale bars = 200 μm

additional short-loop feedback mechanism during the neuro-endocrine control of the thyroid gland; hence, the regulation of TH synthesis. Our postulate does not appear to be so implausible if one considers other theories such as the potential role of brain-derived TSH-R which was proposed by Hosaka and co-workers (Hosaka et al. 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 et al. (1995) performed electron-microscopy studies to localise GLUT1 to the CNS endothelium and astrocytes of the BBB in humans and monkeys (Morgello et al. 1995). The site of GLUT1 localisation suggests its involvement in glucose and glucose metabolite transport to astrocytic and neuronal areas. Thus, there appears to be evidence that supports Hosaka et al. (1992) who proposed a direct role of TSH in the regulation and facilitation of glucose uptake into brain cells (Hosaka et al. 1992). Our finding of a significant TSH-R reduction in bipolar limbic neurons leads one to speculate that a reduced expression of TSH-R in limbic neurons will likely result in decreased ligand-receptor interaction and this may directly affect the glucose uptake for utilisation by neuronal cells, thereby contributing to altered neuronal functioning within mood-regulatory centres of human brain and this may be associated with disturbed mood. Interestingly, this correlates with previous reports describing neuro-inflammation due to microglial alterations in depressed/suicidal bipolar subjects, where microglial alterations lead to reduced glucose uptake and metabolism, thus directly effecting normal neuronal functioning in patients (Cotter et al. 2002; Steiner et al. 2008; Holmes et al. 2018).

An alternative, clinically-related school of thought is that the presence of limbic-derived TSH-R may provide an extra-thyroidal target for auto-immunity: Patients with AITD display immuno-reactivity towards 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 TSH-R resulting in over-activity of the thyroid gland and presentation of classical goiter (Davies et al. 2002; Szkudlinski et al. 2002; Ai et al. 2003). In contrast, inhibitory-type anti-thyroid antibodies bind to TSH-R in hypo-thyroidism (Hashimoto's disease), causing cell-mediated atrophy of thyroid follicular cells. This results in 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 speculate that during AITD, inhibitory-type auto-antibodies associated with Hashimoto's disease, may recognise and agonise TSH-R expressed in limbic neurons leading to cell-mediated atrophy or inactivation of limbic neurons with a consequential reduction in TSH-R levels. Interestingly, here we report reduced TSH-R expression in bipolar limbic neurons which may be related to the loss of limbic neurons or diminished neuronal activity (due to thyroid auto-immunity such as in Hashimoto's disease) and this may contribute towards the development of symptoms of mood dysregulation as observed in BD. 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 preceeding paragraph, decreased ligand-receptor interaction may lead to reduced glucose uptake in limbic neurons, thus depriving limbic regions of an energy source and creating an imbalance affecting mood regulation.

The neuro-pathology of diminished neuronal functioning or neuronal atrophy, suggests a neuro-degenerative aetiology in BD. This is particularly controversial since current evidence implicating neuronal structural and functional changes in BD pathophysiology is limited and does not provide enough

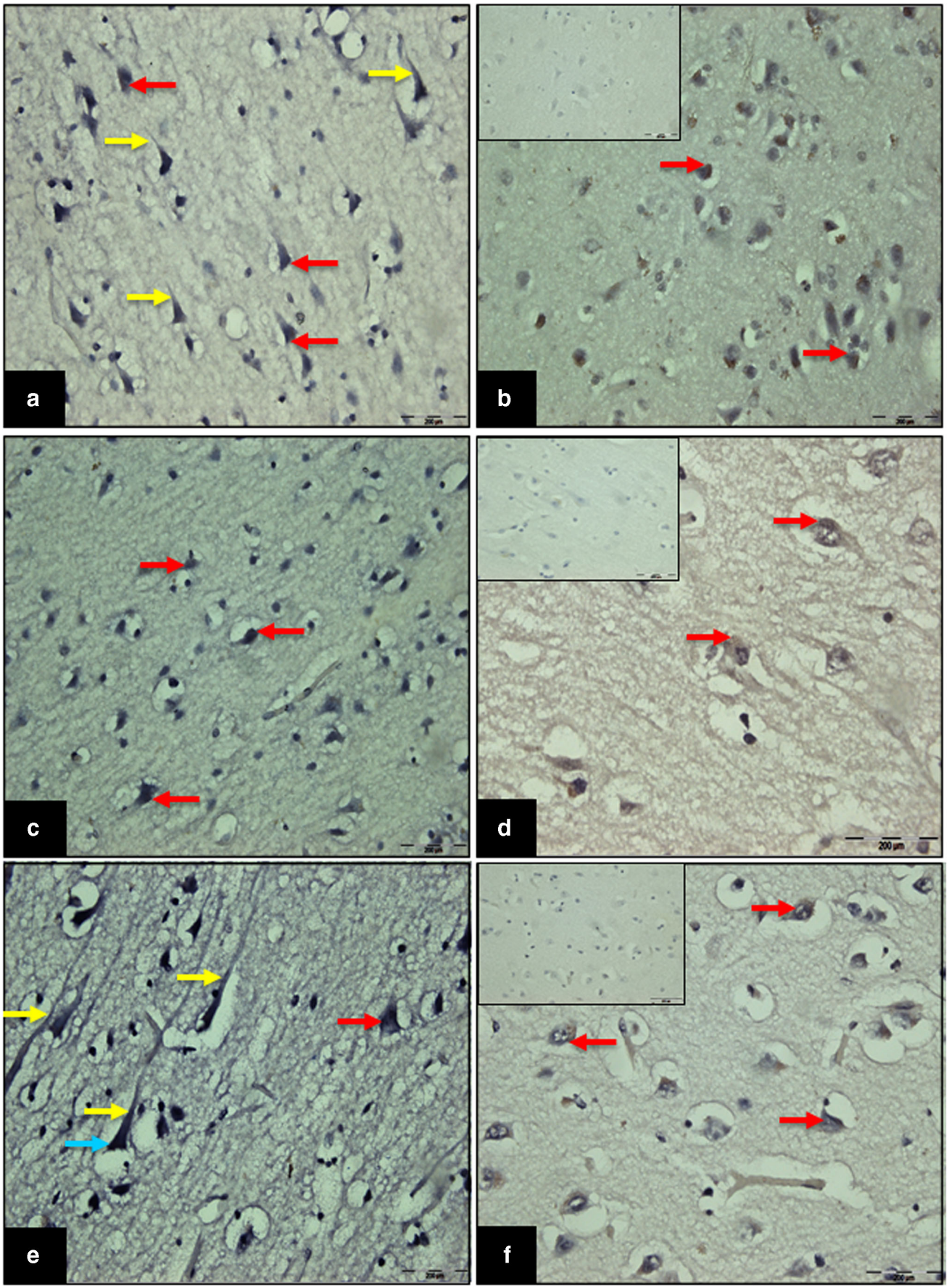
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Table 3 Semi-quantitative comparison of in-situ RT-PCR cellular labelling intensity and extent of regional distribution of TSH-R mRNA in normal and bipolar brain limbic regions

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%

Table 4 RTqPCR data calculations to determine TSH-R gene regulation in bipolar brain limbic regions

Limbic Regions	Sample Number	TSH-R C_T Mean	GAPDH C_T Mean	Δ C_T Mean	ΔΔC_T	Fold difference $2^{-\Delta\Delta CT}$
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	2.90E-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

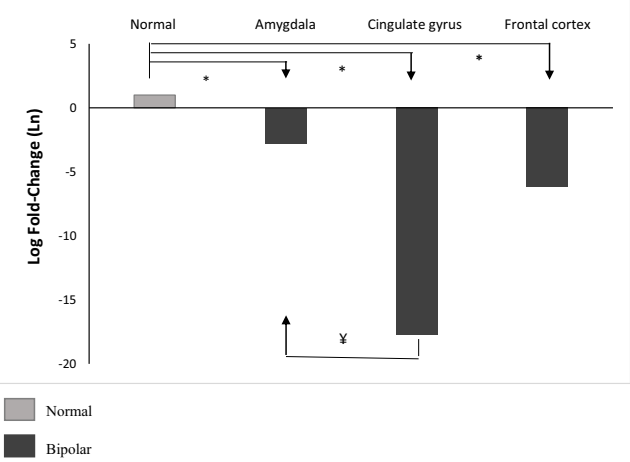
C_T: threshold cycle for target amplification

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 $C_T$sample - Δ $C_T$calibrator

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



* 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

Fig. 6 *Log median (Ln)* expression fold-change differences of TSH-R mRNA in bipolar brain limbic regions. Statistical comparisons demonstrates the down-regulation of TSH-R gene expression in all bipolar groups when compared to the normal control group. 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

support to classify BD as a neuro-degenerative type disorder. However, our study suggests that neuronal alterations may be due to changes in thyroidal status in BD. Our correlation of reduced thyroid protein expression levels in bipolar limbic regions with previous structural (Rajkowska et al. 2001; Lopez-Larson 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), 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) and histological (Rajkowska et al. 2001; Strakowski et al. 2005) neuro-imaging findings of reduced cortico-limbic volumes and activity during BD, provides evidence supporting neuro-degeneration of mood-controlling limbic structures in BD.

Table 5 Log median (*Ln*) quantification of TSH-R gene expression fold-change differences in bipolar brain limbic areas

TSH-R mRNA Expression			
Limbic Regions (Bipolar)	Log Fold-change (Ln)		
	Log median (*Ln*)	*p25*	*p75*
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: *p25* and *p75*
Fold-change vs normal calibrator sample (=1)

Moreover, biochemical studies that describe altered neurochemical and metabolic neuronal marker abnormalities in cortico-limbic regions of bipolar subjects, further implicate neuro-degenerative processing in BD pathophysiology (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 TSH-R/TG in limbic components of bipolar human brain, 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 normal neuronal functioning in primary mood regulatory centres. 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 BD does warrant merit.

Conclusions

Association between thyroid auto-immunity and neuro-psychiatric disorders is well-documented. However, there exists limited literature linking AITD, specifically with clinical features of BD. The present study identifies this lack of available research and explores a possible association between these two disorders, through the extra-thyroidal localisation of TSH-R and TG in major limbic regions of human brain. In order to summarise the relevance of this limbic-derived thyroid protein expression in providing potential therapeutic targets in future BD research endeavours, consider the following arguments:

We propose important neuro-physiological roles of TSH-R/TG in mood control areas of human brain, thus suggesting that reduced TSH-R/TG expression may contribute to altered mood and bipolar pathophysiology. Further, we attribute symptoms of mood dysregulation in BD 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 neuro-degeneration.

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 2016). Our study suggests that neuronal alterations may be due to changes in thyroidal status in BD. 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 BD pathophysiology. Nevertheless, our findings of reduced bipolar limbic thyroid protein expression correlates well with neuro-imaging study reports that demonstrate reduced cortico-limbic volumes and neuro-physiological activity during BD, and is, therefore, suggestive of degenerative processing in limbic brain. This may very well provide an alternate mechanism for a neuro-degenerative aetiology in BD.

An area that has yet to be explored is the fact that we have demonstrated both TSH-R and TG proteins within neurons of the same limbic regions. Some preliminary evidence of this may be contained in a recent genome-wide analysis study that investigated genetic variants in thyroid disease with an unclear link to neuro-psychiatric diseases (Teumer et al. 2018). In the traditional hypothalamo-pituitary-thyroid axis, TSH-R and TG represent significant role players in TH synthesis. It is therefore tempting to speculate that stimulation of TSH-R expressed in limbic neurons may initiate intra-cellular processes to synthesize and modify TG into T_3 and T_4 (the classical TH produced by the thyroid gland). However, there is no evidence to suggest that the presence of TG-like proteins, in those same limbic neurons that co-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 TH synthesis such as thyroid peroxidase (TPO), and sodium-iodide symporter (NIS). We therefore wish to further extend our investigation to determine the limbic expression of TPO and NIS in normal and bipolar human brains.

Acknowledgements The authors thank Kogie Moodley from the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal for providing technical assistance, and Cathy Connelly from the Biostatistics Unit, Department of Family Medicine and Public Health, University of KwaZulu-Natal for providing statistical assistance.

Funding The study was funded by the College of Health Sciences, University of KwaZulu-Natal and Medical Research Council of South Africa.

Compliance with ethical standards

Conflict of interest There was no potential conflict of interest relevant to this article.

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