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SEX- AND AGE-DEPENDENT EFFECTS OF THYROID HORMONE ON GLIAL MORPHOLOGY AND FUNCTION

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Abstract. Thyroid hormones (THs) are essential for the development and function of the central nervous system (CNS), not only for neuronal cells but also for glial development and differentiation. In adult CNS, both hypo- and hyper-thyroidism may affect psychological condition and potentially increase the risk of cognitive impairment and neurodegeneration including Alzheimer's disease (AD). We have reported non-genomic effects of tri-iodothyronine (T3) on microglial functions and its signaling *in vitro* (MORI et al., 2015). Here we report the effects of hyperthyroidism on glial cells *in vivo* using young and old male and female mice. Immunohistochemical analyses showed glial activation are sex- and age-dependent. We also injected fluorescent-labeled amyloid β peptide (A β 1-42) intracranially to L-thyroxine (T4)-injected hyperthyroid model mice and observed sex-dependent microglial phagocytosis *in vivo* as well. These results may partly explain the gender- and age-dependent differences in neurological and psychological symptoms of thyroid dysfunction.

Keywords: thyroid hormones; microglia; astrocytes; hyperthyroidism; amyloid β peptide; phagocytosis.

Introduction

There is a close relationship between the endocrine system and the central nervous system (CNS). Among hormones closely related to the nervous system, thyroid hormones (THs) are critical for the development and function of the CNS (BERNAL, 2000, 2015; DI LIEGRO, 2008; GOMES et al., 2001; PORTERFIELD and HENDRICH, 1993; STENZEL and HUTTNER, 2013; ZOELLER and ROVET, 2004); not only for neuronal cells but also for glial cells (GARCIA-SEGURA et al., 1996; MOHACSIK et al., 2011), especially for their development and differentiation (BAXI et al., 2014; BILLON et al., 2001; DEZONNE et al., 2015; GOMES et al., 1999; JONES et al., 2003; LIMA et al., 2001).

L-thyroxine (T4) is the major TH secreted by the follicular cells of thyroid gland and taken up to astrocytes after transported into the brain through transporters (BERNAL, 2000; BERNAL et al., 2015; BRAUN et al., 2011; HENNEMANN et al., 2001; SCHWEIZER and KOHRLE, 2013; WIRTH et al., 2014). TH transport into astrocytes is also dependent on the H⁺ concentration inside the cells, involving a mechanism linked to the activity of the Na⁺-H⁺ exchanger (BESLIN et al., 1995). T4 in astrocytes is de-iodinated by type2-deiodinase (D2) to produce T3 (DI LIEGRO, 2008; GUADANO-FERRAZ et al., 1999; GUADANOFERRAZ et al., 1997). T3 is then transported out to the brain parenchyma (BERNAL et al., 2015), being apparently released from astrocytes.

Brain function depends on an intimate neuron to glia and glia to neuron signaling (DEZONNE et al., 2013; GOMES et al., 1999; MENDES-DE-AGUIAR et al., 2008; MORTE and BERNAL, 2014; NIEDERKINKHAUS et al., 2009). Thyroid hormone may have protective effects non-genomically or genomically on neurons and glial cells in the setting of acute brain ischemia (LIN et al., 2011). Therefore, any impairment of TH supply to the developing CNS causes severe and irreversible changes in the overall architecture and function

of the human brain, leading to various neurological dysfunctions (DI LIEGRO, 2008; DUNTAS and BIONDI, 2013; HENRICHS et al., 2010). Although the importance of THs on development and various functions of CNS have been reported, precise actions of THs on glial cells have not yet been well explored. We previously investigated the effect of THs on primary cultured microglia and their intracellular signaling (MORI et al., 2015). T3-induced microglial migration and phagocytosis as well as morphological changes appeared to be non-genomic effect, which seem to be relevant to regulation of proliferation and motility of endothelial cells and certain tumor cells (P. J. DAVIS et al., 2016; P. J. DAVIS et al., 2008; KALYANARAMAN et al., 2014; LEONARD, 2008). There are cytoplasmic TH receptors (TRs) in addition to nuclear TRs; T3 might interact with a plasma-membrane-associated TH receptor α variant (KALYANARAMAN et al., 2014), and with cytoplasmic TH receptor β (MARTIN et al., 2014), while T4 interacts with integrin $\alpha\beta_3$ (BERNAL, 2007; CHENG et al., 2010; P. J. DAVIS et al., 2016; P. J. DAVIS et al., 2008), though both nongenomic and genomic effects can overlap in the nucleus (P. J. DAVIS et al., 2016). In astrocyte T4 alters actin polymerization and iodothyronine deiodinase activity through non-genomic pathway, which may also contribute to the normal brain development through multiple signal transduction pathways (KOIBUCHI, 2013). In the present study, in addition to the analyses of TH action in cellular level, morphological and functional changes of glial cells in response to THs were analyzed in T4-injected animal, i.e. hyperthyroid mouse model.

Methods

Animals

The study was approved by the Animal Research Committee of Kyushu University and carried out in accordance with

the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experimental procedures were based on the Guidelines of the Committee for Animal Care and Use of Kyushu University. Hyperthyroidism was induced by intraperitoneally injecting T4 (0.3 mg/kg) 4 times during 2 weeks.

Immunohistochemical Analysis

Mice were anesthetized by pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline. The brain was removed, postfixed in the same fixative and placed in 20% sucrose solution for 24 h at 4 °C. Transverse brain sections (30 μm) were sliced by a HM 550 cryostat (Micro-edge Instruments Co., Tokyo, Japan), then incubated with rabbit anti-mouse Iba-1 IgG (1:2000; Wako) and mouse anti-mouse GFAP IgG (1:2000; Millipore) in 10% block ace for 18 h at 4 °C. The sections were incubated with Alexa fluor 488 conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) and Alexa fluor 568 conjugated goat anti-mouse IgG (1:1000; Molecular Probes) for 3 hr at room temperature. Sections were mounted on coverslips with PermaFluor Aqueous Mounting Medium (Thermo Scientific, Yokohama, Japan). The sections were analyzed using a fluorescence microscope (BZ9000, Keyence, Japan).

In Vivo Intracortical 5-FAM-Labeled Aβ1-42 Delivery

Male/female C57/BL6J hyperthyroid model mice (8

weeks old) were anesthetized with pentobarbital (12.5 mg/kg) and placed in a stereotactic apparatus. A 2 μl Hamilton syringe with a 25 gauge needle was inserted into the right cortex through a small hole drilled through the skull, and then 2 μl of fluorescently labeled 5-FAM-labeled Aβ1-42 (1 mg/ml) was injected.

For intracortical injection, the microsyringe was inserted into brain at the following coordinates; anterior, 0.6 mm; lateral, 1.6 mm; and dorsoventral, 0.8 mm. n = 3 - 4 animals were used per experimental group. 3 days interval after microinjection of Aβ1-42, each mouse was anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS; 80 mM Na2HPO4, 20 mM KH2PO4, 150 mM NaCl, pH 7.4). The brain was removed, postfixed in the same fixative and cryoprotected for 24 h in 20% sucrose solution at 4 °C. Microglia in the sections containing lesion were immunohistochemically stained with rabbit anti-mouse Iba-1 IgG (1:2000; Wako) and Alexa fluor 568 conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) as mentioned below. The sections were analysed by a fluorescence microscope (BZ9000, Keyence, Japan).

Results

Both microglia and astrocytes were morphologically activated with abnormal level of THs. We made hyperthyroid model mice with young and old mice

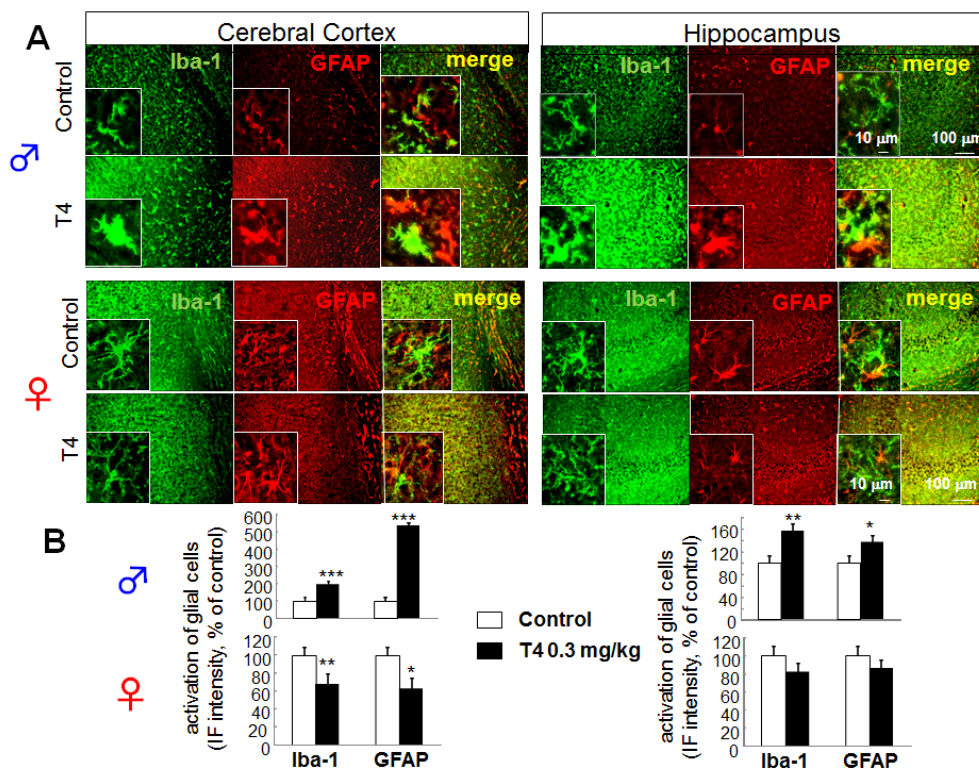


Figure 1. Sex-dependent glial activation in hyperthyroid model of young mice. A. Differential glial activation in cerebral cortex and hippocampus of hyperthyroid mouse model. Double immunofluorescence staining of Iba-1 and GFAP in slice preparation (30 μm) from cerebral cortex and hippocampus of T4-treated hyperthyroid model and control (vehicle-treated) mice in young (8 week old) male (upper panels) and female (lower panels). B. The relative fluorescence intensity of Iba-1 and GFAP immunoreactivity in cerebral cortex and hippocampus of young male (upper panels) and female (lower panels). **p*<0.05, ***p*<0.01, ****p*<0.005 vs control (Scheffe's test)

and observed the effects of hyperthyroidism on glial cells. Interestingly the effects of THs were sex- and age-dependent.

Sex-Dependent Effects of Hyperthyroidism on Microglial and Astrocytic in Cerebral Cortex and Hippocampus

To determine whether change in TH level affects microglia *in vivo*, we investigated the effects of hyperthyroidism on microglia and astrocytes in the cerebral cortex and hippocampus by immunohistochemical analysis. In the hyperthyroid model using young (8 week old) male mice, retraction of processes and enlargement of cell body of microglia and astrocytes were observed in cerebral cortex and hippocampus, especially in dentate gyrus (DG) and CA3 region (Fig. 1A). As a result, the Iba-1 and GFAP immunoreactivities were significantly increased in both regions (cortical immunoreactivity; $197.41 \pm 16.86\%$ of control for Iba-1, $534.38 \pm 70.77\%$ of control for GFAP) (hippocampal immunoreactivity; $155.43\% \pm 12.03$ of control for Iba-1, $136.11 \pm 11.11\%$ of control for GFAP) (Fig. 1B, upper panels). This microglial and astrocytic activation was not observed in young (8 weeks old) female hyperthyroid mice (cortical immunoreactivity; $66.92 \pm 5.68\%$ of control for Iba-1, $62.20 \pm 12.36\%$ of control for GFAP) (hippocampal immunoreactivity; $81.61\% \pm 9.91$ of control for Iba-1, $85.37 \pm 9.98\%$ of control for GFAP) (Fig. 1B, lower panels).

Age-Dependent Effects of Hyperthyroidism on Microglial and Astrocytic Activation in Cerebral Cortex and Hippocampus

Though glial activation was observed in young male mice, the Iba-1 and GFAP immunoreactivities in cerebral cortex were decreased in old (>1 year old) male hyperthyroid mice (Fig. 2A), especially in posterior area of parietal association cortex (PTLp), primary somatosensory cortex (SSp), primary auditory cortex (AUDp) and dorsal/ventral part of auditory cortex (AUDd, AUDv). The cortical immunoreactivity was; $83.34\% \pm 4.26$ of control for Iba-1, $52.43 \pm 3.93\%$ of control for GFAP. On the other hand, in hippocampus, only GFAP immunoreactivity, but not Iba-1 immunoreactivity, decreased significantly in old male mice ($100.07 \pm 5.71\%$ of control for Iba-1, $88.22 \pm 2.76\%$ of control for GFAP) (Fig. 2B upper panels). In female mice, no apparent change was observed in both young and old mice, though statistically significant decrease in Iba-1 immunoreactivity in hippocampus was shown (Fig. 2B lower panels).

Sex-Dependent Effects of Hyperthyroidism on Microglial Phagocytosis *In Vivo*

To examine the effect of THs and microglial activation in young male mice on microglial phagocytic function *in vivo*, we microinjected 5-FAM-labeled A β 1-42 intracortically to control and hyperthyroid mice to observe microglial phagocytosis of A β 1-42 *in vivo*. Phagocytosis of 5-FAM-

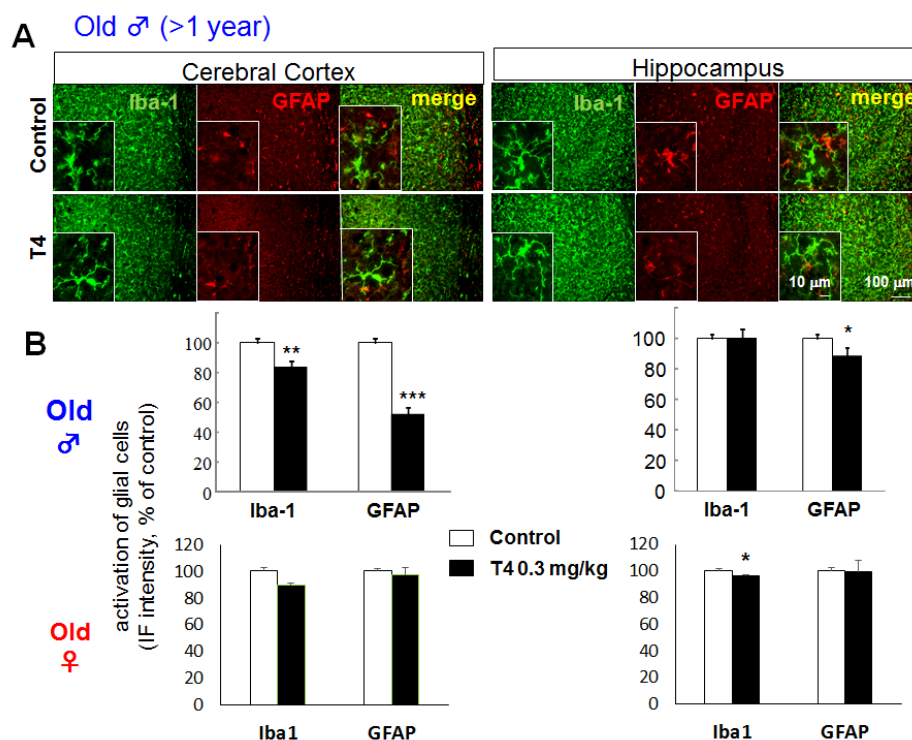


Figure 2. Glial activation in hyperthyroid model is not observed in aged mice even in male. **A.** Differential glial activation in cerebral cortex and hippocampus of T4-treated hyperthyroid model and control (vehicle-treated) mice in old (> 1 year old) male. There is no activation in glial cells in old male mice compared to young male mice (Fig. 1). **B.** The relative fluorescence intensity of Iba-1 and GFAP immunoreactivity in cerebral cortex and hippocampus of old male (upper panels) and female (lower panels). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs control (Sheffe's test)

labeled A β 1-42 by microglia was facilitated in young male hyperthyroid mice compared to the control group (Fig. 3, upper panels). However, this enhancement of microglial phagocytosis was not observed in young female hyperthyroid mice (Fig. 3, lower panels).

Discussion

Hyperthyroidism is an overactivation of thyroid gland, which lead to excessive production of THs. The prevalence of subclinical hyperthyroidism ranges from 1 to 15%, while subclinical hypothyroidism from 3 to 16% in individuals aged 60 years and older (BIONDI and COOPER, 2008). Even at subclinical level, hyperthyroidism in the elderly is suggested to increase the risk of cognitive decline, dementia and AD remarkably (KALMIJN et al., 2000; VAN OSCH et al., 2004; WIJSMAN et al., 2013). In addition, both hypothyroidism and hyperthyroidism, can cause psychiatric disorders such as schizophrenia, bipolar disorder, anxiety and depression. However, the role of microglia and astrocytes in this relationship between thyroid dysfunctions and neuropsychological disorders is largely unknown (NODA, 2015). Therefore, the effects of THs on microglia and astrocytes that act as major TH metabolism and local T3 production regulator were observed. The THs-sensitive regions of the brain are cerebral cortex and hippocampus (FONSECA et al., 2013; GUADANOFERRAZ et al., 1997). We made hyperthyroid mouse models using mice with different sex and age, then measured immunofluorescent intensity of Iba-1 and GFAP in each cerebral cortex and hippocampus as indicators of microglial and astrocytic activation.

In the present study, experimental hyperthyroidism of young (8 weeks old) male mice induced retraction of processes and enlargement of cell body of microglia and astrocytes in cerebral cortex and hippocampus (Fig. 1A), and significant increase in the Iba-1 and GFAP immunoreactivities (Fig. 1B). These results show that

THs induce the morphological change and activation of microglia and astrocytes *in vivo*, as well as *in vitro*. Astrocyte is the major player of TH metabolism in the brain, which controls local T3 production by deiodination of T4. Therefore, this result suggests that elevated circulating and transported T4 stimulated brain astrocytes, might contribute to an increase in local brain T3 production, and provoked activation of microglia.

Contrary to what was observed in male, these morphological changes and activation of microglia and astrocytes were not observed in young (8 week old) female hyperthyroid mice (Fig. 1A and 1B, lower panels). Moreover, in old (>1 year old) male hyperthyroid mice, a significant decrease in Iba-1 and GFAP immunoreactivities in cerebral cortex was observed (Fig. 2A, 2B upper panels).

To examine whether this microglial activation induced by hyperthyroidism affect the microglial function *in vivo*, we performed intracortical microinjection of 5-FAM-labeled A β 1-42 to control and hyperthyroid mice, then observed microglial phagocytosis of A β 1-42 within the brain by immunohistochemical analysis. Microglial phagocytosis of 5-FAM-labeled A β 1-42 was facilitated in young male hyperthyroid mice compared to the control group (Fig. 3, lower panels). However, similar to the results of glial activation, this enhancement of microglial phagocytosis was not observed in young female hyperthyroid mice (Fig. 3, lower panels). In aspect of sexual difference, it is reported that low and high thyrotropin levels were associated with an increased risk of AD in female but not in male (TAN et al., 2008). Therefore this differential activation of microglia and astrocytes in hyperthyroid model mice suggests that THs regulate their function through complex mechanisms, involving various factors, for example change in serum concentration of insulin-like growth factor II (TADA et al., 1994b) or apolipoproteins (TADA et al., 1994a), in addition to sex- and age-dependent hormonal changes. Alternatively TH transporter expression might change with age or sex as reported in liver (ENGELS et al., 2015).

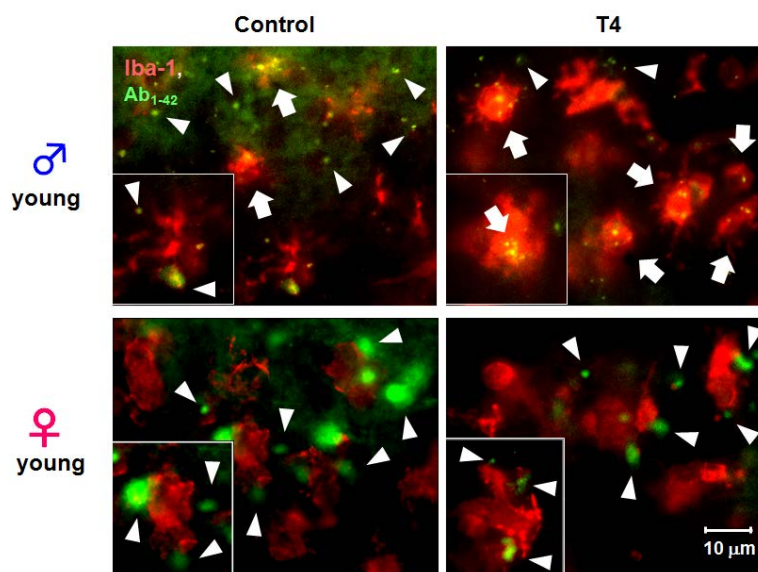


Figure 3. Sex-dependent microglial phagocytosis in hyperthyroidism *in vivo*. Typical images 5-FAM-labeled A β 1-42 (green) and microglia (red, anti-Iba1) in cerebral cortex from control (vehicle-treated) or T4-treated hyperthyroid model mice in young male (upper panels) and female (lower panels). Extracellular fluorescent-labeled A β 1-42 is observed as green (white arrow head), while phagocytosed A β 1-42 within microglia can be observed as yellow particles (white arrow) (scale bar, 10 μ m)

Age and/or sex characteristics of the thyroid hormone synthesis or function was reported decades ago (DAINAT et al., 1984; F. B. DAVIS et al., 1982; DUCASSOU et al., 1980; ERFURTH et al., 1984; EVERED et al., 1978; GEORGIEV and PETKOV, 1986; GRANDHI and BROWN, 1975; GREGERMAN, 1963; HANSEN et al., 1975; HEGEDUS et al., 1983; IVERSEN and PEDERSEN, 1979; KAPITOLA et al., 1969; LOSKUTOVA, 1974; NAKAI et al., 1981; SEGAL et al., 1982; TENORE et al., 1980; TROUT, 1974; WENZEL et al., 1974; WILKE, 1983; WILSON and JOHNSON, 1964; YOUSEF and LUICK, 1971). Gender dependence in the hormone content, including T3, of the immune cells (CSABA and PALLINGER, 2009) and sex dependence of autoimmune disease (ESTIENNE et al., 2002) were also reported, while no significant differences were observed for prolactin, free T3 and free T4 between the 21-30-year age group and the > 70-year age group (ELMLINGER et al., 2003). Since thyroid dysfunction increases with age, changes in THs levels in the elderly could be a factor affecting the development of neurodegenerative diseases including Alzheimer's disease (VILLANUEVA et al., 2013). Nevertheless, differential activation of glial cells in the brain has been totally unknown. The current findings will give us a hint to understand the molecular basis of the gender- and age-dependent phenomena due to TH abnormalities.

Age-dependent phenomena are also reported for the effect of neurotransmitter. For example, glutamate-dependent neuroglial calcium signaling differs between young and adult brain. It is because astrocytic expression of mGluR5 is developmentally regulated and is undetectable after postnatal week 3 (SUN et al., 2013). Age-specific localization of NMDA receptors on oligodendrocytes and age-dependent process in ischemic injury to white matter are also reported (BALTAN, 2009, 2014, 2015; BALTAN et al., 2008). Overall, we may need to highlight the importance of age and gender for better understanding of neurological disorders and in any successful therapy.

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Conflicting interests: The authors declare no conflicting interests.

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List of abbreviations:

AβP	amyloid β peptide
AD	Alzheimer's disease
CNS	central nervous system
D2	type2-deiodinase
DG	dentate gyrus
FAM	fluorescein amidite
GFAP	glial fibrillary acidic protein
Iba-1	ionized calcium-binding adapter molecule
IgG	immune globulin G
mGluR	metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
TH	thyroid hormone
T3	3,5,3'tri-iodo-L-thyronine, also known as tri-iodothyronine
T4	3,5,3',5'tetraiodo-L-thyronine, also known as L-thyroxine