VARIATION IN THYROID HORMONE CONCENTRATION IN BRAIN MICRODIALYSATES FROM FREELY-MOVING ADULT RATS

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A thesis submitted to the Graduate School- Camden

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Masters of Science

Graduate Program in Biology

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Camden, New Jersey

May, 2009

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Abstract

**VARIATION IN THYROID HORMONE CONCENTRATION IN BRAIN
MICRODIALYSATES FROM FREELY-MOVING ADULT RATS**

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Thyroid hormone has genomic and non-genomic actions in the developing and
mature human. The focus of this research was to find if there was a measurable amount
of thyroid hormone in the extracellular fluid of the adult rat brain and how it varied over
time. To study the levels of thyroid hormone concentration in the adult brain, stereotaxic
surgery was performed to implant a microdialysis cannula and electrodes for the study of
electroencephalogram (EEG), electromyogram (EMG). The stylet blocking the
microdialysis cannula was removed and the probe was inserted at the beginning of the
study for sample collection. EEG and EMG waves were recorded concurrently. Artificial
cerebrospinal fluid (aCSF) was run through the inlet tube of the probe and sample was
obtained from the outlet using a refrigerated fraction collector. Collected samples were
analyzed by high pressure liquid chromatography (HPLC), liquid chromatography mass
spectrometry (LC/MS) and radioimmunoassay (RIA) for presence of the thyroid
hormone. Samples run through the C18 column on the HPLC gave a peak for thyroid
hormone at a retention time of 4.1minutes. Similar results were generated when the
microdialysate samples were analyzed using LC/MS. The LC of the microdialysate gave
the peak at the retention time of 4.1 minutes. This peak was further studied for
confirmation by MS ionization. MS gave a fragmentation pattern which corresponded to
the breakdown products of the thyroid hormone. The RIA kit with thyroid hormone

standards prepared in aCSF was used for the detection of the thyroid hormone concentration. The level of thyroid hormone in samples varies over time. The variation in thyroid hormone level over time was compared to the sleep data obtained concurrently. The variation seen in the thyroid hormone concentration over time was detected by HPLC, LC/MS and RIA. This variation suggests some possibility of a mechanism of release of thyroid hormone in adult brain. This finding would be consistent with the potential signaling role of TH suggested by earlier experiments performed in our lab.

Acknowledgement

I owe my gratitude to Dr. J.V.Martin for guiding me from working in lab to writing this thesis. I am also thankful to Dr. P.J. McIlroy and Dr. W. M. Saidel for being my committee member and offering me their wisdom and expertise with utmost kindness and compassion. I would also like to thank James Boulden for his expert guidance with the software needed for my research. My appreciation is given to Sulbha, Maria, Jon'elle, Shirin, Brittany, Alfonso and Shannon for making the day-to-day experience more entertaining. Last but not the least, thanks to my husband, Neeraj Choudhary and my son Neil Choudhary for the love and support, without their support this thesis could not have been possible.

Dedication

To my family

Neeraj and Neil Choudhary

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Introduction

1. Thyroid hormone: - Thyroid hormone (TH) is important for normal growth and development of human. In early developmental, it is required for brain development**.** In the adult, TH helps in maintenance of metabolic stability by regulating oxygen requirement, body weight and intermediary metabolism. Deficiency of TH in early developmental results in condition called cretinism, a mental and growth retarded effect. Deficiency in the adult results in an accumulation of mucopolysaccharides in subcutaneous tissues and other organs, a condition termed myxedema. Hyperthyroidism or excess TH during growth leads to protuberant eyes and enlarged thyroid gland or goiter. Grave's disease is caused when auto antibodies against thyroid stimulating hormones are produced, which stimulates thyroid gland for thyroid hormone production and release. This results in increased thyroid hormone level in circulation. Other causes of hyperthyroidism are toxic uninodular or multinodular goiter.

1.1 Thyroid hormone synthesis and secretion:-

TH is released in the blood from the thyroid gland, located just below the larynx in humans. Thyrotropin-releasing hormone (TRH) is released from the hypothalamus. TRH stimulates cells termed thyrotrophs within the anterior pituitary to release thyroid stimulating hormone (TSH). TSH stimulates release of thyroid hormone from thyroid gland. TSH binds to the TSH receptors present on thyroid gland. This binding stimulates adenylate cyclase catalysed cyclic adenosine monophosphate (cAMP) production, phospholipase C activation and calcium mobilization. TSH activates a sodium iodide symporter which helps to actively take up iodide in the thyroid follicle cell. Iodoamino acids are precursor amino acids for TH production within the matrix of thyroglobulin.

Iodination of tyrosine residues within the cell results in iodoamino acids like diiodotyrosine and monoiodotyrosine. Thyroxin (T_4) and triiodothyronine (T_3) are formed within thyroglobulin by a coupling reaction catalyzed by thyroid peroxidase, involving two diiodotyrosyl residues or a monoiodotyrosyl and a diiodotyrosyl residue respectively (Nilsson, 2001). Thyroglobulin is a soluble protein present in the lumen of a thyroid follicle in the thyroid gland. The TH formed is released when TSH stimulates the thyroid gland. TSH regulates hormone production, storage and secretion by the thyroid gland (Griffin, 2004).

1.2 Thyroid hormone transport and tissue delivery:-

Thyroid hormone released by the thyroid gland gets attached to thyroid hormone-binding globulin (TGB) (Terry, 1992), transthyretin, albumin (Petitpas, 2003) and other proteins in the blood. A very small amount of TH in the picomolar range is found to be free of binding protein in the serum (Shi, 2002). Thyroid hormone bound to protein is transported to tissue. Before entering the tissue, TH dissociates from the binding proteins. The binding proteins are necessary for maintenance of the TH half life in the serum. Movement of TH into the tissue cell from circulation takes place by plasma membrane carriers present on the plasma membranes. The transport process is energy dependent and requires adenosine triphosphate (ATP). Various TH transporters have been identified to transport TH to intracellular sites. They are sodium/taurocholate co-transporting polypeptides, various organic anion transporters, and the L-amino acid transporters (Friesema, 2001).

1.3 Activation and inactivation of thyroid hormone:-

Thyroid hormone is released from the thyroid gland mainly as T_4. The two chemically possible forms of T_4 are the L- and D-isomers. The L-isomer is the only product of thyroid gland *in vivo* (Schwartz, 1983). Also, the L-isomer is more potent in eliciting thyroid hormone nuclear receptor binding and function (Oppenheimer, 1985). Most circulating T_3 is produced by monodeiodination of T_4 in peripheral tissues (Braverman, 1970). T_3 is the most active form of TH for the nuclear thyroid hormone receptor. Three types of deiodinase enzymes are responsible for TH activation and inactivation. Type 1 deiodinase (D1) has both inner-and outer-ring deiodinase and converts T_4 to T_3 and $5,5',3'$-reverse-triiodothyronine (r T_3) to $3,3'$-diiodothyronine (T_2). This D1 enzyme is responsible for the circulating T_3 in liver, kidney and brain. D1 along with D3 works in deiodinating T_3 to another less active form, 3, 5-diiodothyronine (T_2) (Toyoda, 1994). Type 2 deiodinase (D2) has outer ring activity and is thought to be an important source of intracellular T_3 in certain tissues and brain as well as circulatory T_3. In humans D2 is found in the pituitary, brain, brown adipose tissue, thyroid gland, placenta and both skeletal and cardiac muscle. Type 3 deiodinase (D3) has inner ring activity and is important for inactivating T_4 and T_3. It converts T_4 to rT_3, a less active iodothyronine, thereby deactivating it (Visser, 1982). D3 is present in brain, placenta and skin. D3 is enhanced in brain by TH excess and decrease by TH deficiency. The opposite regulation of D3 and D2 activities helps in maintenance of T_3 concentration in the excess and deficiency of TH. D1 is sensitive to propylthiouracil (PTU) whereas D2 and D3 are insensitive to PTU. There are other decarboxylated metabolites of thyroid hormone that are present in our body.

1.4 Genomic action of thyroid hormone: -

The classical genomic actions of TH are mediated by the thyroid hormone nuclear receptors (TR) that have a high affinity for T_3 (Samuels *et al.,* 1973). In addition to binding T_3, a true TR must also bind to a thyroid response element (TRE), a specific DNA sequence in the promoter region of thyroid responsive gene (Harvey *et al.,* 2002). The TREs is a direct repeat of AGGTCA with 4 nucleotide spacer. The binding of TR to TRE is independent of T_3. Binding of T_3 and TR to TRE results in an alteration of gene transcription (either activation or suppression). When TR is bound to TRE in the absence of T_3, it silences the gene transcription. On the other hand when T_3 is bound to TR it dissociates a co-repressor complex and recruits a co-activator complex containing histone acetyltransferase. This dissociation results in the initiation of transcription of TR responsive gene (Tsai, 1994). The TH nuclear receptor is responsible for its effect in almost all tissues of the body except adult brain.

Genomic effects are mediated by the action of TH on the expression and activity of enzymes. Some of the genomic actions are

1. TH stimulates synthesis of membrane Na+, K+ -ATPase (Lei *et al.,* 2004). The increase in ATPase number increases energy expenditure and oxygen consumption. TH binding to a mitochondrial nuclear receptor stimulates calorigenesis.

2.TH regulates energy metabolism by stimulating expression of uncoupling proteins. Uncoupling proteins allow the return of protons to the mitochondrial matrix, bypassing ATP synthase and therefore uncoupling oxidative phosphorylation. Fatty acid oxidation

takes place with generation of heat but no ATP production, thus increasing basal metabolic rate (BMR) (Bianco *et al.,* 1987).

3. While TH stimulates protein synthesis, an excess of TH accelerates protein catabolism, leading to increased nitrogen excretion (Griffin, 2004).

4.Thyroid hormone stimulates glycogenolysis and gluconeogenesis by enhancing the action of epinephrine due to increased expression of the adrenergic receptor (Griffin, 2004).

5. TH increases the breakdown of lipids and fatty acids by regulating expression of the appropriate enzymes (Oppenheimer *et al.,* 1987).

6. TH has a synergistic role with growth hormone and somatomedins in bone formation and maturation (Lakatos *et al.,* 2003). Hypothyroidism is associated with increased bone fracture risk. Hence, TH affects growth.

7. The molecular action of thyroid hormone is important for growth and development of brain in the early developmental stages. (Schwartz *et al.,* 1993; Oppenheimer *et a,l*1997).

1.5 Non-Genomic action of thyroid hormone: -

Genomic actions take place when a ligand binds to a nuclear receptor to regulate gene transcription, whereas non-genomic actions are independent of the gene expression and the action of ligand is mostly confined to plasma membrane receptors. Non-genomic actions regulate membrane channels (Incerpi *et al.,* 1999, Sun *et al.,* 2000), protein trafficking, (Zhu *et al.,* 1998, Maruvada *et al.,* 2003) and signal transduction (Lin *et al.,* 1999, Davis *et al.,* 2000, Lin *et al.,* 2003). Non-genomic actions of TH take place on mitochondria, the actin cytoskeleton, certain cytoplasmic proteins and ribosomes (Davis

et al., 1996). In addition non-genomic actions of thyroid hormone are known to increase activities of Na^+/H^+ exchange (Incerpi *et al.,* 1999), sodium current (Sun *et al.,* 2000), inward rectifying K^+ current (Sakaguchi *et al* , 1996), and Ca^{2+} - ATPase activity (Smith *et al* ,1989). Evidence shows involvement of TH in regulation of mitogen activated protein kinase (MAPK) (Davis *et al.,* 2000), protein kinase C (Lin *et al.,* 1997), and phospholipid dependent protein kinase (Lawrence *et al.,* 1989).

2. Thyroid hormone action in brain: -

Thyroid hormone has genomic and non-genomic actions in the brain. Genomic actions of TH are responsible for early brain development whereas non-genomic actions are mostly found in adult brain (Sarkar 2003, 2004, 2006).

2.1 Genomic action of thyroid hormone in brain:-

Thyroid hormone is important for brain growth during early developmental stages (3rd month of gestation-birth). Cretinism, a disease associated with severe mental retardation, is associated with hypothyroidism during development. Hypothyroid prenatal brain appears to be normal morphologically but the hypothyroid individual suffers from mental retardation, ataxia and spasticity (Anderson *et al.,* 2001). Thyroid hormone binds to a neonatal TR and regulates transcription of genes that are important in neurological development. In the developing brain, thyroid hormone is responsible for the expression of synaptotagamin related gene1 (Srg 1) that mediates synaptic structure and activity (Thompson *et al.,* 2000), cortical cellular size and spacing (Legrand *et al.,* 1976), decreased axonal density and dendritic shaft in Purkinje cells of the cerebellum (Rabie,

1977), and regulation of myelination of nerves by controlling the expression of myelin associated glycoprotein (Rodriguez-Pena *et al.,* 1993).

2.2 Non-genomic actions of thyroid hormone: -

There is some evidence that supports non-genomic actions of TH on neuronal tissues. A study done by Bruno (2003) on rat brain showed that TH acts indirectly on a G-coupled receptor and alters intracellular ATP hydrolysis. ATP is considered an excitatory neurotransmitter in the adult brain. The study showed that the presence of T_4 in synaptosomes from adult hippocampus and cerebral cortex inhibits adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) hydrolysis. The inhibition of ATP hydrolysis by T_4 gives ATP more time for receptor binding and activation.

Submicromolar doses of TH have been showed to stimulate the binding of $[^{35}S]$t-butylbicyclophophorothionate, a ligand that binds the convulsant site of $GABA_A$ in highly washed rat brain membrane, while a higher concentration (10 µM) of TH inhibited the binding of ligand (Martin *et al.,* 1996). Thyroid hormone and pregnenolone sulfate have similar antagonistic effects at the $GABA_A$ receptor. Also, through molecular modeling, it has been shown that T_3 and pregnenolone sulfate have similar structure and volume suggesting a possible shared binding site. (Martin *et al.,* 2004). More work has been done on the effect of T_3 on the recombinant $GABA_A$ receptor expressed in *Xenopus* oocytes. Pharmacological doses of T_3 in the absence of GABA have shown to induce a Cl^- ion current that is similar to that evoked by GABA binding. At lower, physiological

levels, T_3 acts as a noncompetitive inhibitor in presence of a GABA. (Chapell *et al* , 1998). A recent study shows alteration in protein phosphorylation by thyroid hormone *in vitro*. Sarkar (*et al.,* 2006) found that incubation of synaptosomal lysate of adult rat brain with radiolabeled [γ-^{32}P]-ATP in presence of nanomolar concentrations of exogenous thyroid hormone resulted in incorporation of labeled phosphate into synaptosomal protein. Since the synaptosomes have little or no cell nuclear contamination, the effect on phosphorylation must not be genomic.

3. Physiological action of thyroid hormone in adult brain: -

Dratman (1983) did a study on subcellular fractions of brain at different intervals during development of the rat. Radiolabeled [^{125}I] T_3 was injected intravenously into the developing rat and the subcellular fractions were used for study. Higher concentrations of label were detected in the nuclear fraction during development, label shifted to the synaptosomal fractions in adults. The finding suggests that once neurological development has reached an adult stage, thyroid hormone localization changes from nucleus to neuropil in adult brain.

TH is present in the developing and adult brain. TH is released into the blood by the thyroid gland, and is then transported to the brain. Thyroid hormone circulating in the blood must cross the blood brain barrier to enter brain tissue. Autoradiographic study of serial brain sections after intrathecal and intravenous injection of radiolabeled TH showed that thyroid hormone crosses the blood brain barrier and

choroid plexus: cerebrospinal fluid interfaces before reaching other areas of the brain (Dratman *et al.,* 1978).Thyroid hormone circulating in the blood crosses the choroid

plexus barrier to enter cerebrospinal fluid (CSF). Choroid plexus is responsible for the synthesis of the protein transthyretin (TTR). TTR synthesized in the brain appears to be controlled separately from that of liver TTR.TTR helps in the transport of hormone from blood to brain, as free hormone is found in higher concentration of in CSF than in the blood. TTR binds to the hormone and maintains its level (Schreiber *et al.,* 1990). In a recent study done by Palha (2000) on TTR null mice, it was found that TTR might be responsible for TH transport and maintenance in the choroid plexus. Study showed that, TH transport to different part of brain region is TTR independent. Also, TTR is not responsible for maintenance of TH level in the brain.

As mentioned, Dratman (1976) found that TH is concentrated in the cell nucleus of a developing rat brain and is found in nerve terminals or synaptosomes of an adult brain.

The transport mechanism of TH in adult brain was studied by Gordon *et al.* (1999). Gordon used a specific locus coeruleus lesioning agent, N-(2- chloroethyl)-N-2-bromobenzylamine hydrochloride (DSP-4) in her study. She did an immunohistochemical study on rat brain treated with DSP-4, and found that there was a reduction in labeled TH present in the nerve terminals. The loss of labeled TH in the nerve terminals suggests the anterogade axonal transport of TH. Axonal transport is a characteristic route of neurotransmitters.

When lysed by osmotic shock, synaptosomes release thyroid hormone (Dratman *et al.,* 1978). Calcium-dependent release of thyroid hormone from synaptosomes has also been seen once under depolarizing concentrations (Mason *et al.,* 1993). This calcium dependent release was difficult to confirm (Mason, personal communication to J.V. Martin).

Thyroid hormone concentrations present in the terminal and synaptic clefts are uncertain. In peripheral tissues, nuclear bound T_3 comes from the plasma pool whereas the supply of physiologically active hormone T_3 in brain depends mainly on the cellular uptake and intracellular deiodination of T_4. Type II 5' deiodinase (D2) is responsible for intracellular deiodination of T_4 (Crantz *et al.,* 1982).

There is a circadian variation in the level of thyroid hormone concentrations and type II 5' iodothyronine deiodinase (D2) in brain areas. The enzyme activity of D2 increases during the dark period of the day resulting in the biosynthesis of T_3 during the dark phase of photoperiod (Barrios *et al.,* 1997). There is a difference in the concentration level of thyroid hormone in various parts of the brain. Pinna (1999) extracted iodothyronine from up to 11 regions of rat brain and used radioimmunoassay (RIA) for detection of T_4 and T_3. He found that there were high concentration of T_3 (0.5-4 pmol/g of homogenate) from in pituitary, midbrain, and hypothalamus as compared to other brain areas. The level of T_4 (1-4 pmol/g of homogenate) was seen higher in medulla, septum, olfactory bulb and striatum compared to other regions in brain.

Other thyroid hormone metabolites present in the brain regions were 3, 5-T_2, 3, 3'-$T_2,$ rT_3 and T_2S in the femtomolar range. Tissue concentrations of 3, 5 T_2 showed circadian variation closely paralleling those of T_3 in the brain regions (Pinna *et al.,* 2002).

Homeostasis in brain tissue is important for proper functioning of brain. There are numerous signs of nervous dysfunction in both hypothyroid and hyperthyroid individuals. Hence, it is possible that small changes in the amount of TH can produce a significant change in the behavior and nervous system function. Dratman (1983) found that the levels of thyroid hormone in adult brain are resistant to hypothyroidism or

hyperthyroidism. The study suggested that homeostatic mechanisms must be present in the brain that helped in maintaining the concentrations of these hormones in brain. The coordinated regulation of D2 and D3 activities in the brain appeared to help in maintaining neuronal T_3 level under hypothyroidal conditions. In the hypothyroidal situation there was an increase in the activity of D2 which catalyzes the conversion of T_4 to T_3, whereas a decrease in activity of D3. D3 catalyzes the breakdown of T_3 .The coordinated action of these two deiodinase enzymes helped the brain to maintain TH levels (Kundu, 2006). Thyroid hormone from the synaptosome and nerve terminals could stimulate some membrane receptors, suggesting a non-genomic action of thyroid hormone, like a neurotransmitter or neuromodulator (Dratman *et al.,* 1996, Martin, Sarkar, 2003, 2004, 2006). Such a role of TH would explain the need for high levels of homeostatic regulation to maintain levels of T_3 in nerve terminals.

3.1 Influence of thyroid hormone on sleep and electroencephalographic (EEG) measures of brain activity:-

A variety of studies show a relationship between thyroid hormone and sleep. The increased numbers of awakenings during non-REM (rapid eye movement) sleep in the hypothyroid condition in adult rat (Carpenter and Timiras, 1982). Studies in hypothyroid animals have shown a significant increase in the integrated EEG wave amplitude not only in total sleep and non-REM sleep but also in waking (Gull, 1989). Salin-Pascual (1997) found a significant increase in deep non-REM sleep in rats that had been surgically thyroidectomized as compared to sham-operated controls.

Microinjection of T_3 to different brain areas of adult rat was done by Zhang and Martin (2001). WhenT_3 microinjected to the medial preoptic area, an increase in REM sleep was observed. Injections at the median-preoptic nucleus significantly inhibited the non-REM sleep, whereas injections to the diagonal band of Broca did not alter EEG-defined sleep. Sleep-active neurons are GABAergic and may have an opposite effect on the wake-promoting aminergic neurons. (McGinty and Szymusiak, 2003)

Methods

1. Stereotaxic Implantations

For microdialysis studies, the model organisms were young adult male Sprague Dawley rats (Hilltop Lab Animals, Scottdale, Pa). A rat was anesthetized by intramuscular injection of ketamine (70 mg/kg) and xylazine (6 mg/kg) into the gastrocnemius muscle. Once the rat was anesthetized, 0.1ml of atropine (0.54 mg/ml) was injected subcutaneously and rat was placed on a stereotaxic device with that the head was centered. A medial incision was made to expose the cranial surface. Around the edge of the incision, 0.5 ml of xylocaine was injected. A hole was drilled using a 0.0125" bit for implantation of CMA/11 guide cannula on the rat skull. The coordinates for cannula were 0.5mm medial-lateral (bilateral) -0.2mm anterior-posterior and -7.1mm dorsal-ventral with respect to bregma for medial preoptic area. The implanted cannula was encased in the dental acrylic. Rats were checked daily after surgery for 7 days. At this time rats have recovered sufficiently for sample collection.

2. EEG Measurements

For EEG recordings, a hole was partially drilled in each quadrant of the skull surface for placement of four stainless steel screws. Each screw had a 2-3cm length of Teflon coated stainless steel wire attached with an Amphenol socket at the end. Two additional wires with stripped ends were implanted in the dorsal musculature for electromyographic (EMG) signals. The entire wire assembly was connected to a connector and encased by dental acrylic. The edges of wounds were checked daily for

7 days before including rats in the study. For EEG recording, a cable was screwed onto the animal's headset, connecting the implanted electrodes to a multichannel amplifier. Signals from the amplifier are collected on Keithley A/D board and recorded on the hard disk of a computer during the study. During the study three electrophysiological signals (bifrontal EEG, a fronto-occipital EEG and EMG) were displayed on monitor. The spectra obtained during study were later scored using a computer program written by Dr. Martin.

3. Temperature and activity measurements

A Mini-mitter probe was implanted in each rat through a small midventral incision made in the abdominal wall. The Mini-mitter probe monitors body temperature and activity during study. The probe sent a signal which was picked up by the Mini-mitter receivers placed under each rat cage and recorded. Recorded signals were analyzed for activity and temperature data.

4. Collection of microdialysate

Microdialysate was collected using a microdialysis probe. The blocking guide cannula implanted in the rat skull during surgery was replaced by the CMA/11 microdialysis probe, which has a cuprophane membrane. The inlet capillary was attached to a microdialysis pump which pumped artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 0.5 mM KH_2PO_4, 27 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$ and 10 mM glucose; pH 7.2) at a flow rate of 0.16 µl/min for

stabilization and 2µl/min for sample collection. Outlet tubing was attached to a refrigerated fraction collector for sample collection and storage.

5. Analysis of microdialysate

5.1 High Pressure Liquid Chromatography (HPLC)

5.1.1 Preparation of Hormone solutions

3, 3', 5 triiodothyronine sodium salt [T_3] was dissolved in a 50% aqueous methanol-0.5% sodium hydroxide solution.

5.1.2 Instrumental conditions

Microdialysates were analyzed on the ESA CoulArray HPLC. Different compounds present in a microdialysate were separated on a C18 column by linear gradient elution of 20-100% of mobile phase B [acetonitrile: 0.10M sodium phosphate: methanol, 60:30:10(v/v/v) adjusted to pH 3.0], mobile phase A [0.5 M sodium phosphate: methanol, 99:1 (v/v) adjusted to pH 3.0] at a flow rate of 0.5 ml/min. Compounds were eluted at different retention times and the eluted compounds were detected on CoulArray electrodes set at different voltages (0, 180, 240, 360, 620, 680, 740 mV).

5.2 Mass Spectrometry

5.2.1 Preparation of Hormone solutions

T_3 sodium salt was dissolved in 10% (v/v) ammonium hydroxide solution in filtered water.

5.2.2 Instrumental conditions

An Agilent HPLC/Triple Quadrapole Mass Spectrometer (HPLC/MS) system was used for sample analysis. An Eclipse Plus C18 column with dimensions 30 x 2.1mm was used for chromatographic separations using a linear gradient from 100% mobile phase A (0.1% formic acid in water) to 100% mobile phase B (0.1% formic acid in acetonitrile) for a 40 min run at a flow rat of 0.4 ml/min. Mass spectrometry was performed using electrospray ionization at 325° C with 50 psi nebulizer pressure, 10 L/min of drying gas flow, 4000 V capillary potential and 140 V fragmentor potential, at a 25-1500 acquisition mass range. The collision energy for MS/MS was 20 V.

5.3 Radioimmunoassay (RIA)

A TRK31 radioimmunoassay kit (Diagnostic Products Corporation) was used for measurement of free and bound L- T_3 in the microdialysate. The limit for detection for free L-T3 is as low as fM concentrations. For a standard curve, thyroid hormone salt samples were prepared in aCSF.

6. Histology

Each rat underwent microdialysate collection and EEG recording for a given experimental protocol. After the sample collection the rat was injected with 1-2 cc of sodium pentobarbital intraperitoneal and perfused transcardially with phosphate buffered saline (0.9% NaCl) followed by 3.7 % formalin in phosphate buffered saline. For perfusion the right vena cava of sedated rat was cut, a needle was inserted into the left ventricle and heart was flushed with saline until the ears turn white. Saline was

switched to 10 % formalin and flushed until muscle contraction stopped. The rat was then decapitated, the brain removed and stored in the 3.7 % formalin and 10% sucrose solution. Brain sectioning was carried out on a freezing microtome and sections were mounted on chrome-alum coated slides. The slides were stained with cresyl violet. The microdialysis cannula track was then localized by light microscopy, and the information catalogued using a PC.

7. Correlation analysis

Data obtained from all the four rats were analyzed for correlation. To look if there is any correlation present between TH concentration and movement, TH concentration and temperature the data obtained for each rat were analyzed by Graph pad prism.

Other type of correlation was done in which all the four rat's data for TH concentration were normalized to the 1pm-4pm value, this normalized data were then combined and Bonferroni's *post hoc* comparison test was performed. Similarly the temperature and activity data for all the four rats studied. The temperature data were normalized to 1am-am values and activity to 4am-7am values.

Results

The presence of extracellular thyroid hormone in a rat's brain was demonstrated in three different ways. The first method used for analysis of microdialysate from a rat brain was HPLC, which detected the hormone using coulometric electrodes. Different compounds in the microdialysate elute at different retention times therefore giving different peaks in a chromatogram. Peak height is directly related to the concentration of the compound in the sample. The second method for analysis of microdialysate from rat brain was MS, which first separates out different compounds using an HPLC column then fragments the compounds into daughter compounds with different mass-to-charge ratio. The last method for analysis was radioimmunoassay, which gives measurements of thyroid hormone concentration in microdialysate using radioactive iodine on T_3.

1. Detection of thyroid hormone in microdialysate

The presence of thyroid hormone T_3 in microdialysate was determined using a HPLC C18 column by gradient elution. Separated compounds were eluted with different retention times and the eluted compounds were detected on 8 different CoulArray electrode channels. Electrode potentials were set at 8 different voltages (0, 180, 240, 300, 360, 620, 680, 740 mV). T_3 caused a response predominantly on channel 6 (620 mV) (see figure 1). The retention time for T_3 averaged 4.1 minutes. A standard curve for T_3 was used to determine the concentration of T_3 in the microdialysate from rat 5. As a test of peak purity, spiking with excess unlabeled hormone was done. For spiking, 10 µg/ml of T_3 was added to the microdialysate of rat 5. No shouldering was seen in the chromatogram supporting the identity of the peak to be T_3 (see figure 2). Presence of

shoulder indicate that there is a different chemically similar compound present in the sample other than the compound added to spike the sample.

2. Determination of thyroid hormone L-T3 by Mass Spectrometry (MS)

The presence of T_3 in microdialysate was examined using MS. The total ion chromatogram (TIC) of microdialysate and standard T_3 concentration (see figure 3) showed similar results to that described previously for HPLC with coulometric detection (see figure 1). The retention time for T_3 was 4.1 min from the TIC an ion with mass-to-charge ratio of 652 was extracted to obtain the extracted ion chromatogram (EIC). An EIC of mass-to-charge ratio (m/z) 652 (see figure 4) showed a single peak at 4.1 minutes for microdialysate and standard. A full MS scan of compound with m/z 652 having a retention time of 4.1 minutes gave similar spectra (see figure 5) for microdialysate and standard showing m/z of 651.9 in MS. MS/MS on m/z 652 of the 4.1-minute peak gave a matching spectra for standard and microdialysate. Different m/z fragments were obtained by product ion scan (MS/MS) on 652 (see figure 6), which correspond to expected ion fragments for T_3 (i.e. 606.9 and 479).

2.1 Relationship of thyroid hormone, temperature, activity and sleep in rat.

Rat 6

Microdialysis was carried out at a flow rate of 0.16µl/min for 6 hours (11:50 am - 5:50 pm) to equilibrate the rat (rat 6) brain. Actual sample collection was started at an increased flow rate of 2µl/min after the 6 hours of pre-equilibration. Samples collected were at 30 minute intervals for about 1.5 days .The time vs. concentration of T_3, activity

and temperature curve for rat 6 after 6 hours of pre-equilibration is shown in figure 7. Concentration decreased in the beginning from 19.2 nM to approximately 2 nM with variations thereafter. Correlation analysis was done for T_3 with temperature and movement. There was no significant correlation seen between T_3 and temperature (see figure 8).The Spearman r value for correlation was 0.1192 and the P value was 0.4580.T_3 and movement correlation was also non-significant with the Spearman r value = 0.1769 and the P value = 0.2879 (see figure 9).

Rat 8

Microdialysis was carried out at a flow rate of 2 µl/min for 24 hours and 30 minutes (11:00 am -11:30 am) from rat (rat 8) brain at 30 minutes interval. Actual sample collection was without any pre-equilibration. The time vs. concentration, activity and temperature curve for rat 8 (see figure 10) shows T_3 levels in microdialysate. The T_3 concentration started high, at 5.4 nM then decreased and finally leveled out. Correlation analysis was done for T_3 with temperature and movement. There was no significant correlation seen between T_3 and movement. (see figure 11).The Spearman r value for correlation was 0.06368 and the P value was 0.6638. There was significant correlation seen between T_3 and temperature. The correlation gave Spearman r value = 0.3233 and the P value = 0.0266 (see figure 12).

Rat 9

Microdialysis was carried out at a flow rate of 2 µl/min for 840 minutes (11:00 am -12:30 am) from rat (rat 9) brain at 30 min intervals. The actual sample collection was started at an increased flow rate of 2µl/min without any pre-equilibration. The time vs. concentration, activity and temperature curve for rat 9 (see figure 13) showed T_3 levels in

microdialysate. At the beginning there was a decrease in the level of T_3 concentration from 7.6 nM to 1 nM which equilibrates to lower concentration. Correlation analysis was done for T_3 with temperature and movement. There was no significant correlation seen between T_3 and temperature. The Spearman r was 0.06462 and P value was 0.7538 (see figure 14).On the other hand, there was significant correlation found between T_3 and movement. The correlation had a negative slope with the Spearman r value = -0.4422 and P value = 0.0393. (see figure 15)

Rat 10

Microdialysis was carried out at a flow rate of 0.16µl/min for 6 hours (7:38pm-7:38 am) to equilibrate the rat (rat 10) brain. Actual sample collection was started at an increased flow rate (2µl/min) at 7:38 AM. Samples collected were at 30 min intervals for 12 hours (7:38am-7: 38pm).The time vs. T_3 concentration, activity and temperature curve for rat 10 is shown in figure 16. The concentration of T_3 in microdialysate was as higher as 15nM in the starting which gradually decreases over time. Correlation analysis was done for T_3 with temperature and movement. There was no significant correlation found between T_3 and temperature and the Spearman r was -0.2122 and P value was 0.2880 (see figure 17). T_3 and movement showed no significant correlation. The Spearman r value was -0.1784 and P value was 0.3732 (see figure 18).

3. Determination of thyroid hormone L-T3 by radioimmunoassay.

Microdialysis was carried out for 3 hours at a flow rate of 2µl/min after 12 hours of pre-equilibration at a flow rate of 0.16µl/min. Samples were collected at 5 min intervals. Collected samples were pooled to get 10 min collections. Collected samples were

analyzed by radioimmunoassay to get T_3 levels (see figure 19 top graph). During sample collection EEG signals were recorded and scored signals gave a curve (see figure 19 bottom graph) to correlate sleep with T_3 levels. The concentration of T_3 in the microdialysate of a freely moving rat varies over time. The recorded EEG and EMG also show variation in the rapid eye movement (REM) sleep and non-REM sleep cycle over time.

Discussion

1. Thyroid Hormone- Potential Neurotransmitter or Neuromodulator.

The preceding results show T_3 as a potential chemical-like signal in adult brain. A neurotransmitter is a chemical substance that (1) is synthesized in the presynaptic cell body or terminal neuron, (2) transported to nerve terminal through an axonal or anterogade transport (if synthesized in cell body), (3) released from the nerve terminal in the synaptic cleft following depolarization of the membrane in the presence of Ca^{+2} (4) has parallel effects on the membrane receptors of the postsynaptic neurons and (5) gets inactivated by reuptake or enzymatic (proteolytic) breakdown. For T_3 to be a neurotransmitter, it should have all the characteristic a neurotransmitter has. Previous studies have provided evidence for some of the above mentioned criteria

(1) TH has a high concentration in the nerve terminals (Dratman *et al.,* 1982)

(2) T_4 is transported to the brain by crossing blood brain barrier and is concentrated in the brain (Dratman, 1978).TH can be transported by axonal transport to the nerve terminal (Gordon, 1999). The high concentration of 5' deiodinase in the nerve terminal leads to production of T_3 from T_4 (Kundu, 2006)

(1, 5) TH release from the synaptosome is seen by osmotic shock (Dratman, 1978).

(3) There was a calcium dependent release (difficult to confirm) (Mason, 1993).

(4) Several effects of T_3 have been observed on the $GABA_A$ receptor in vitro and there are few findings that show that T_3 alters phosphorylation of several proteins (Sarkar, 2005)

(5) There are deiodinases found in the brain that are responsible for inactivation of T4 and T3 (Kundu, 2006).

However the weakest support in the previous literature was for characteristic (3). My research was specially designed to examine the possible release of T3 from adult rat brain tissue.

2. Presence of thyroid hormone in cerebrospinal fluid.

Exogenous T_3 injected on HPLC column gave a peak with a retention time of 4.1 min. When freshly collected microdialysate was injected, the CoulArray Win program detected and labeled a peak T_3 based on characteristic retention time and maximal electrode response within the array (channel 6, 620 mV) (see figure 1). To further verify that the unknown peak was of T_3, 10 ng of exogenous T_3 was added to the microdialysate (see figure 2). The peak that eluted at 4.1 min was labeled as T_3 by the CoulArray Analysis program. The peak size was bigger compared to peak obtained from microdialysate without any exogenous T_3.The peak grew in size without any shoulder; this supports the conclusion that the existing peak is of T_3. That is, if the peak labeled T_3 was not actual T_3 but some other chemically similar compound, the exogenous T_3 would be likely to have eluted to the left or right of the peak forming a peak shoulder. This was not the case. The HPLC data supported the conclusion that the peak detected in the microdialysate was actual endogenous T_3.

Microdialysate was further analyzed on LC/MS. A 10 ng sample of exogenous T_3 was injected and the major peak eluted at 4.1 minutes. When freshly collected microdialysate was injected, the resultant peak coincided with the major peak of standard T_3 in TIC (see figure 3). A full MS scan was performed for both the standard T_3 and the microdialysate peak eluting at 4.1 minutes. The base peak for both scans was 652 m/z because the instrumental conditions in MS protonate the injected compound, making the

molecular ion T_3H+ (652 m/z) (see figure 5). An EIC at 652m/z was then performed for the standard and microdialysate. The EIC for both chromatograms gave identical results (see figure 4). The 652 m/z peaks for both standard and microdialysate coincided with each other at a retention time of 4.1 minutes. Finally, a PI scan was done for ion at the 4.1-minute peak. The standard T_3 and microdialysate show identical fragmentation patterns and the peaks obtained after fragmentation can be explained to be generated from the parent compound T_3H+ (see figure 6). The major peaks obtained after fragmentation were 651.9, 606.9, 592.6, 507.9, 479,352.9, 380.9, 225.9 and 253.9. The fragment with molecular weight 606.9 would be obtained after decarboxylation of 651.9 molecular weight parent compound. Similarly fragment with molecular weight 592.6 was after the deamination and decarboxylation of T_3H+. The molecular weight compound 507.9 was obtained from the deamination and deiodination of the parent compound. A fragment at 479.9 was generated by the deiodination and decarboxylation of the 651.9 parent compound. On the other hand the decarboxylation and dideiodination of the parent compound leads to production of the 352.9 molecular weight fragment. Similarly, the fragment of molecular weight 380.9 and 225.9 corresponded to the deaminated dideiodinated 651.9 and decarboxylated trideiodinated 651.9 respectively. Similarly, fragment 253.9 was the trideiodinated and deaminated T_3H+. Based on the fragmentation pattern obtained, the LC/MS results showed that the 652 m/z ion is the protonated T_3H+ ion in the microdialysate confirming that the peak obtained is of T_3.

3. Variation in thyroid hormone concentration in cerebrospinal fluid with time.

   Microdialysate samples were collected from four different rats at different circadian times for a duration of 5-24 hours in the preliminary studies. Collected samples were

analyzed on MS for thyroid hormone levels in the microdialysate overtime. Each rat showed moment-to-moment variations in thyroid hormone.

The results obtained from rats with 6 hours pre-equilibration at a flow rate of 0.01 ul/min show more variations in thyroid hormone concentration over time (see figure 7 and 16), whereas the result from rats without any pre-equilibration period show less variation in thyroid hormone concentration over time (see figure 10 and 13). The microdialysate for two rats with pre-equilibration time were collected at different phases of the light-dark cycle. Microdialysate from rat 6 was collected mostly during the dark phase (7pm-7am) whereas samples were collected from rat 10 during the light phase (7am-7pm). Samples collected during the light phase (see figure 16) seemed to show more variation in thyroid hormone concentrations compared to the dark phase (see figure 7).

As this was a preliminary study, some rats have 6 hrs data while others have 24 hrs data. A statistical study was done by combining all the rat data with a 3-hr interval. The statistics show a variation in thyroid hormone concentration with time. More thyroid hormone was found in 10am-1pm samples (see figure 20). The analysis of variance shows that there are significant differences in the T_3 levels by time of day [F = 4.742, d.f 144 (total), 137 (residual), 7 (treatment),]. One caveat is that the results do have high T_3 level at the beginning of each study. This is thought to be an artifact which is caused by probe insertion in the tissue. Furthermore this was a preliminary study with 4 rats studied at 4 different time point and flow rates. However, these studies indicate a variation in the T_3 level over time.

More statistical analysis was done with temperature and movement data for the four rats. The rats' data were combined in a 3-hr interval manner and then the combined data were analyzed for temperature and movement.

1. The statistics show that a significant variation in temperature and movement with respect to time of day.

2. The analysis of variance shows significant variation in temperature over time [F = 109.0, d.f 5759 (total), 5752 (residual), 7 (treatment),] (see figure 22).

3. The results show that there is a peak in temperature at the end of the light phase and at the beginning of the dark phase.

4. The previous study by Roberto Refinetti (1989) demonstrated the body temperature fluctuations. Rats are high at night compared to their body temperature at the day.

5. Our study seems to have some similarity with the Roberto study and with a circadian body temperature rhythm.

6. The study showed more activity at 4am-7am interval, which is consistent with the nocturnal nature of rats.

7. The analysis of variance shows a significant variation in the activity with time [F = 34.86, d.f 5759 (total), 5752 (residual), 7 (treatment),] (see figure 21).

4. Possible relation of thyroid hormone and sleep.

Another study was done with microdialysate collected from rats. Microdialysate was analyzed by radioimmunoassay for detection of T_3 and concurrently recorded EEG and EMG data were scored. The scored sleep study and thyroid hormone concentration over time were plotted against time (see figure 19). In this preliminary study, thyroid hormone concentration appears to be inversely related to the percent sleep. The study shows that higher concentration of thyroid hormone in microdialysate might lower the sleep response in rat. These preliminary studies throw some light on relationship of thyroid hormone and sleep suggesting that thyroid hormone release in brain extracellular fluid might regulate sleep.

In summary, the retention time of the peak obtained from the microdialysate on HPLC matches that of exogenous T_3. Results obtained from LC/MS unequivocally identify the peak generated from microdialysate as that of endogenous T_3. This was proved by TIC and EIC which gave the same retention time for both standard T_3 and the microdialysate peak. The fragmentation pattern obtained by LC/MS clarifies the finding by generating the daughter fragments of T_3H^+. In order to further confirm the previous findings, the microdialysate samples were analyzed by RIA, the analysis supported the previous results. The LC/MS and RIA showed that there was an initial drop in the T_3 level which later on varies over time. The sleep study showed that there was variation in sleep pattern which might possibly be related to the variation in T_3 levels. The 24 hrs study for temperature and more activity for 4 different rats showed that there was a circadian rhythm in these measures. There was higher body temperature and more activity during the night compared to the daytime. The study with T_3 showed more T_3 concentration

during the early light phase. No significant correlation was found for T_3 with temperature and activity except in the case of one rat.

In all, some interesting possible relationships between T_3 release and physiological parameters have been noted and will be the subject of continuing studies.

The presence of thyroid hormone in microdialysate itself is a notable finding, as this has not been previously documented. Variations in thyroid hormone concentration in extracellular fluid could result from a release of thyroid hormone, like a neurotransmitter is released from brain cells. Dratman (1996) observed the accumulation of thyroid hormone in nerve terminals, and Mason (1993) saw the release of calcium dependent thyroid hormone release from nerve terminal once. Based on these data and other data regarding plasma membrane receptor effects(Martin 2004, Sarkar 2006), thyroid hormone could have a neurotransmitter or neuromodulator like action in adult mammalian brain.
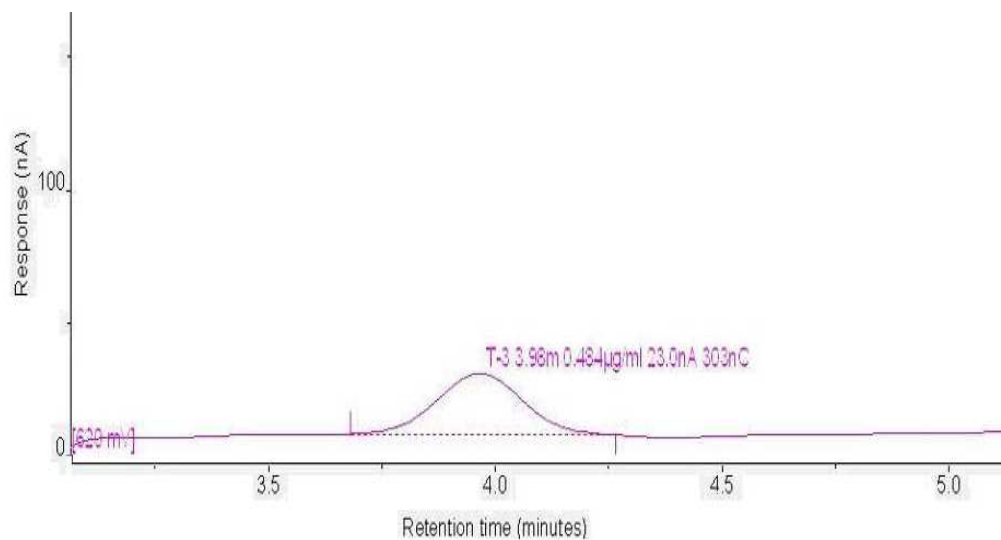
Figure 1- Chromatogram of 10 µl microdialysate from rat 5 at the 620Mv channel.

The microdialysate was run through HPLC C18 column for separation by linear gradient

elution and separated samples were detected at different coulometric electrodes. The peak

of thyroid hormone was obtained at 620 mV channel with a retention time of 3.98

minutes. Axes are expanded to demonstrate the shape of peak.

Chromatography conditions were specified in the methods.

T-3 3.78m 20.2µg/ml 961nA 29.1µC

Response (nA)

500

[620 mV]

0

3.5   4.0   4.5   5.0

Retention time (min)
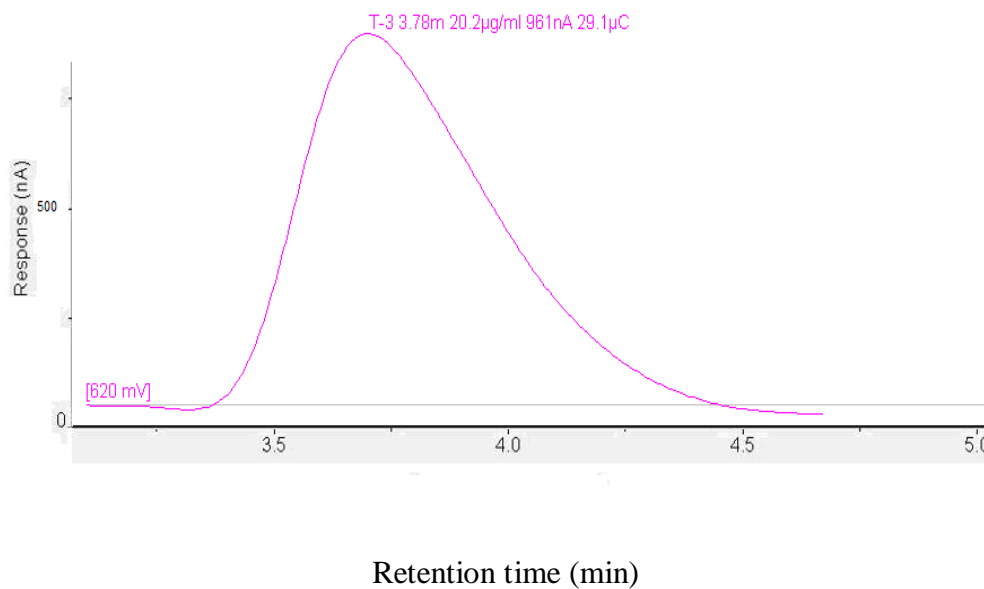
Figure 2- Chromatogram of spiked microdialysate sample from rat 5.

Microdialysate obtained from rat 5 was spiked with additional 10 ug/ml of exogenous L-T$_3$. The peak shown is at channel 6 (620 mV electrode) which is the prominent electrode for L-T$_3$.

Chromatography conditions were as specified in the methods.
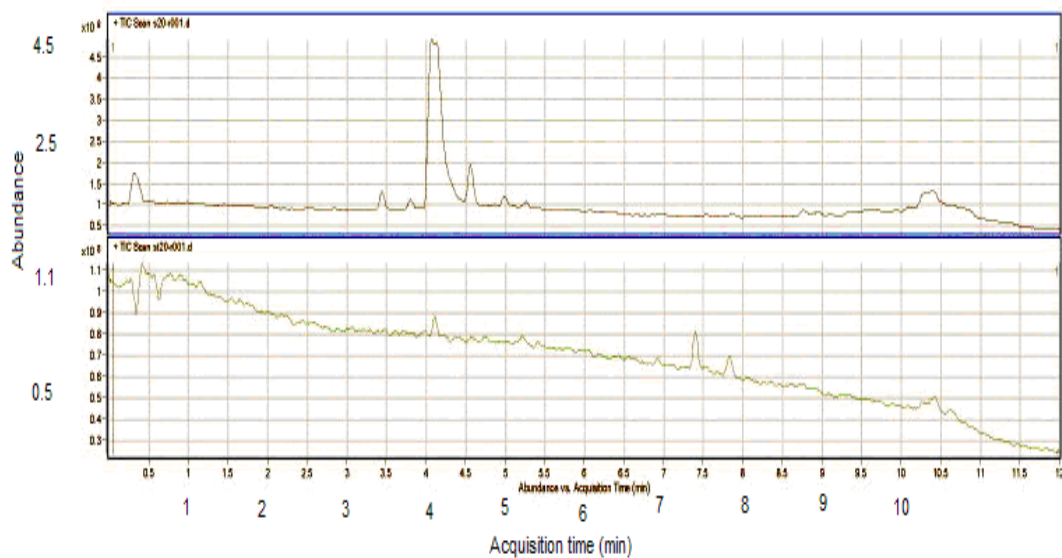
Abundance vs. Acquisition time (min)

Figure 3-Total ion chromatograms (TIC).

The retention time (4.1 min) of the major peak in standard L-T$_3$ (top chromatogram) matched a peak in microdialysate collected from rat brain (bottom chromatogram). Chromatography conditions were as specified in the methods.
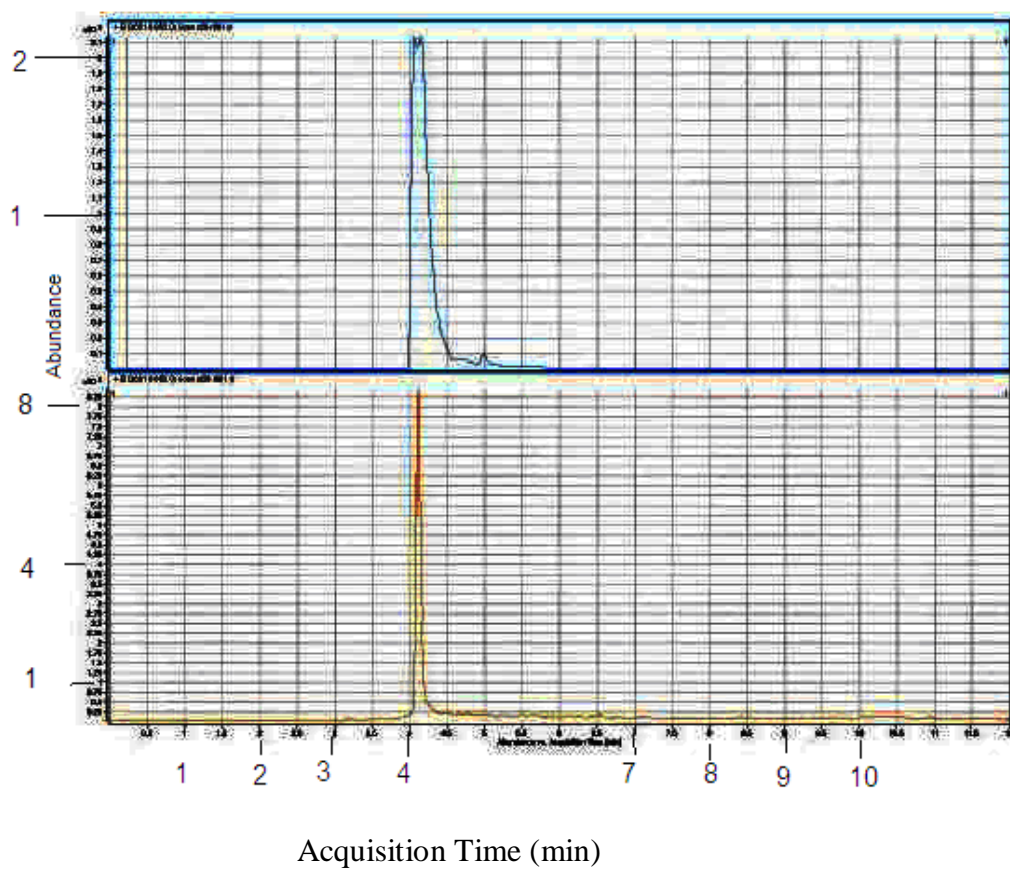
Figure 4- Extracted ion chromatogram (EIC) of mass-to-charge ratio (m/z) of 652.

The peak obtained from thyroid hormone standard (top chromatogram) and the peak of microdialysate from rat brain (bottom chromatogram) has the same retention times. Chromatography and MS conditions were as specified in the methods.

Mass-to-Charge (m/z)
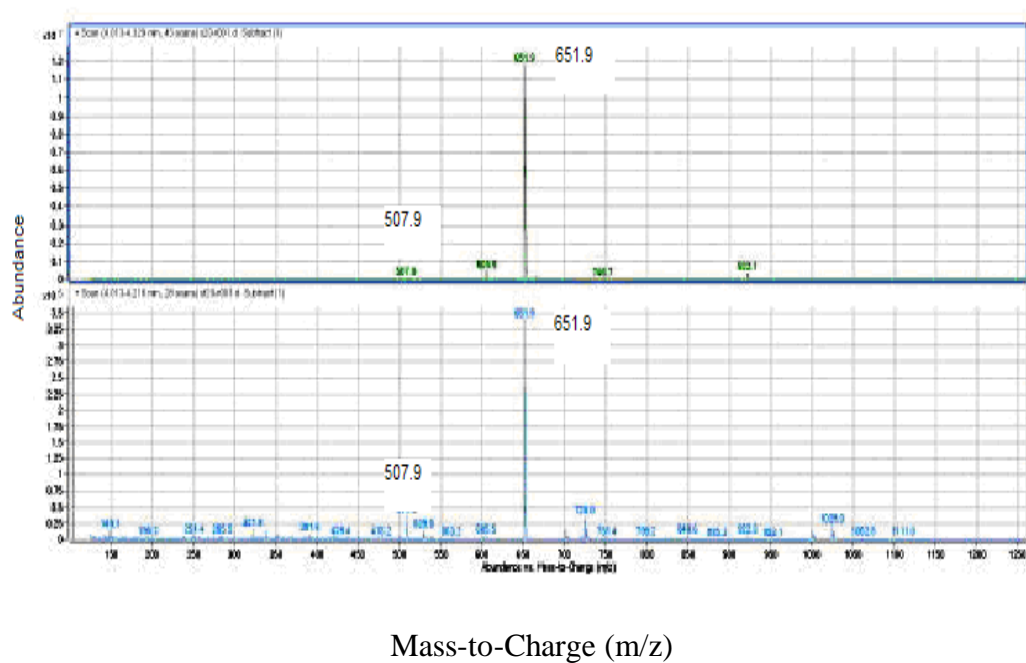
Figure.5- Full Mass Spectrometry (MS) scan of a peak eluting at 4.1 minutes.

Top: Chromatogram of standard thyroid hormone,

Bottom: Microdialysate from rat brain.

MS conditions were as specified in the methods.
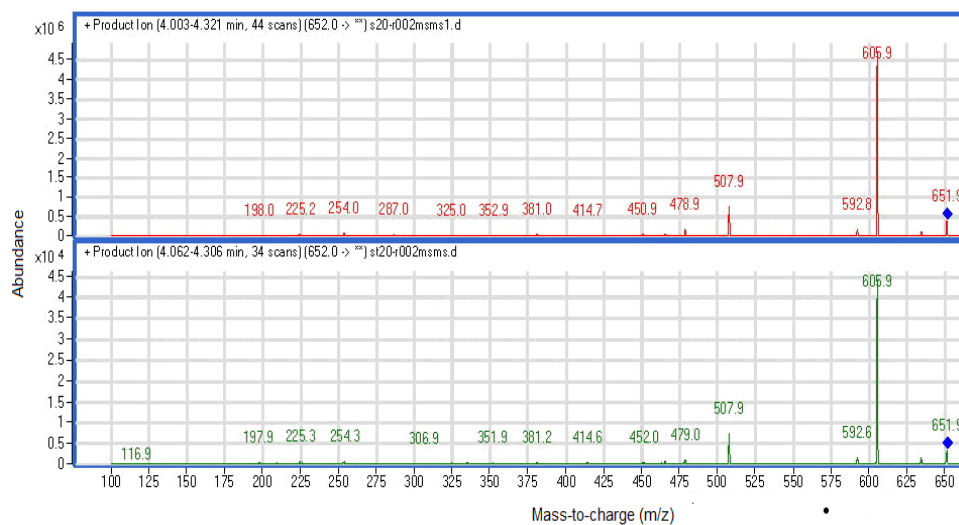
Figure 6- MS/MS on m/z 652 at retention time of 4.1 minutes.

Top: Chromatogram of standard thyroid hormone,

Bottom: Chromatogram of microdialysate from brain.
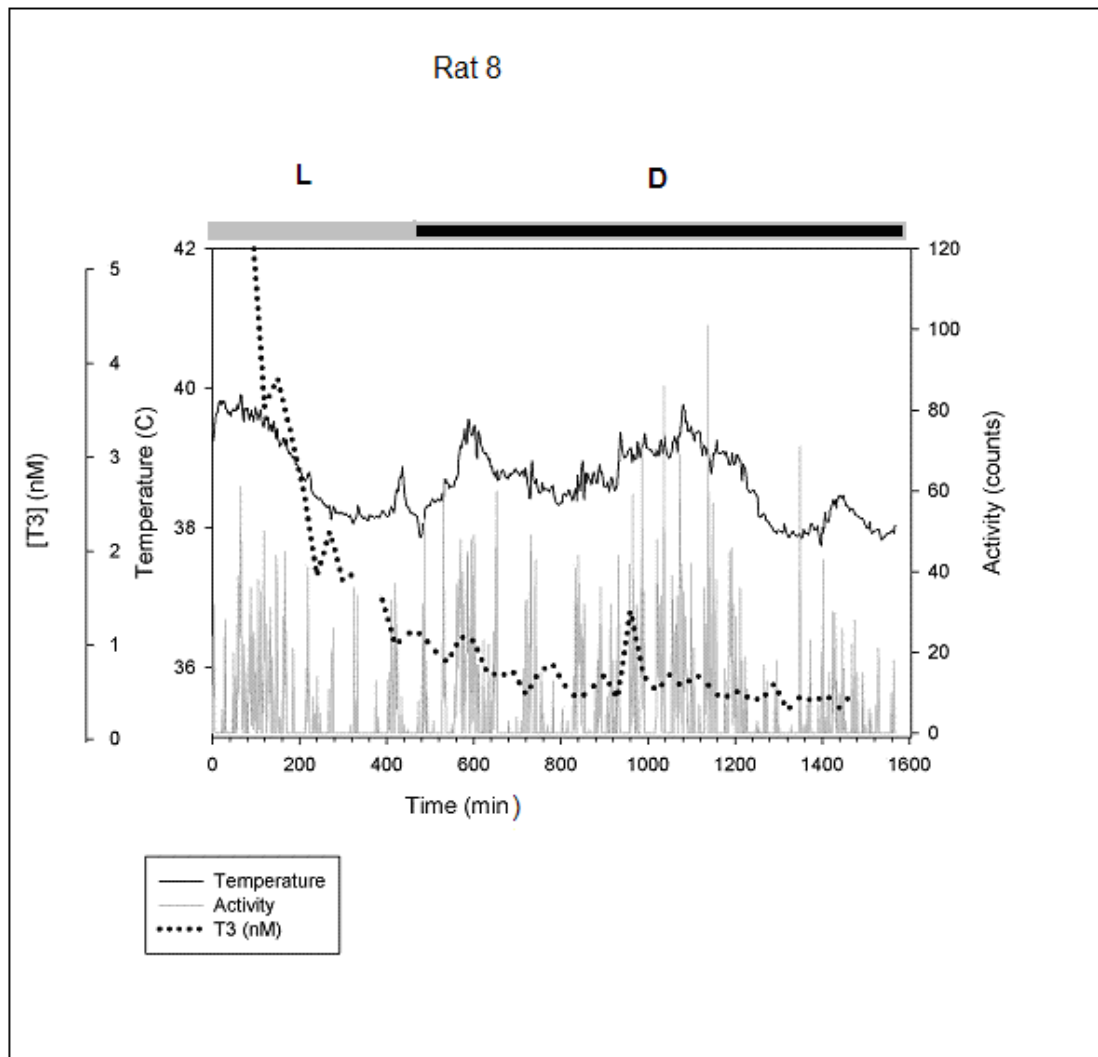
MS conditions were as specified in the methods.
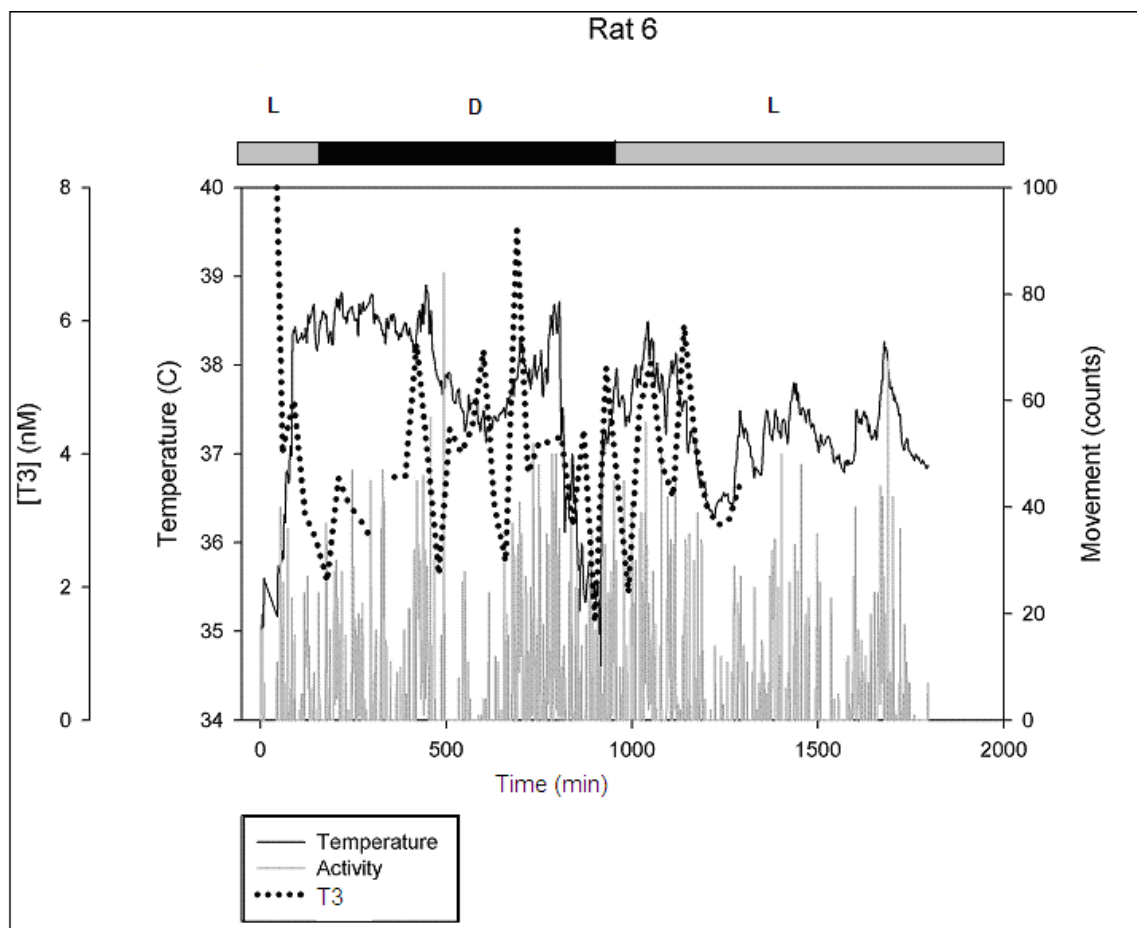
Figure 7- L- T_3 concentration, activity and temperature of rat 6 during microdialysis.

Points represent data collected at a flow rate of 2ul/min after 6 hours of pre-equilibration

at a flow rate of 0.16 ul/min.

D is period of darkness (7 pm-7am).

L is period of light (7am-7pm).
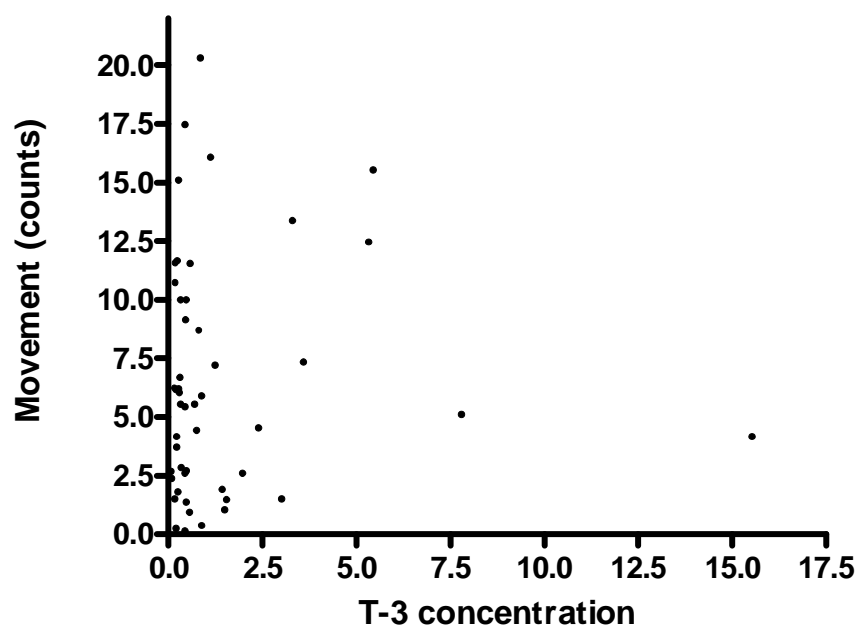
Experimental conditions were as specified in the methods.
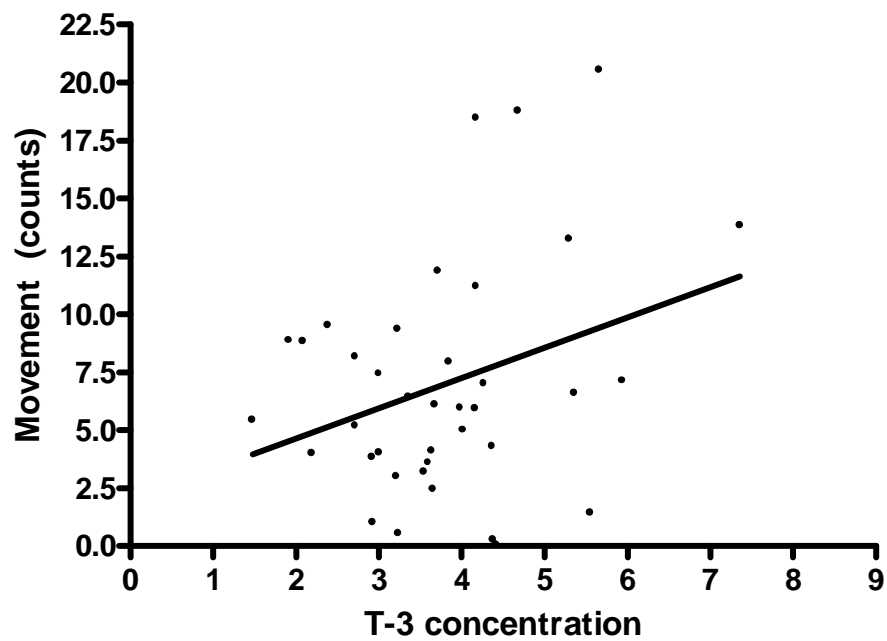
Figure 8- Correlation analysis between activity and T_3 of Rat 6.
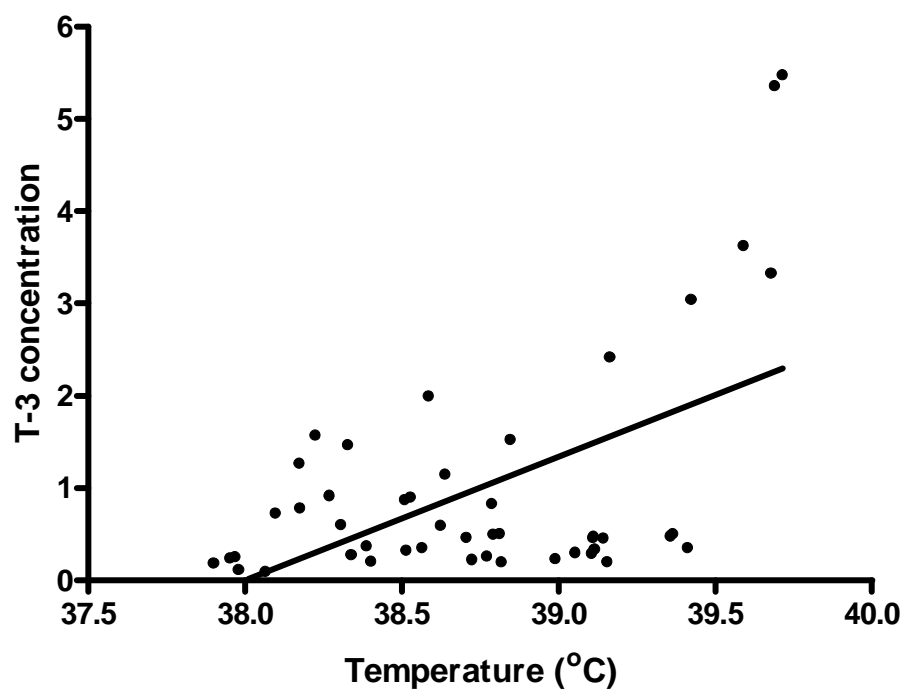
Spearman r = 0.1769, P value = 0.2879
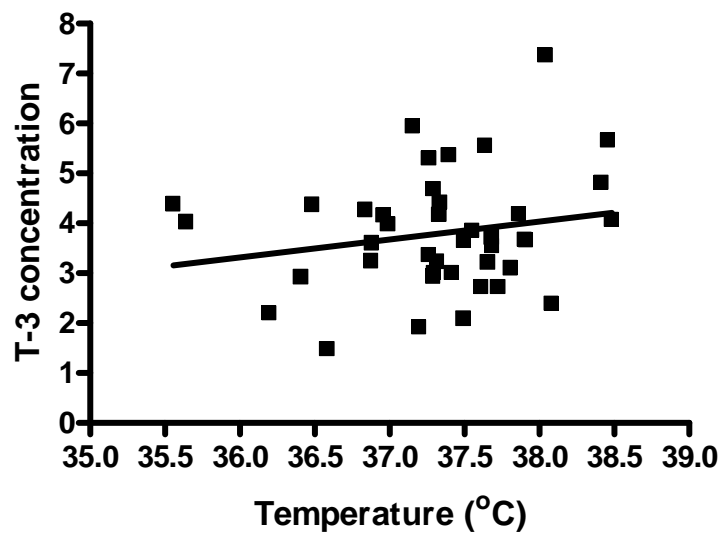
Figure 9-Correlation analysis between temperature and T-3 of rat 6.

Spearman r = 0.1192, P value = 0.4580

Figure 10- Variations in L- T_3 concentration, activity and temperature of rat 8 during

microdialysis.

Points represent data collected at a flow rate of 2 µl/min without pre-equilibration at flow

rate of 0.16 ul/min.

D is period of darkness (7 pm-7am). L is period of light (7am-7pm).

Experimental conditions were as specified in the methods.

Figure 11- Correlation analysis between activity and T-3 of rat 8.

Spearman r = 0.06368, P value = 0.6638

Figure 12- Correlation analysis between temperature and T-3 of rat 8.
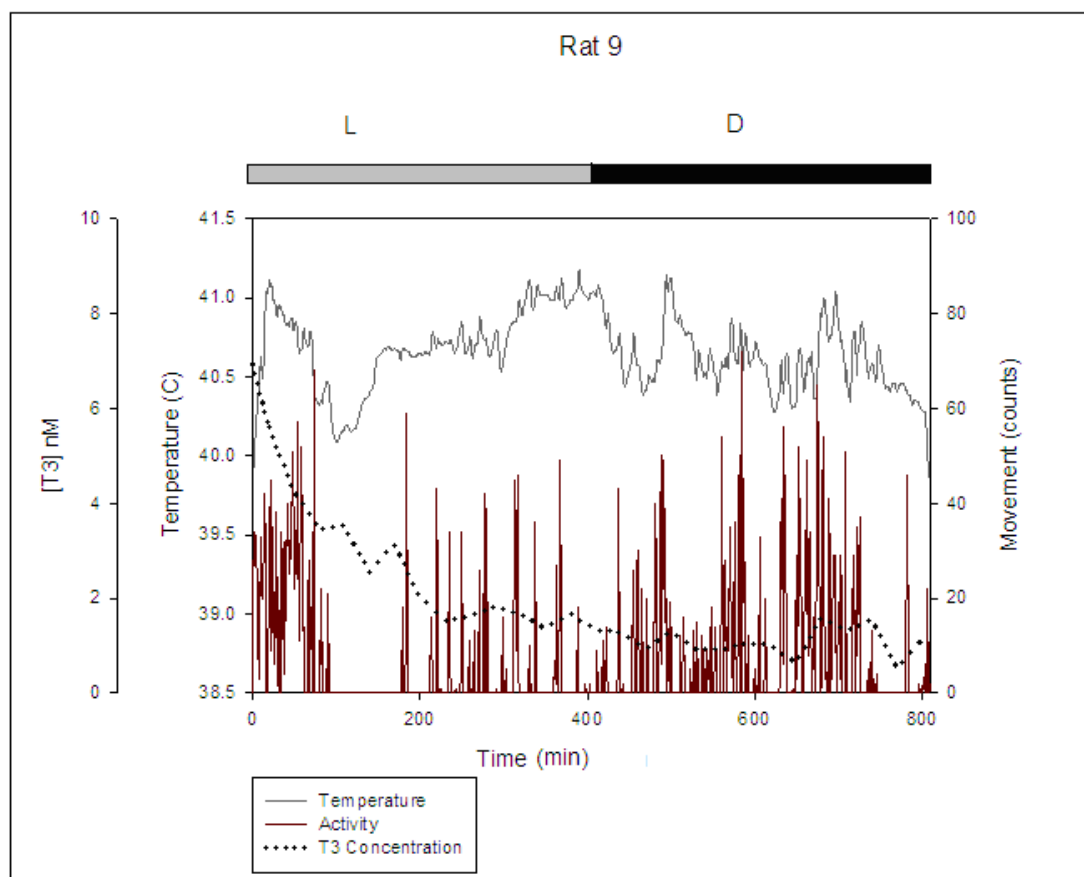
Spearman r = 0.3233, P value = 0.0266

Figure 13- Variations in L- T_3 concentration, activity and temperature of rat 9 during microdialysis.

Points represent data collected at a flow rate of 2ul/min without pre-equilibration at a flow rate of 0.16 ul/min.

D is period of darkness (7 pm-7am). L is period of light (7am-7pm).

Experimental conditions were as specified in the methods.

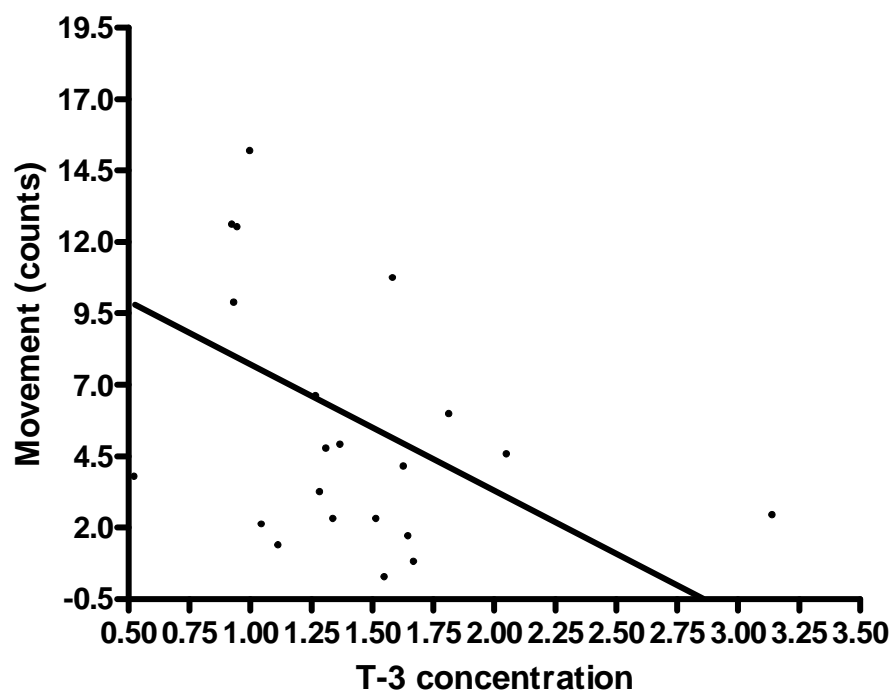Figure 14- Correlation analysis between activity and T-3 of Rat 9.
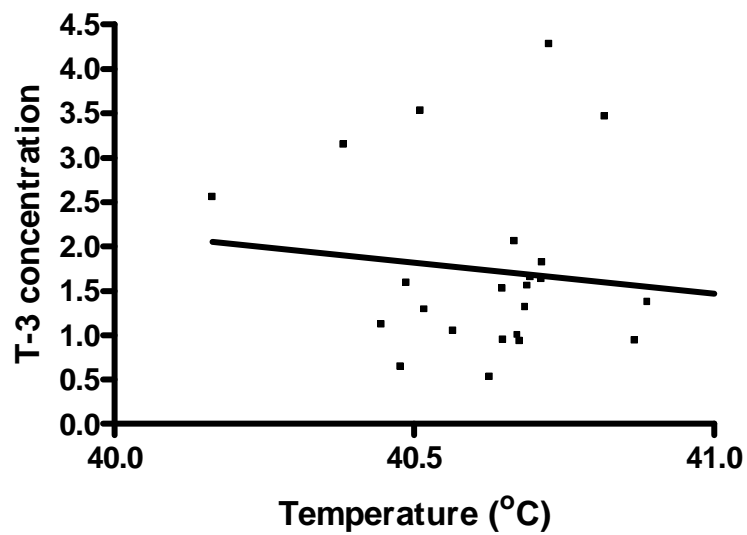
Spearman r = - 0.4422, P value = 0.0393

Figure 15- Correlation analysis between temperature and T-3 of Rat 9.

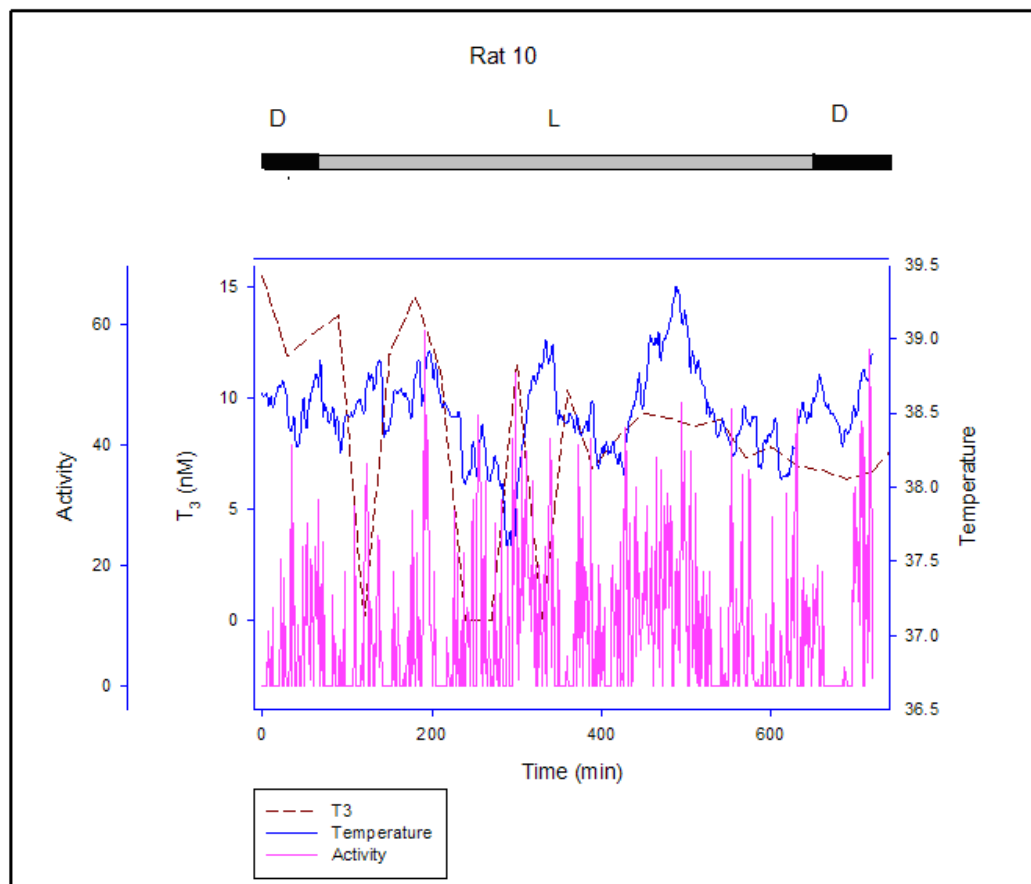Spearman r = 0.06462, P value = 0.7538

Figure 16- Variations in L- T_3 concentration in microdialysate collected from rat 10.

Points represent data collected at a flow rate of 2ul/min after 6 hours of pre-equilibration

at a flow rate of 0.16 ul/min.

D is period of darkness (7 pm-7am).

L is period of light (7am-7pm).

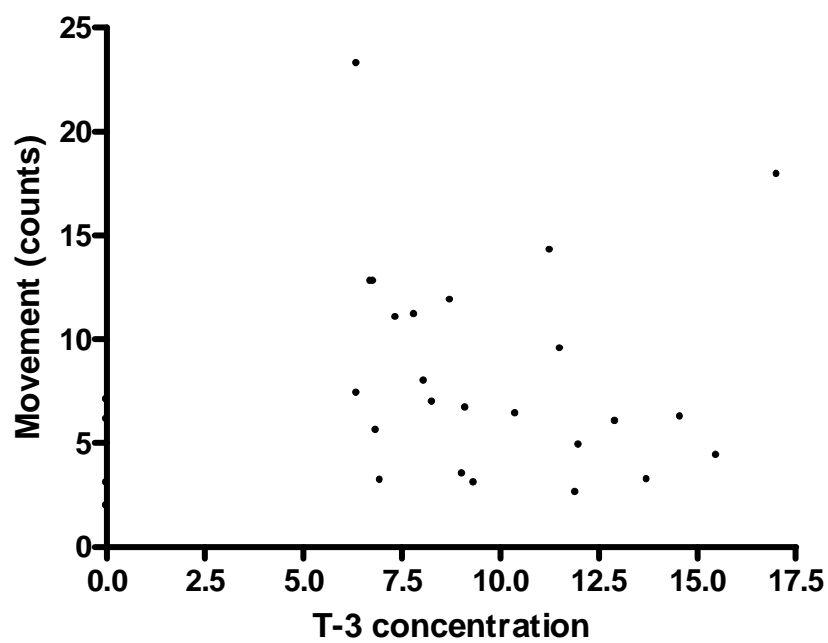Experimental conditions were as specified in the methods.

Figure 17- Correlation analysis between activity and T-3 of Rat 10.
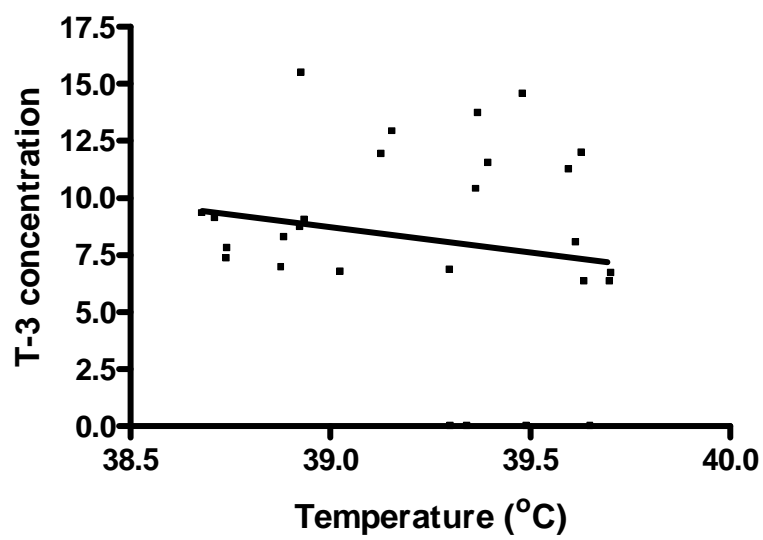
Spearman r = - 0.1784, P value = 0.3732

Figure 18- Correlation analysis between temperature and T-3 of Rat 10.

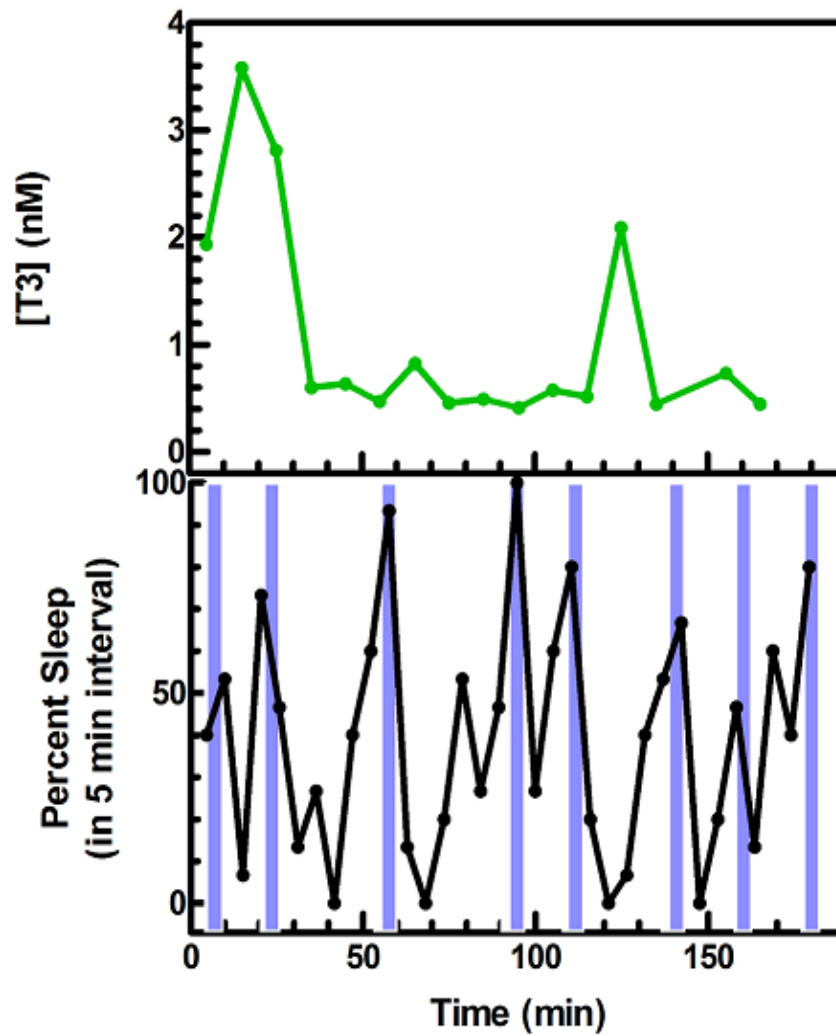 Spearman r = - 0.2122, P value = 0.2880

Figure 19- Determination of L- T_3 in microdialysate from rat and concurrent EEG-defined sleep and waking as a function of time.

Top graph: Concentration of L- T_3 in microdialysate (5 minutes fraction over a 3 hour period).

Bottom graph: Percent of epochs scored as REM and NonREM sleep in 5 min intervals in same rat.
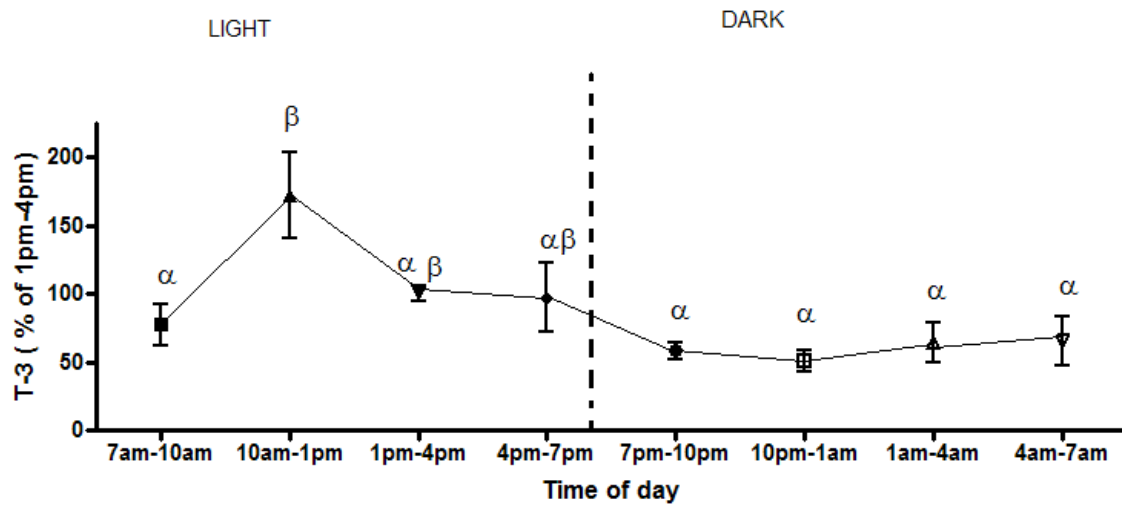
Figure 20- Combined T$_3$ vs. time of day.

Points with different symbols (α, β) at the top are significantly different from each other at $p < 0.05$ by Bonferroni's *post hoc* comparison.

Figure 21- Combined activity vs. time of day.

Points with different symbols (α, β) at the top are significantly different from each other

at $p < 0.05$ by Bonferroni's *post hoc* comparison.

Figure 22- Combined temperature vs. time of day.

Bars with different symbols (α, β, δ, ε) at the top are significantly different from each other at p < 0.05 by Bonferroni's *post hoc* comparison.
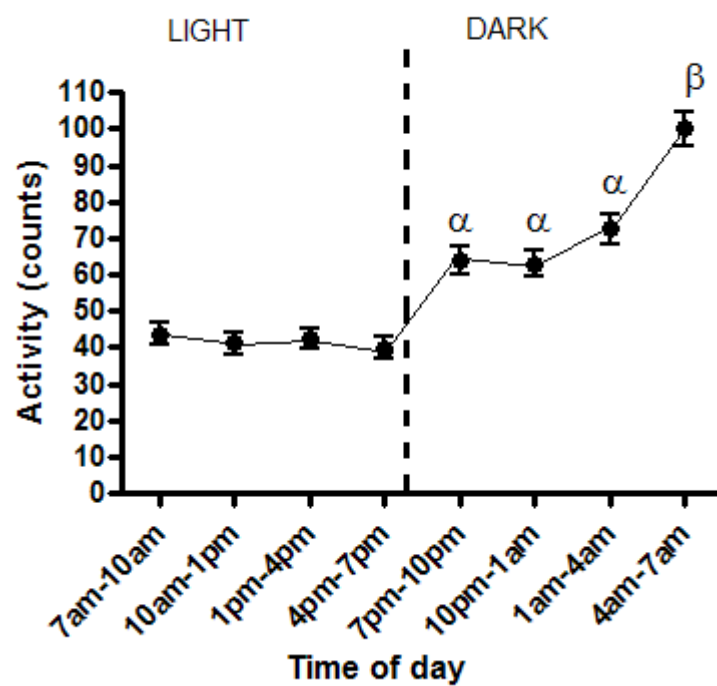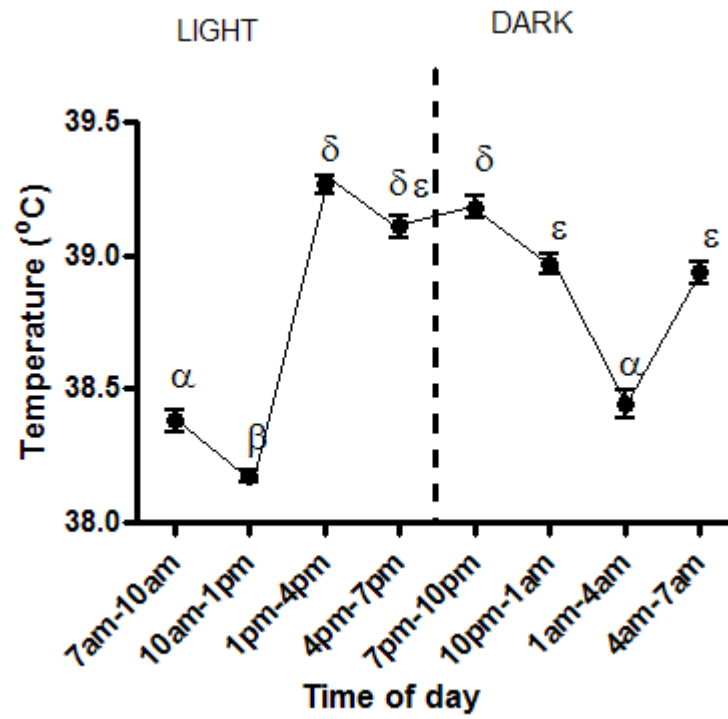
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