Effects of acute microinjections of thyroid hormone to the median preoptic nucleus of adult male rats on total sleep, loco-motor activity, and core body temperature

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ABSTRACT OF THESIS

Effects of acute microinjections of thyroid hormone to the median preoptic nucleus of adult male rats on total sleep, loco-motor activity, and core body temperature

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One clinical effect of dysthyroidism is often the disruption of normal sleep. The hypothalamus, more specifically the Median Preoptic Nucleus (MePO) contains highly restricted neuroanatomical clusters of sleep-active neurons. These neurons express $GABA_A$ receptors, which have previously been implicated in the onset and maintenance of sleep. T_3 has been shown to inhibit the actions of benzodiazepines in electrophysiological experiments. The current experiment served to investigate possible non-genomic action of T_3 microinjections to the MePO on sleep behavior over a series of 48-hour intervals, comprised of one 24-hour control and one 24-hour experimental interval. Injections to the MePO, of varying doses of T_3 caused significantly different response effects on REM, non-REM, core-body temperature, and locomotor activity. The date indicated that T_3 at 1 µg and 3 µg T_3 injections caused a significant decrease in Total Sleep (TS) ($P<0.0001$ and $P< 0.0001$ respectively). T_3 yielded a significant decrease in non-REM sleep (NREMS) for the doses of 1 µg, and 3 µg injections of T_3 ($P <0.0001$ and $P<0.0001$ respectively along with an increase in REM sleep (REMS) in doses of 1 µg, and 3 µg T_3 injections ($P<0.03$ and $P<0.0001$ respectively) within 24 hours of injection. These effects may be due to possible neurotransmitter or neurosteroid-like properties of T_3 in the brain, or they may be from inhibitory protein phosphorylation effects found in previous experiments. Since the effects of T_3 were demonstrated after acute injections, the data is consistent with possible non-genomic actions of thyroid hormones in the adult brain.

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Introduction

A primary clinical observation of hypothyroid and hyperthyroid adult patients is disturbed sleep. [71,73] Clinicians observed that hypothyroid patients complain of lack of sleep.[56,70] A major symptom of hypothyroidism is hypersomnolence[50] in some patients.[19] According to health-related quality of life (HRQL) evaluations of different treatments for patients with thyroid disorders. Questions still remain as to which treatment for thyroid disease is most effective. [68] Patients with treated thyroid disease also mentioned suffering from a wide variety of symptoms including disturbance in sleep.[5,11] Generally, electroencephalographic (EEG) studies have confirmed a difference in sleep patterns during thyroid disturbance in humans and also after experimental manipulation of thyroid function in animals. However, the details do not follow a general consensus.[7,16,17,30] Patients with hyperthyroidism showed a decrease in non-REM sleep (NREMS), particularly stages 3 and 4.[71,73] Rats which were rendered hypothyroid with treatment by 6-n-propyl-2-thiouracil (PTU) showed significant increases in integrated EEG wave amplitude in total sleep (TS), NREMS, and wakefulness compared to euthyroid rats.[25] One of the major systemic effects resulting from prolonged deprivation of sleep in animals is lower blood circulation of thyroid hormones in the hypothalamus[g]. Rats with chronic sleep deprivation have consistently exhibited a drop in thyroid hormone levels.[4,20,21].

The effects of thyroid hormones in adults are noticeably distinct from the effects on development. It has previously been noted that postnatal rats show an increasing concentration of L-triiodothyronine (T_3) within synaptic terminals (more rapidly than the accumulation of synaptosomal protein) accompanied by a decrease in the concentration of thyroid hormone in neuronal nuclei.[15] The location of T_3 accumulation following peripheral administration was the

noradrenergic centers where norepinephrine co-localizes.[14] Axoplasmic transport delivers T_3 through the medial forebrain bundle to the adrenergic projection sites. When L- thyroxine (T_4) is administered intravenously it enters specific regions of the rat brain by a saturable mechanism and it's concentration increases over time in synaptosomal fractions. [14] Once secreted from the thyroid gland, T_4 is deiodinated in nerve terminal fractions by brain enzymes 5'D-II and 5D-III[12,13,24,67] Within the brain, 5'D-II activity increases during the dark cycle, indicating a potential link to circadian processes, such as locomotor activity and sleep.[6]

The majority of thyroid hormone in blood plasma is bound to albumin, thyroxin binding globulin, or transthyretin, with the remainder, which is a small amount, circulating freely as T_3 and T_4.[26] Specific transporters such as Monocarboxylate Transporters (MCT)8, MCT10, and Organic Ion Transporter 14 (OATP), which have been characterized in the adult mammalian central nervous system and blood brain barrier are important in allowing thyroid hormone to actively cross the cell membrane.[67] Since T_3 is a derivative of a hydrophilic amino acid tyrosine, the formal charge on the amino acid would seemingly not allow it to cross the hydrophobic core of the plasma membrane and has been confirmed experimentally.[26] The adult brain resists hyperthyroidism and hypothyroidism, even when the rest of the body has abnormal levels of thyroid hormone.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and exerts its effects mainly through the $GABA_A$ receptor.[8] This receptor consists of five subunits, the most common stoichiometric combination being the $_1$: $_2$:$_2$ subunits.[48] In the hypothalamus, the site of many sleep-regulatory nuclei, the $_1$ $_2$ $_2$ subunit is commonly

expressed.[55,72] The $GABA_A$ receptor complex is one of the major cellular sites for the action of hypnotic drugs. Sleep in rats has been shown to be influenced by agonists, antagonists, and inverse agonists of the $GABA_A$ receptor.[36,52] Inverse agonists bind to the same binding site on a receptor as an agonist and cause the reverse of the constitutive response of the receptor.[28] More specifically -carbolene-3 carboxylate-t-butyl ester (CCT) is an inverse agonist with the ability to cross the blood brain barrier. It binds to the $GABA_A$ receptor at the benzodiazepine site and decreases sleep.[43]

Intraperitoneal (IP) administration of gaboxadol, a $GABA_A$ agonist, has recently been found to increase NREMS duration and enhanced delta activity (0.5-4 Hz) during the light (resting) period or in the dark (active) period.[32,33] GABA agonists such as muscimol (which binds to the GABA site) and midazolam (which binds to the benzodiazepine site) were shown to have varying effects on sleep indicating that activation of different sites on the $GABA_A$ receptor complex affect the state of sleep and sleep EEG distinctively[32]. A neurosteroid known to interact with the $GABA_A$ receptor, 5 -pregnane-3 , 21-diol-20-one (3 ,5 -THDOC), had potent dose-dependent positive effects on the initiation of sleep and sleep maintenance.[43] Hypnotic effects have also been induced by progesterone and its derivatives[34,35] The GABA-negative neurosteroid pregnenolone sulfate also affects sleep.[10,12] Picrotoxin which is a $GABA_A$ antagonist that binds near the Cl- channel significantly decreases NREMS and increases wakefulness when microinjected into the Medial Preoptic Area (MPOA). Neurotransmission by GABA in the MPOA is spontaneously active in the regulation of hypnotic function which includes REM and NREMS and more specifically is mediated by the $GABA_A$ receptor.[1]

$GABA_A$ receptors have been found to be influenced by low micromolar or high nanomolar doses of thyroid hormones on several measures of receptor binding and activity.[3,50] There are at least two different sites in synaptosomes to which L-T_3 specifically binds *in vitro*.[46,47,63] One or both of these sites may be responsible for modulatory effects on neurotransmitter receptors. TH's effect on membrane receptors resembles the actions of GABA-negative neurosteroids.[2] In a recent study, the injection of varying doses of T_3 and T_4 greatly decreased the currents due to the effects of N-methyl-D-aspartate on hippocampal (NMDA) receptors which suggests another example of a non-genomic action of thyroid hormones.[39] *In vitro* studies of rat brain synaptosomal lysates treated with nanomolar concentrations of L-T_3 showed phosphorylation of proteins within minutes.[58] Some endogenous steroids have GABA positive effects on $GABA_A$ receptor function while others are GABA negative and inhibit its function as Thyroid Hormones do at micromolar concentrations.

Two-electrode voltage clamp experiments showed that L-T_4 and L-T_3 greatly reduced the Cl^- currents in specific subtypes of $GABA_A$ receptor subunits expressed in Xenopus oocytes.. The $_1{}_2{}_2$ subtype had a 50 percent reduction in GABA stimulated Cl^- current when treated with 20 M concentration L-T_3. However receptors comprised of the $_6{}_2$ subtype did not express any sensitivity to the hormone. The $_1{}_2$ subtype did show sensitivity to the TH receptor, suggesting that a gamma subunit is not required for the effects of TH. The rapid effect of thyroid hormone on the activity of the $GABA_A$ receptor *in vitro* is an indication of thyroid acting in a non-genomic manner[8] rather than a long-term slower-acting genomic mechanism. These *in vitro* studies have shown the non-genomic activity of thyroid hormone indisputably, due to the fact that none of the synaptosomal fractions contained cell nuclei.[60,64] $GABA_A$ receptors, more

specifically the ones comprised of the $_1$ $_2$ $_2$, subunits are sensitive to both T_3 and the inverse agonist CCT, which was mentioned above to decrease sleep. Since the $GABA_A$ receptors are expressed in important sleep-regulatory regions in the preoptic area, it is possible that T_3 could also disrupt sleep.

A key brain region in the circuitry of sleep regulation is the preoptic area.[58] Highly restricted neuroanatomical clusters of sleep-active neurons have been demonstrated in the MePO and ventrolateral region of the preoptic area.[22,66] GABAergic neurons are known to be expressed in the median preoptic nucleus (MePO).[49] These GABAergic neurons have generally reciprocal connections with wake-promoting aminergic systems such as the locus coeruleus (LC), tuberomammillary nucleus (TMN), and dorsal raphe nuclei (DRN).[49] Hypocretin, also known as orexin, is produced by a group of wake-active neurons clustered in the perifornical region of the lateral hypothalamus (PFLH).[57] The lateral hypothalamus has reciprocal connections with the sleep-active preoptic area nuclei.[23,56]

Studies of the anatomical specificity of the effects of $GABA_A$ receptors were performed using triazolam, a highly potent benzodiazepine hypnotic drug.[43] Special sensitivity to hypnotic effects of triazolam microinjections at low amounts (0.25 g and 0.50 g) were demonstrated in the medial preoptic area (MPOA) without any significant changes in core body temperature, which suggests that $GABA_A$ receptors in this brain region play a key role in the mechanism of sleep regulation and further confirming that the hypothalamus plays a key role in regulating waking and sleep.[51,53] Triazolam, when injected into the DRN, significantly increased wakefulness in rats, which is congruent with previous studies on the effects of GABA agonists

and benzodiazepines at this site. The effect of triazolam was most noticeable in decreasing NREMS and increasing sleep latency within two hours of injection.[53]

The aim of this study was to further explore the relationship between sleep, T_3, and the MnPO. A preliminary study [73] found a significant inhibition in NREMS from the acute microinjection of 2 and 4 g doses of T_3 to the MnPO. The acute effects of T_3 were examined as it would imply a non-genomic mechanism of action.

Materials and Methods

Subjects

Seven male Sprague-Dawley rats ranging from 250-300 g, purchased from Hilltop Lab Animals (Scottsdale, PA) were housed individually in shoebox cages. After surgical implantation of a microinjection guide cannula an electroencephalographic (EEG) and electromyographic (EMG) electrodes, the rats were given at least one week to recover. During the recovery week, rats were inspected to be sure the cannulae were clear and the incision completely healed prior to use in the study. The rats were housed in the chambers for 24 hours before an experiment test. The temperature was maintained at 22.2-23.3°C and relative humidity was controlled at 55%. The lights were on from 7:00 AM until 7:00 PM and off from 7:00 PM until 7:00 AM. The rats were handled for at least 5 days before use to reduce the effects of stress. The procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Rutgers University.

Cannula and electrode implantation

Rats were first positioned in an induction chamber primed with 5% gaseous isofluorane at a flow rate of 2 LPM for 5 and then placed in a stereotaxic device on a heated platform maintained at 37ºC to prevent hypothermia. A midline scalp incision was made, and the skull was cleared of skin and muscle to expose the dorsal-lateral flexures approximately 0.5 cm

anterior of and 1 cm posterior of bregma. Using a 0.125" drill, holes were made in the skull according to the medial-lateral (ML), anterior-posterior (AP) and dorsal-ventral (DV) coordinates of the desired locus with respect to bregma (+0.5 mm ML, -0.2 mm AP, and -7.1 mm DV for MePO). Two 23 ga stainless steel, 1.2 cm long cannulae suspended adjacent to one another were stereotaxically lowered below the surface of the skull according to the DV coordinates of the desired site of the cannulae and cemented in place with a small amount of dental acrylic. A hole was partially drilled in each quadrant (with respect to bregma) of the skull surface for placement of four 0-80 stainless steel screws to be used as EEG electrodes. Each screw was attached to a 4-5 cm length of 0.010" Teflon coated stainless steel wire with an Amphenol socket at the end. The stripped ends of two additional wires were implanted in the dorsal neck musculature to provide EMG electrodes. The exposed ends of these wires were stripped 1 mm and soldered to an Amphenol socket. The entire assembly of cannulae, electrodes and sockets was encased in dental acrylic and the injection guide cannulae were blocked with stylets. Bacitracin was applied topically to the periphery of the incision.

Implantation of E-Mitter Transponder

An E-Mitter Transponder (MINI-MITTER) was implanted through a small midventral incision in the abdominal wall to monitor locomotor activity and core body temperature. A difference in signal strength from the Mini-Mitter indicated movement of the rat which was calculated as activity counts per minute. Temperature was measured by a thermistor which controlled an oscillation circuit. The frequency was related to the temperature of the thermistor. The muscle layer and the skin were closed with vicryl sutures and bacitracin was applied

topically to the wound. The edges of the wound were checked daily for 7 days, at which time rats were healed adequately to be included in the study.

Experimental Procedures

Each rat was tested four times for two consecutive days at weekly intervals in a randomly assigned order of treatment conditions. On Day I (baseline), 0.25 µl of a vehicle solution (30% 0.2 N NaOH diluted with 70% Phosphate Buffered Saline [PBS]) with a pH of 7.4 was injected bilaterally. On Day 2 (experimental), 0.3, 1, 3 or 10 µg of L-3,3'5-triiodothyronine (T_3) dissolved in vehicle solution immediately before use and injected bilaterally. While the rat was quietly restrained, the stylet was replaced with a 26 ga inner cannula extending precisely 1 mm past the end of each of the bilateral guide cannulae. Injections were made at a rate of 0.25µL/min in each cannula for a total of two minutes (0.5 µL). After each injection, the cannula was left in place for an additional 30 seconds to prevent backflow.

Each weekly test was initiated at approximately 9 AM, 2 hours after the beginning of the light phase. Immediately following the injections, each rat was returned to its isolation chamber. The implanted electrodes were then connected to a multichannel amplifier via a shielded cable. This amplifier fed into a Keithley A/D board and the signals were recorded on the hard disk of a Pentium-based computer. EEG, EMG, locomotor activity and core body temperature were monitored for twenty-four hours following the injections The electrophysiological signals, including a bifrontal EEG, a fronto-occipital EEG, and an EMG trace for each rat, were monitored on the display at that time to ensure data was properly being collected. At a later date,

the files were replayed in 30-second intervals for each rat, and a trained investigator, who was unaware of the rat's treatment condition, assigned a stage of consciousness (wakefulness, NREMS or REMS) to each interval according to standard criteria using Spike2 Score software. The core body temperature and locomotor activity were recorded by Mini-mitter receivers under each rat, then registered by the VitalView software program in 1 min-intervals.

Histology

After the completion of data collection and experimentation, the rats were injected with pentobarbital and perfused with formalin to obtain the brains for histology. The location of all probes and cannulae were verified by viewing 40 m histological sections (Figure 6) obtained from a freezing sliding microtome under a light microscope as compared with a photographic atlas of the rat brain.[29]

STATISTICAL ANALYSIS

Statistical analysis was based on 2-way analysis of variance (ANOVA) for effects of dosages and time after injection using GraphPad Prism software. Data analyzed included injection of T_3, effect of time, stage of consciousness, TS, core body temperature, and locomotor activity. All measured data were expressed as the means over each 2-hour interval. Sleep was measured in terms of response according to time and dose against a pooled control. Core body temperature and locomotor activity were analyzed over time against the particular control day.

Results

Effect of T_3 administered to the Median Preoptic Nucleus on Total Sleep (TS)- Figure 1A-D

The effect on TS of 1 g and 3 g T_3 injections (Fig. 1B, and 1C respectively) administered to the MePO were statistically significant ($F_{1, 176} = 32.16$, $P<0.0001$; $F_{1, 204} = 19.31$, $P<0.0001$ respectively). The injection of 1 g T_3 consistently lowered TS compared to injection of vehicle solution over 24 hours. The injection of 3 g T_3 yielded lower periods of TS from two to sixteen hours. From sixteen hours on, the injection did not appear to be as significant. The effect of time was significant for both 1 g and 3 g T_3 injections ($F_{11, 176} = 2.15$, $P<0.02$; $F_{11, 204} = 2.06$, $P<0.03$ respectively), with less sleep during the dark phase than during the light phase. However, the interaction of time and T_3 injection was not significant for 1 g and 3 g T_3 doses ($F_{11, 176} = 0.34$, $P<0.98$; $F_{11, 204} = 0.63$, $P<0.96$ respectively).

No statistically significant effect on TS was observed when 0.3 g and 10 g T_3 injections (Fig. 1A, and 1D respectively) were administered to the MePO ($F_{1, 163} = 2.53$, $P<0.12$; $F_{1, 176} = .0001$, $P<1$ respectively). After the 0.3 g T_3 injection, there was a trend of less TS than the vehicle solution over 24 hours. The effect of time was significant for the 10 g T_3 injection ($F_{11, 176} = 2.07$, $P<0.0006$), but not for the 0.3 g T_3 injection ($F_{11, 163} = 1.40$, $P<0.0006$). No significant interaction between T_3 administration and time was observed for both 0.3 g and 10 g T_3 injections ($F_{11, 163} = 0.09$, $P=1$; $F_{11, 176} = 0.23$, $P<1$ respectively).

Effect of T_3 administered to the Median Preoptic Nucleus on non-REM Sleep (NREMS) Figure 2A-D

The effects of 1 g, and 3 g T_3 (Fig. 2B, and 2C) injections to the MePO were significant on NREMS ($F_{1, 176}$ = 31.22, P<0.0001; $F_{1, 204}$= 59.08, P<0.0001 respectively). The 1 g and 3 g T_3 injections resulted in less NREMS compared to the vehicle solution over 24 hours. A significant effect of time on NREMS was observed with the administration of 1 g and 3 g T_3 ($F_{11, 176}$= 2.54, P<0.006; $F_{11, 204}$ = 2.79, P<0.003 respectively) with less NREMS during the dark cycle. The interaction between 1 g and 3 g T_3 injections and time were not significant ($F_{11, 296}$= 0.31, P<0.99; $F_{11, 204}$ =0.28, P<0.99 respectively).

The injections of 0.3 g and 10 g T_3 (Fig. 2A, and 2D) to the MePO did not have a significant effect on NREMS ($F_{1, 164}$= 2.73, P<0.11; $F_{1, 176}$= 3.07, P<0.09 respectively), although the injection seemed to induce less sleep compared to the vehicle solution in the first 6 hours and from 16 to 20 hours for the 0.3 g T_3 injections. NREMS did vary significantly over time for the 0.3 g and 10 g T_3 ($F_{11, 164}$ = 1.35, P<0.01; $F_{11, 176}$ = 2.69, P<0.004 respectively) with the indication of a circadian rhythm and an overall decrease in the amount of NREMS during the dark phase. The injections 0.3 g and 10 g T_3 and time did not result in a significant interaction ($F_{11, 164}$ = 0.12, P<1; $F_{11, 176}$= 0.80, P<0.64 respectively).

Effect of T_3 administered to the Median Preoptic Nucleus on REM Sleep (REMS)- Figure 3A-D

The effects of 1 g and 3 g T_3 (Fig. 3B, and 3C respectively) injections to the MePO were significant on REMS ($F_{1, 176} = 14.48$, $P<0.003$; $F_{1, 204} = 11.95$, $P<0.0008$ respectively). The 1 g T_3 injection compared to the vehicle solution increased REMS in the first 14 hours. An increase in REMS for the 3 g T_3 injection was detected as compared with the vehicle solution for the first 14 hours of a 24 hour period and the last 6 hours. No significant effect of time on REMS resulted with the administration of 1 g and 3 g T_3 ($F_{11, 176}= 0.88$, $P<0.57$; $F_{11, 204} = 1.17$, $P<0.32$ respectively). The injections of 1 g and 3 g T_3 did not have a significant interaction with time ($F_{11, 176} = 0.62$, $P<0.82$; $F_{11, 204} = 0.42$, $P<0.95$ respectively).

The effects of 0.3 g and 10 g T_3 (Fig. 3A and 3D) injections to the MePO were not significant on REMS ($F_{1, 164} = 1.49$, $P<0.23$; $F_{1, 176} = 0.71$, $P<0.41$). The 0.3 g injection of T_3 appeared to increase the amount of REMS in the first 6 hours after injections and the last 4 hours in a 24 hour period compared to the vehicle solution. There was an increase in REM sleep for the 10 g T_3 injection compared to the vehicle solution in the last 4 hours. The effect of time was extremely significant with the administration of 0.3 g and 10 g T_3 ($F_{11, 164}= 0.89$, $P<0.56$; $F_{11, 176}= 0.55$, $P<0.87$). No significant interaction was observed between 0.3 g and 10 g T_3 injection and time ($F_{11, 164}= 0.62$, $P<0.81$; $F_{11, 176}= 0.37$, $P<0.97$).

Effect of T_3 administered to the Median Preoptic Nucleus on Activity Counts- Figure 4A-D

The effect of 0.3 g T_3 (Fig. 4A) administered to the MePO on activity was highly significant ($F_{1, 363}$= 7.19, P<0.008), resulting in more activity compared to the vehicle solution. The effect was apparent between eight and twenty hours post injections. The effect of time was extremely significant ($F_{10, 363}$ = 13.43, P<0.0001) yielding a consistently higher level of activity during the dark cycle. The effect of the interaction between 0.3 g T_3 and time was not significant ($F_{10, 363}$ = 0.69, P<0.79).

The administration of 1 g, 3 g and 10 g T_3 (Fig. 4B, 4C and 4D respectively) to the MePO did not have a significant effect on activity ($F_{1, 385}$= 0.01, P<0.92; $F_{1, 407}$= 0.66, P<0.40; $F_{1, 385}$= 1.18, P<0.15 respectively). The level of activity for the vehicle solution and 1 g, 3 g and 10 g T_3 injections had similar trends, with insignificant crossover levels of activity. The effect of time, however, was significant with the administration of 1 g, 3 g and 10 g T_3 ($F_{10, 385}$= 12.07, P<0.0001, $F_{10, 407}$= 17.86, P<0.0001; $F_{10, 385}$= 13.43, P<0.0001 respectively), with consistently more activity during the dark cycle. The interaction of injecting 1 g, 3 g and 10 g T_3 with time were not significant ($F_{10, 385}$ = 0.54, P<0.90; $F_{10, 407}$= 0.39 P<0.97; $F_{10, 385}$= 0.94, P<0.47 respectively).

Core Body Temperature- Figure 5A-D

The effect of 0.3 g and T_3 (Fig. 5A) injection to the MePO was significant on core body temperature ($F_{1, 72}$= 11.37, P<0.0009). A decrease in core body temperature was most apparent

from 10 hours on with the 0.3 g T_3 injection compared to the vehicle solution. The 0.3 g T_3 injection yielded a significant effect on core body temperature ($F_{11, 72}$= 4.57, P<0.0001). However, the injection did not result in a statistically significant interaction between administration of 0.3 g T_3 and time ($F_{11, 72}$= 0.52, P<0.95).

No significant effect of 1 g, 3 g and 10 g T_3 (Fig 5B, 5C and 5D respectively) injections to the MePO were observed on core body temperature ($F_{1, 96}$= 2.15, P<0.14; $F_{1, 120}$= 1.85, P<0.30; $F_{1, 120}$= 1.15, P<0.29 respectively). A trend indicated lower core body temperature for the 1 g and 3 g T_3 treatments within 12 hours. For the 10 g T_3 injection, a slight increase in core body temperature was observed from 14 hours on compared to the vehicle solution. The 1 g and 3 g T_3 injections resulted in a significant effect of time on body temperature ($F_{11, 96}$= 2.38 P<0.02; $F_{11, 120}$ = 4.17, P<0.0001 respectively) but not for the 10 g T_3 injection ($F_{11, 120}$= 1.76, P<0.07). There was no interaction between 1 g, 3 g and 10 g T_3 injection and time ($F_{11, 96}$= 0.44, P<0.93; $F_{11, 120}$= 0.81, P<0.64; $F_{11, 120}$ = 0.59, P<0.80 respectively).

Discussion

The intent of this experiment was to explore a possible non-genomic effect of T_3 on brain tissue by following a variety of responses to an acute injection. A previous experiment had established a link between microinjection of T_3 to the MePO and reduced Total Sleep (TS) and Non-REM Sleep (NREMS) in hypothyroid rats.[54]The current experiment repeated those conditions without rendering the animals hypothyroid by the administration of PTU. We investigated the effects of microinjections in euthyroid rats, increasing the availability of T_3 beyond its physiological level.

It is well-established that agonists, inverse agonists, and antagonists of the inhibitory receptor $GABA_A$ decrease effects on sleep.[35,51] The $GABA_A$ receptor is highly expressed in the MPOA,[47] and hypnotic drugs injected into this region cause an increase in sleep.[50,52] Picrotoxin, a $GABA_A$ antagonist which binds near the Cl- channel, significantly decreased NREMS and increased wakefulness when microinjected into the MPOA.[35] Neurotransmission by GABA in the MPOA is spontaneously active in the regulation of hypnotic function which includes REM and NREMS, and more specifically, is mediated by the $GABA_A$ receptor.[1] Other experiments show that T_3 has negative electrophysiological effects on the $GABA_A$ receptor,[8,44] as well as inducing protein phosphorylation of synaptosomal membranes that do not contain cell nuclei.[63] It was reasonable to consider the $GABA_A$-negative effects of T_3 on sleep, given the connection between $GABA_A$ receptors, the MePO, T_3, and sleep.

The results of these experiments relating to TS do not offer support to a $GABA_A$-negative mode of T_3 action in the MePO. According to the sleep-switch model,[57] injection of T3 to the MePO would be expected to increase TS.[57] All significant results of these experiments indicate a decrease in TS when the MePO is injected with T_3, and all non-significant results have trends that show the same effect. Microinjections of T_3 to the MePO cause a decrease in sleep on a short time scale, which could imply a non-genomic mode of action. However, the nature of this non-genomic mode of action remains to be discovered. Furthermore, while it is possible that T_3 may be exhibiting neurotransmitter-like properties backed up by proven characteristics such as the deiodinases available to synthesize T_3 from T4 within the synaptic terminal, it is unknown whether other neurotransmitter traits exist, such as vesicular packing and Ca^{2+}-mediated release into the synapse.

The experiment of Zhang and Martin [73] showed an effect of low doses (2 g, 4 g) of T_3 microinjected into the MePO to decrease NREMS and induce an increase in REM sleep, while higher doses did not yield the same results. Moffett et al., (2009) also confirmed the same findings on NREMS where effects were seen at low doses (1 ug, 3 ug) of T_3. Also, these effects were seen shortly of injection, implying a mode of action that could exclude the genomic.[54,73]

The current results not only corroborate the findings related to NREMS effects of T_3 [54,73]but also reproduce the increase in REM sleep. Additionally, the biphasic effect of T_3 injection on REM sleep was also reproduced in the current experiments. An effect was observed at injections of 0.3 g 1 g and 3 g, but not 10 g T_3. These results are consistent with a preliminary study (Zhang et al.,) wherein there were observable significant increases in REM sleep with doses of 2 g and 4 g T_3, but no significant effects with 8 g or 16 g.

One possible explanation for the biphasic effect lies in the results from Sarkar [59] in which he demonstrated that T_3 rapidly modulates protein phosphorylation in cerebrocortical synaptosomes from adult rat brain. He found after L-T_3 and L-T4 administration to the synaptosomal lysates, an upregulation in protein phosphorylation ascended with increasing doses of T_3. It peaked at 400-600% of the normalized control between 10 and 100 nM T_3 and then fell again to control levels by 1000nM. While the values of T_3 administered to synaptosomal lysate that had an effect were in a different range than those of microinjection of T_3 directly to intact brain tissue, the biphasic effect is conserved. The difference in range may be accounted for by other means, such as a proportional dispersion of T_3 in brain tissue. If applicable, this result may indicate a non-genomic mode of action in the MePO that is based on protein phosphorylation. Sarkar[62] suggested that incubation with T_3 might lead to the phosphorylation of the alpha subunit of Na+-K+ ATPase thereby preventing enzyme activity. He speculated this mechanism possibly being part of $GABA_A$ receptor inhibition.

Lancel[32] established the effects of two $GABA_A$ receptor agonists (muscimol and midazolam) with differing binding sites on EEG studies. The EEG analyses yielded the discovery of two distinct effects on sleep[32], muscimol increased NREMS and REM sleep, midazolam, increased NREMS, but decreased REM sleep. However, as shown previously, thyroid hormone does not appear to have a direct action at the benzodiazepine site on the $GABA_A$ receptor. In HEK cells which expressed the subunits $\alpha_1\beta_2\gamma_2$, flumazenil, an inhibitor of the benzodiazepine recognition site, did not influence the inhibitory effect of L-T_3. Allosteric modulation of benzodiazepines on $GABA_A$ receptor function requires γ subunits, but the ■

subunit is not necessary with thyroid hormone induced responses in recombinant expressed receptors.

The inhibitory effects of T_3 though mimic those of the GABA negative steroid pregnenolone sulfate (PREGS) in many aspects. PREGS and T_3 are structurally similar[33] and are equally potent in the inhibition of GABA responses in a binary subunit combination. These include $\alpha_1\beta_1$ subunits as in ternary combinations made of $\alpha_1\beta_1\gamma_2$ in recombinant receptors which are expressed in Xenopus oocytes. Physiological levels of endogenous thyroid hormone in the synapse may possibly contribute to a tonic steady-state inhibition of $GABA_A$ receptor function in certain classes of neurons, particularly in response to thyroid dysfunction.

This study has shown that microinjection of T_3 into the MePO modified sleep stages in a distinct pattern. Total and NREMS decreased while REMS decreased over a short time scale. These findings are consistent with a previous experiment[54] in their effects on sleep in hypothyroid rats as well as their biphasic properties, which extend from behavior to the actions of protein phosphorylation.[59] The most apparent effects on TS was from the injection of 1 and 3 g T_3 to the MePO which caused a decrease in TS as was the case for the hypothyroid rats. NREMS was significantly decreased when 1, 3 and 10 g were injected to the MePO which was the same effect seen in the hypothyroid rats. A significant increase in REMS was detected when the rats were injected with 0.3, 1 and 3 g T_3. When compared directly to the hypothyroid rats, the euthyroid rats displayed less NREMS. However, the euthyroid rats also displayed, more REMS which in turn led to increasing TS compared to the hypothyroid rats. The current experiment has also illustrated the effect of microinjection of T_3 into the MePO on core body

temperature with 0.3 g T_3 treatment. The effects on core body temperature paralleled those on activity. The effect of 0.3 g T_3 injection on activity was significant. The administration of 1, 3 and 10 g did not show a significant effect on activity but had a significant effect on time which indicated a circadian rhythm, with more activity during the dark phase.

Dratman[15] previously been noted that postnatal rats have a geometrically increasing concentration of L-triiodothyronine (T_3) within synaptic terminals (more rapidly than the accumulation of synaptosomal protein) accompanied by a decrease in the concentration of thyroid hormone in neuronal nuclei. The location of T_3 accumulation following peripheral administration was the noradrenergic centers where norepinephrine co-localizes.[14] Axoplasmic transport delivers T_3 through the medial forebrain bundle to the adrenergic projection sites. Proposed similarity in actions between NE and T3 may explain effect seen of microinjections to the MePO.

Figures:

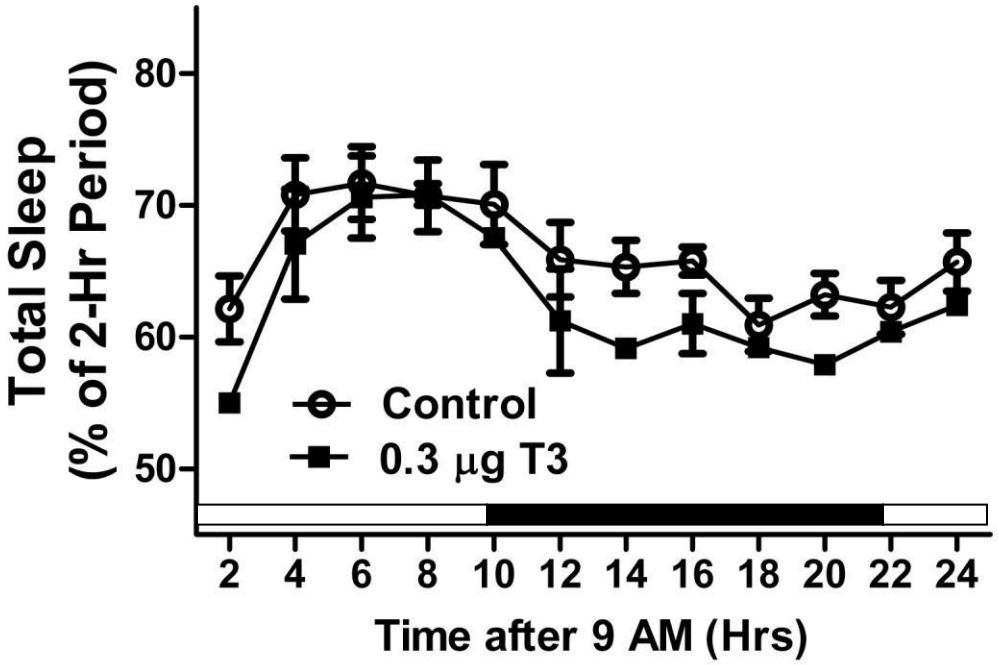


Figure 1A: Effect of microinjection of 0.3 g T_3 to the MePO on TS. Four rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 0.3 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of TS during each two-hour interval, and the error bars indicate standard errors of the mean (SEM). Two-Way ANOVA did not show a significant effect of 0.3 g T_3 injection on TS ($P<0.12$), nor was there a significant effect of time ($P<0.18$). There was no significant interaction between administration of 0.3 g T_3 and time ($P=1$)

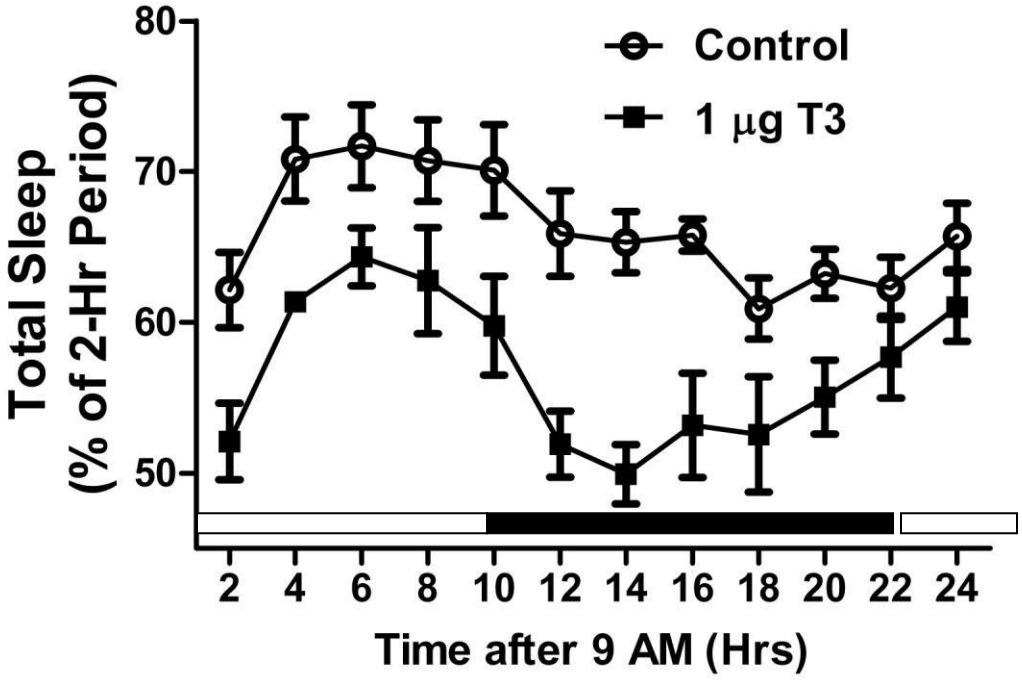


Figure 1B: Effect of microinjection of 1 g T_3 to the MePO on TS. Five rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 1 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of TS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA indicated an extremely significant effect of 1 g T_3 injection ($P<0.0001$) along with a significant effect of time ($P<0.02$). However, there was not a significant interaction between administration of 1 g T_3 and time ($P<0.98$)

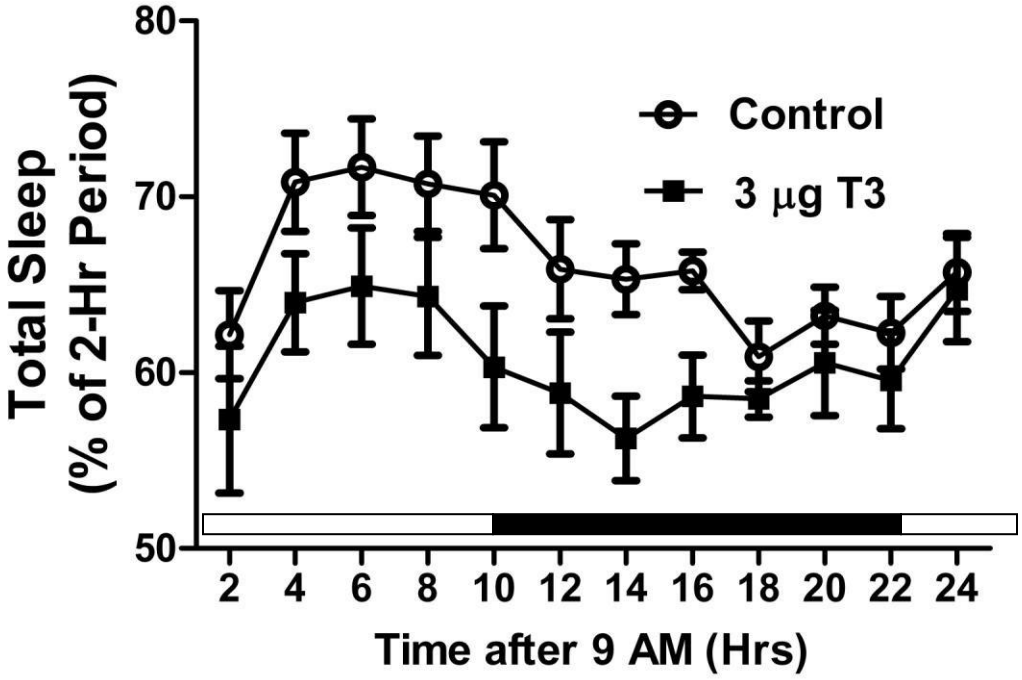


Figure 1C: Effect of microinjection of 3 g T_3 to the MePO on TS. Six rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 3 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of TS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA indicated a significant effect of 3 g T_3 injection ($P<0.0001$).. There was a significant effect of time ($P<0.03$), however there was not a significant interaction between administration of 3 g T_3 and time ($P<0.96$)

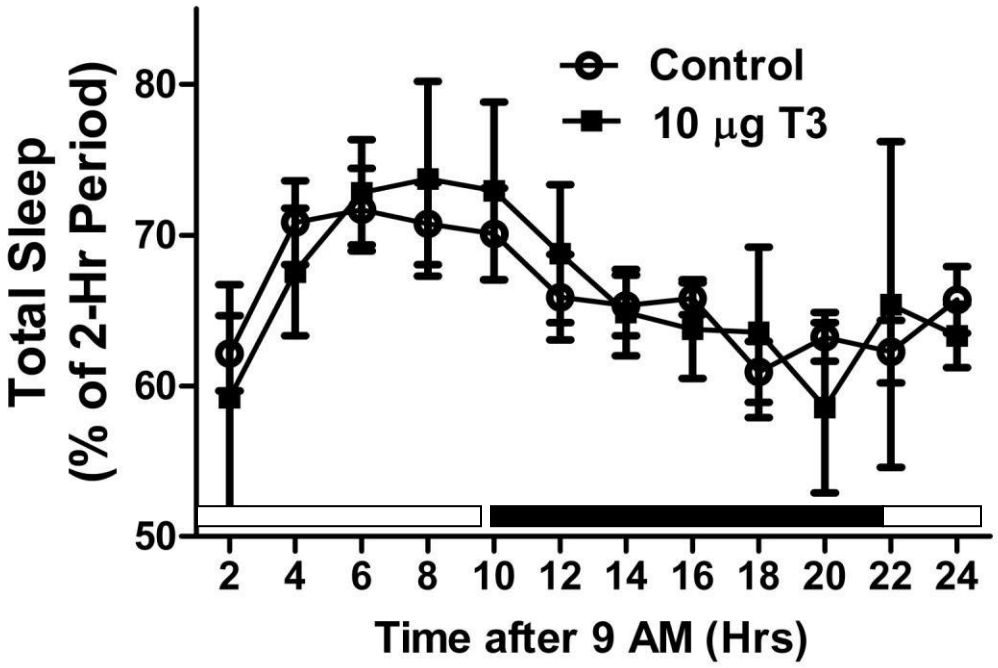


Figure 1D: Effect of microinjection of 10 g T_3 to the MePO on TS. Six rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 10 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of TS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA did not show a significant effect of 10 g T_3 administration ($P<1$). There was a significant effect of time ($P<0.03$), however there was no significant interaction between administration of 10 g T_3 and time ($P<1$).

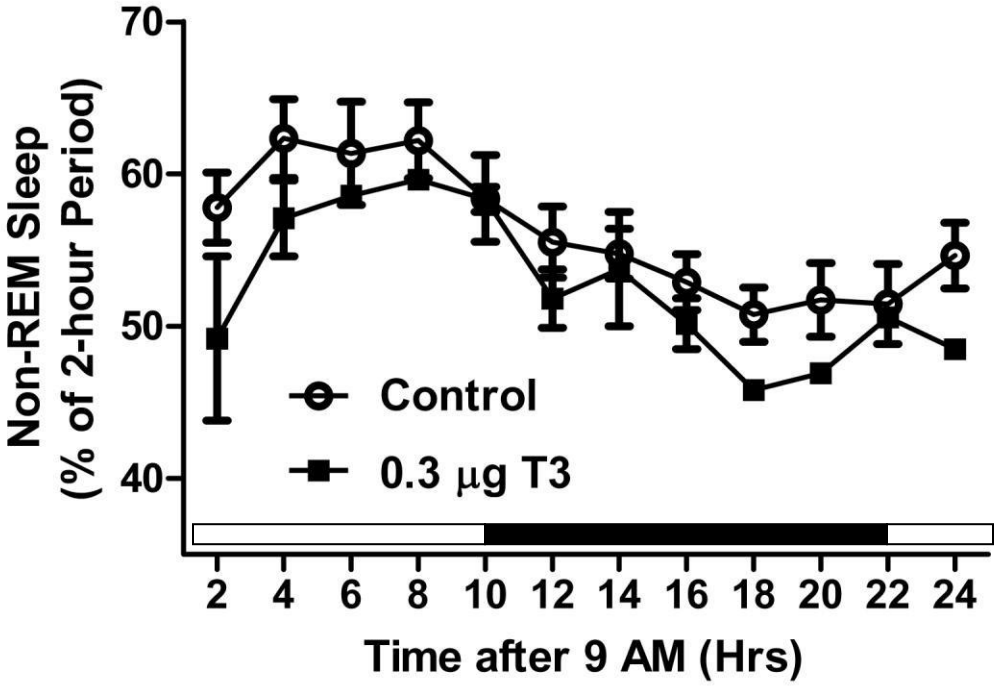


Figure 2A: Effect of microinjection of 0.3 g T_3 to the MePO on NREMS. Six rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 0.3 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of NREMS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA did not show a significant effect of 0.3 g T_3 ($P<0.11$). There was no significant effect of time ($P<0.21$) nor was there a significant interaction between administration of 0.3 g T_3 and time ($P<1$).

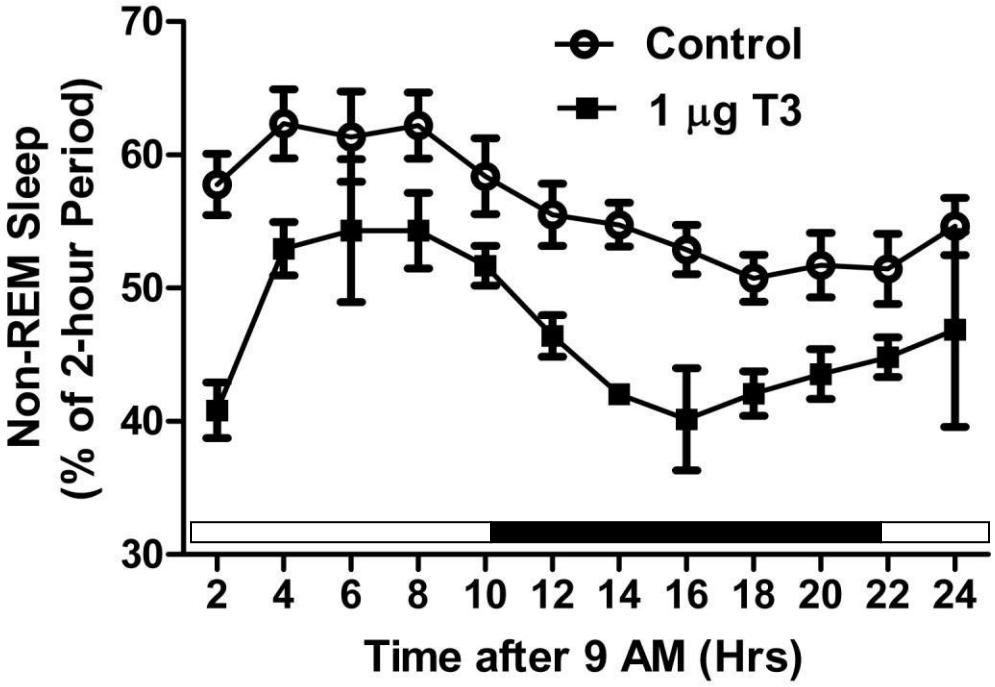


Figure 2B: Effect of microinjection of 1 g T_3 to the MePO on NREMS. Six rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 1 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of NREMS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA showed an extremely significant effect of 1 g T_3 injection ($P<0.0001$). There was a significant effect of time ($P<0.006$), however there was no a significant interaction between administration of 1 g T_3 and time ($P<0.99$)

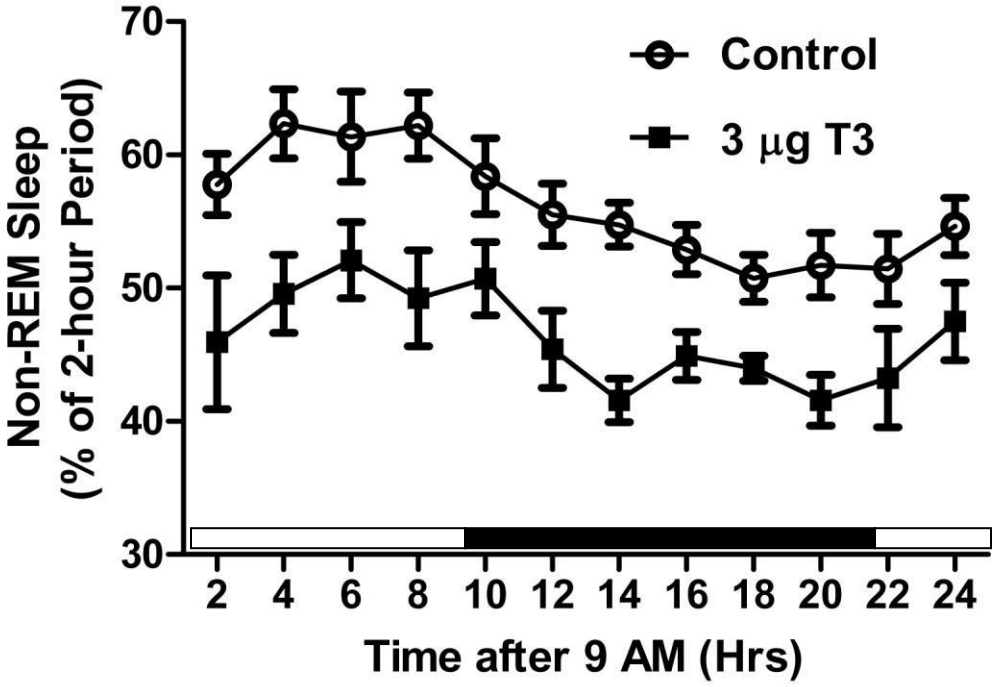


Figure 2C: Effect of microinjection of 3 g T_3 to the MePO on NREMS. Six rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 3 g T_3 dissolved in PBS (closed square). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of NREMS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA showed an extremely significant effect of 3 g T_3 injection ($P<0.0001$). There was a highly significant effect of time ($P<0.003$), however there was no a significant interaction between administration of 3 g T_3 and time ($P<0.99$)

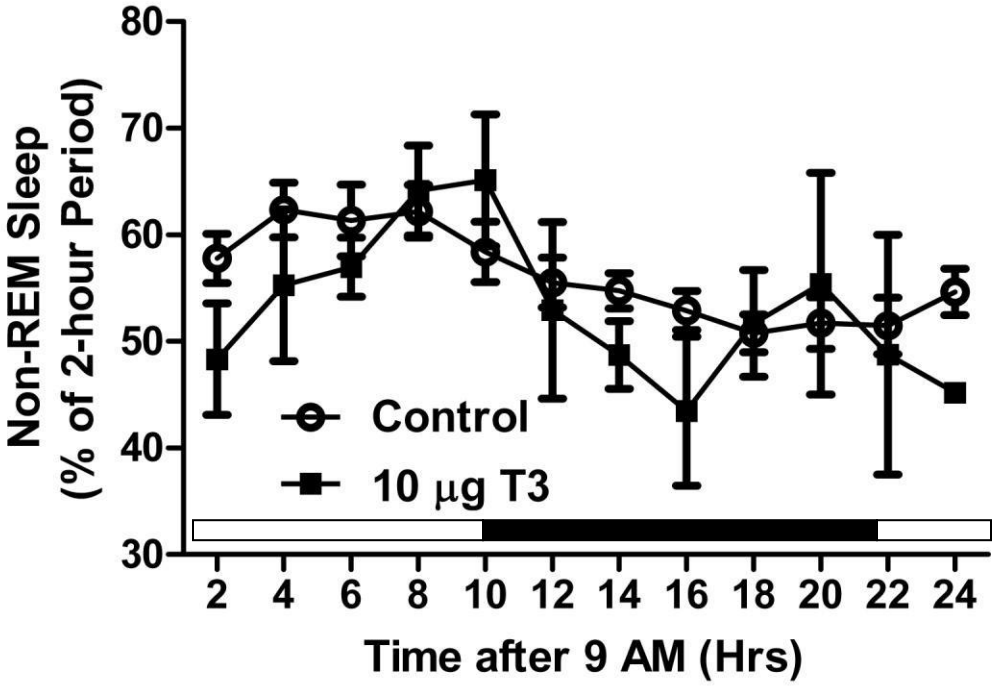


Figure 2D: Effect of microinjection of 10 g T_3 to the MePO on NREMS. Six rats were given injections of PBS (open circle). After twenty-four hours, rats were injected with 10 g T_3 dissolved in PBS (closed squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points indicate the mean percentage of NREMS during each two-hour interval, and the error bars indicate SEM. Two-Way ANOVA did not show a significant effect of 10 g T_3 injection ($P<0.09$). There was a highly significant effect of time ($P<0.004$), however there was no a significant interaction between administration of 10 g T_3 and time ($P<0.64$)

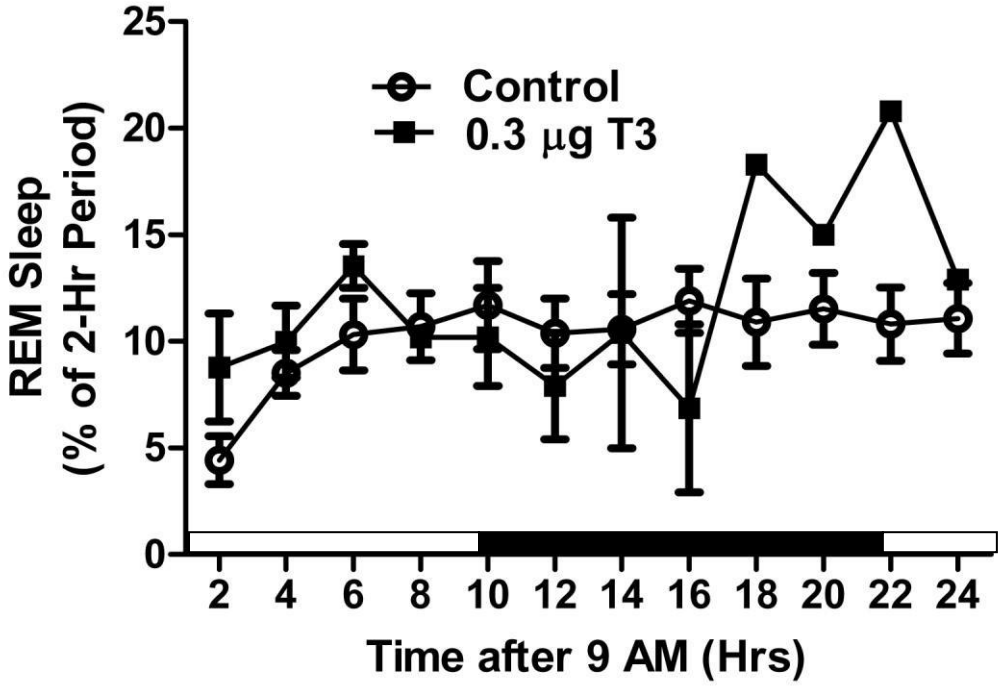


Figure 3A: Effect of 0.3 g microinjection T_3 to the MePO on REMS. Six rats were given injections of PBS to the MePO (open circles). After twenty-four hours, rats were injected with 0.3 g T_3 dissolved in PBS (filled squares). The X-axis represents the time after injection, beginning two hours into the light cycle. The data points represent the mean percentage of REMS during each two-hour interval and the error bars indicate SEM. Two-Way ANOVA did not show a significant effect of 0.3 g T_3 treatment ($P<0.23$). There was not a significant effect of time ($P<0.56$), nor was there a significant interaction between treatment and time ($P<0.81$)

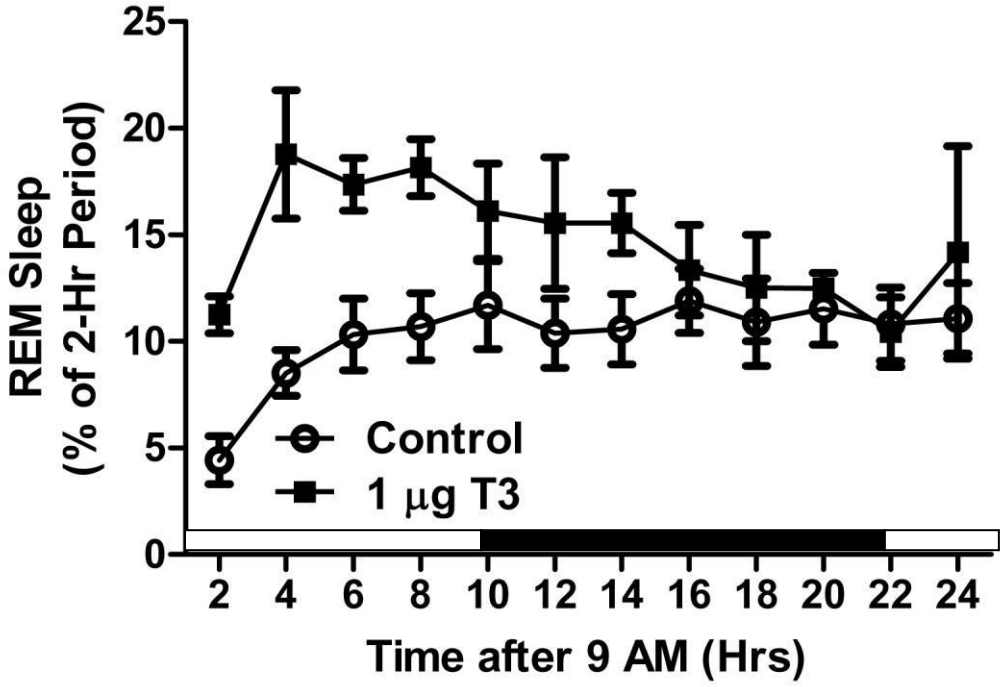


Figure 3B: Effect of 1 g microinjection T_3 to the MePO on REMS. Six rats were given injections of PBS to the MePO (open circles). After twenty-four hours, rats were injected with 1 g T_3 dissolved in PBS (filled squares). The X-axis represents the time after injection, beginning two hours into the light cycle. The data points represent the mean percentage of REMS during each two-hour interval and the error bars indicate SEM. Two-Way ANOVA showed an extremely significant effect of 1 g T_3 treatment ($P<0.0003$). There was not a significant effect of time ($P<0.57$), nor was there a significant interaction between treatment and time ($P<0.82$).

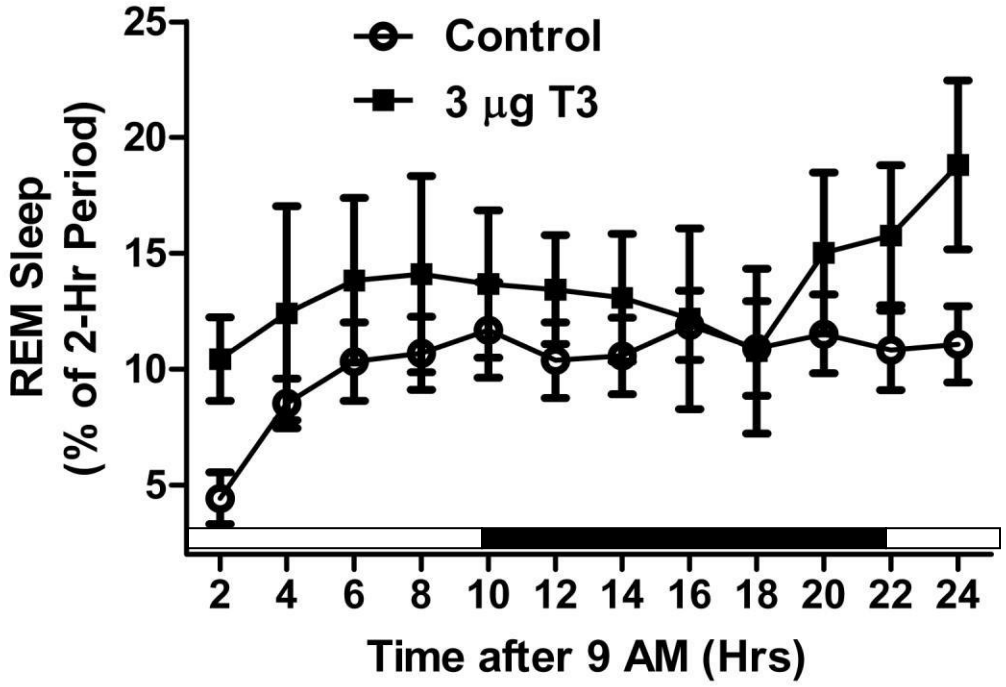


Figure 3C: Effect of 3 g microinjection T_3 to the MePO on REMS. Six rats were given injections of PBS to the MePO (open circles). After twenty-four hours, rats were injected with 3 g T_3 dissolved in PBS (filled squares). The X-axis represents the time after injection, beginning two hours into the light cycle. The data points represent the mean percentage of REMS during each two-hour interval and the error bars indicate SEM. Two-Way ANOVA showed an extremely significant effect of 3 g T_3 treatment ($P<0.0008$). There was no significant effect of time ($P<0.32$), nor was there a significant interaction between treatment and time ($P<0.95$).

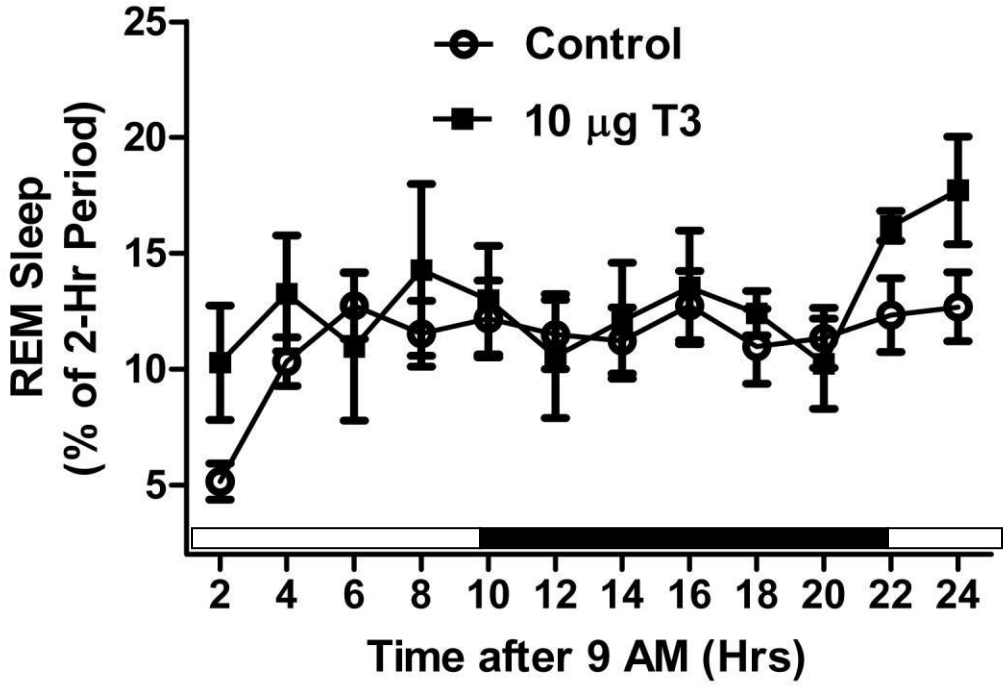


Figure 3D: Effect of 10 g microinjection T_3 to the MePO on REMS. Six rats were given injections of PBS to the MePO (open circles). After twenty-four hours, rats were injected with 10 g T_3 dissolved in PBS (filled squares). The X-axis represents the time after injection, beginning two hours into the light cycle. The data points represent the mean percentage of REMS during each two-hour interval and the error bars indicate SEM. Two-Way ANOVA did not show a significant effect of 10 g T_3 treatment ($P<0.41$). There was no significant effect of time ($P<0.87$), nor was there a significant interaction between treatment and time ($P<0.97$).

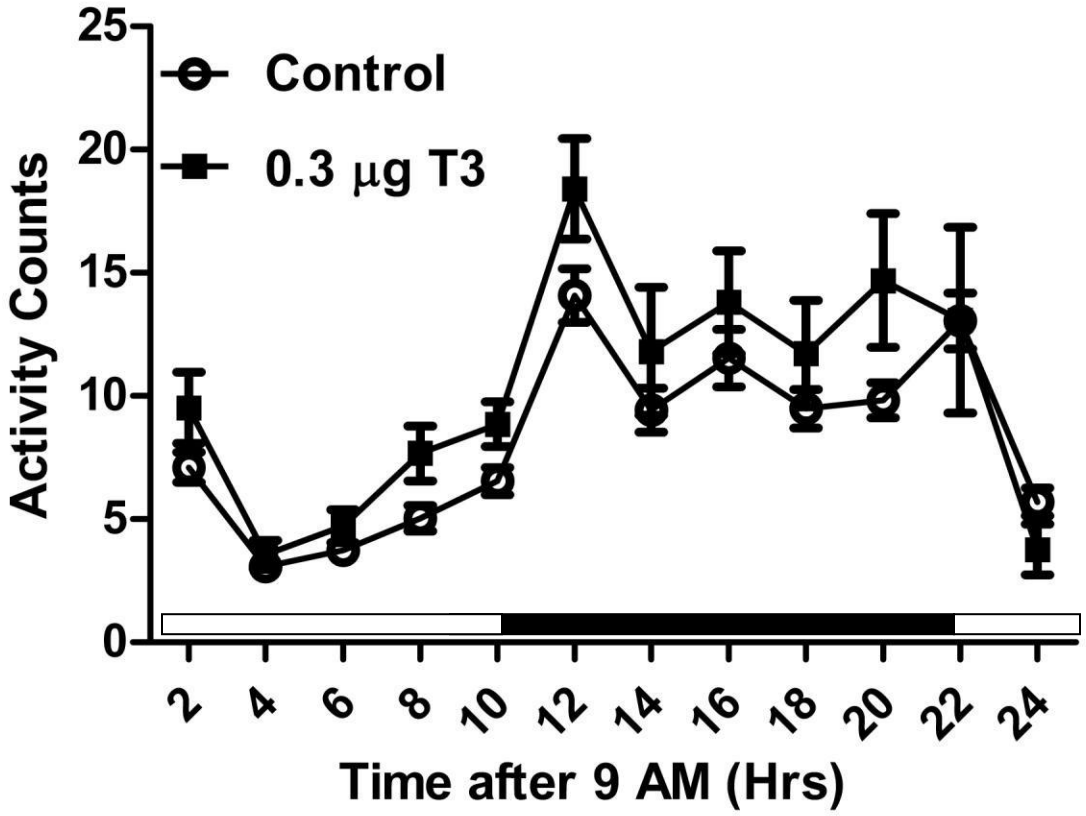


Figure 4A: Effect of microinjection of 0.3 g T_3 to the MePO on locomotor activity. Six rats were given injections of PBS (control; open circle). After twenty-four hours, rats were injected with 0.3 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent mean activity counts in a two-hour interval and the error-bars indicate SEM. Two-Way ANOVA showed a highly significant effect of 0.3 g T_3 treatment ($P<0.004$). There was an extremely significant effect of time ($P<0.0001$), however there was not a significant interaction between treatment and time ($P<0.79$).

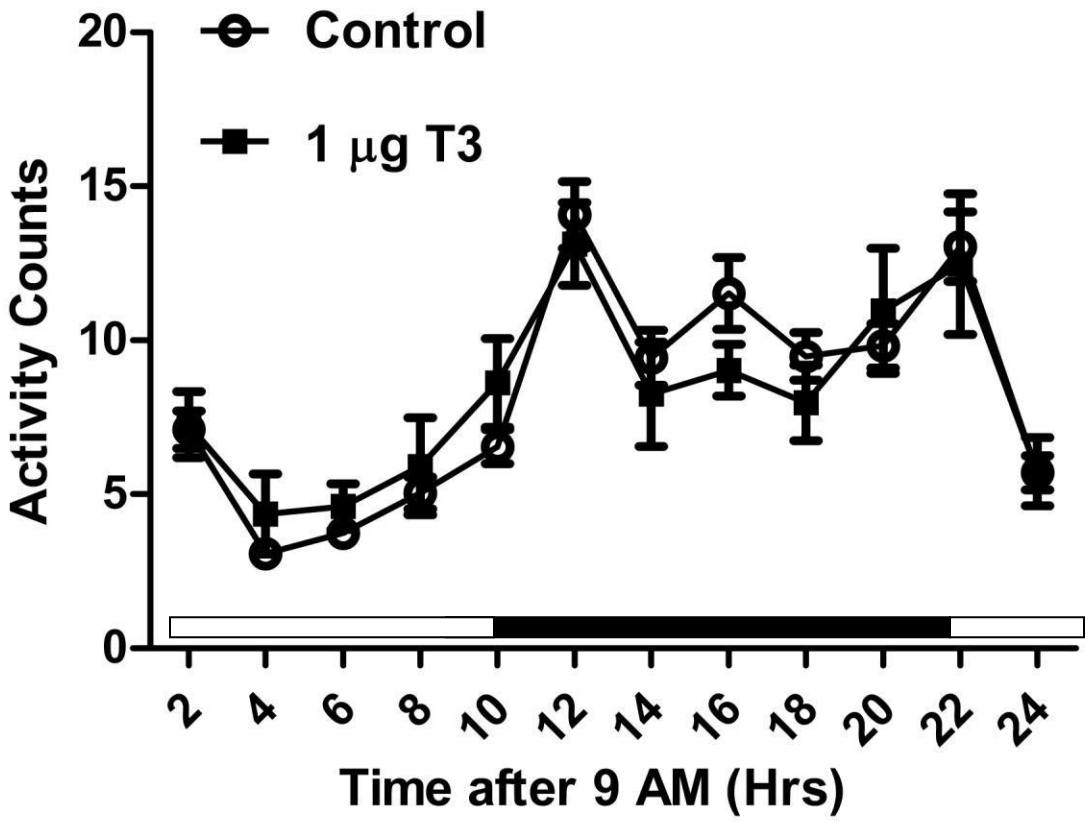


Figure 4B: Effect of microinjection of 1 g T_3 to the MePO on locomotor activity. Six rats were given injections of PBS (control; open circle). After twenty-four hours, rats were injected with 1 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent mean activity counts in a two-hour interval and the error-bars indicate SEM. Two-Way ANOVA did not show significant effect of 1 g T_3 treatment ($P<0.92$). There was an extremely significant effect of time ($P<0.0001$) indicating a similar trend in both the control and dose, however there was not a significant interaction between treatment and time ($P<0.90$).

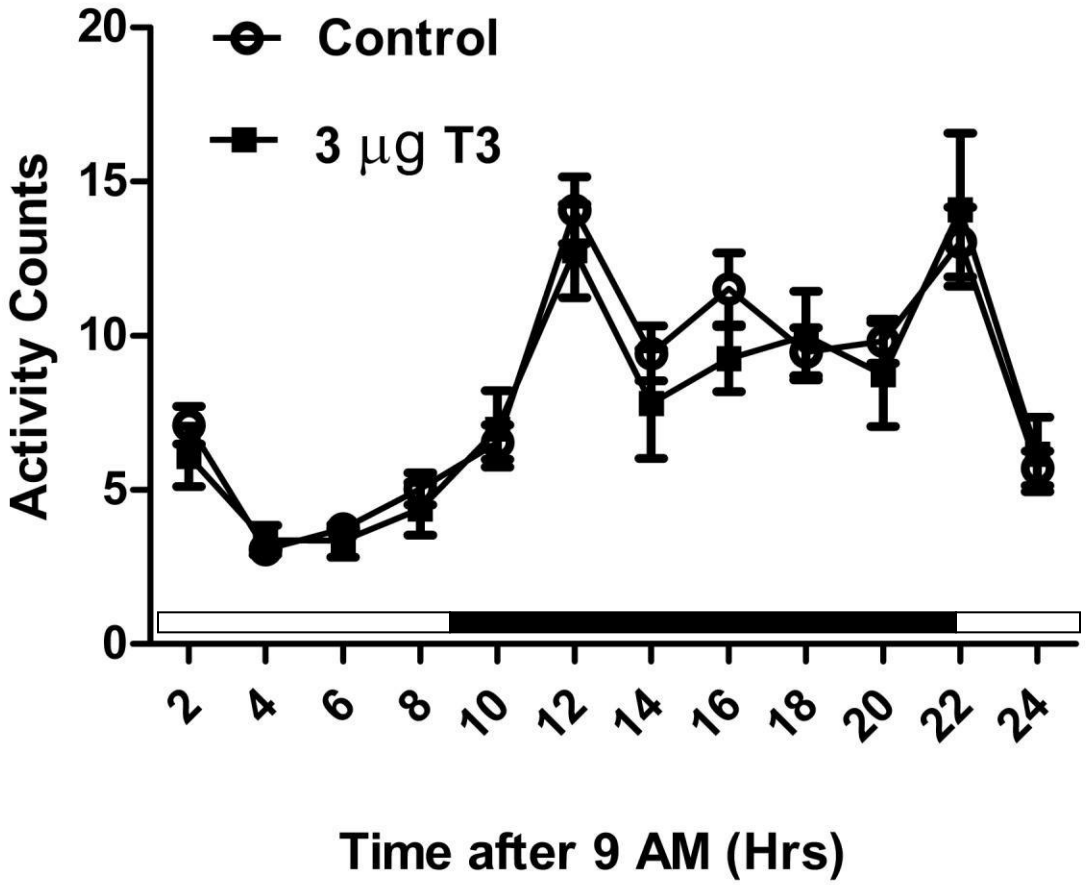


Figure 4C: Effect of microinjection of 3 g T_3 to the MePO on locomotor activity. Six rats were given injections of PBS (control; open circle). After twenty-four hours, rats were injected with 3 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent mean activity counts in a two-hour interval and the error-bars indicate SEM. Two-Way ANOVA did not show a significant effect of 3 g T_3 treatment ($P<0.40$). There was an extremely significant effect of time ($P<0.0001$), however there was not a significant interaction between treatment and time ($P<0.79$).

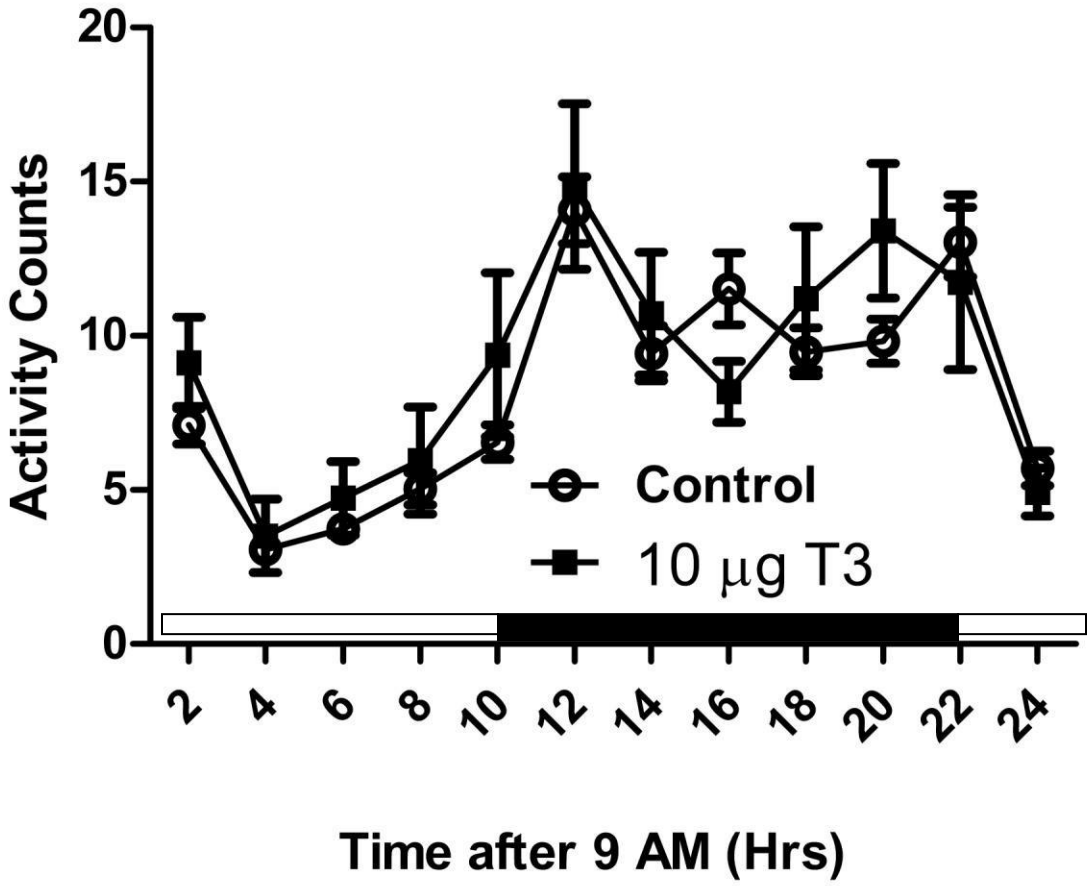


Figure 4D: Effect of microinjection of 10 g T_3 to the MePO on locomotor activity. Six rats were given injections of PBS (control; open circle). After twenty-four hours, rats were injected with 10 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent mean activity counts in a two-hour interval and the error-bars indicate SEM. Two-Way ANOVA did not show a significant effect of 10 g T_3 treatment ($P<0.15$). There was an extremely significant effect of time ($P<0.0001$), however there was not a significant interaction between treatmen and time ($P<0.47$).

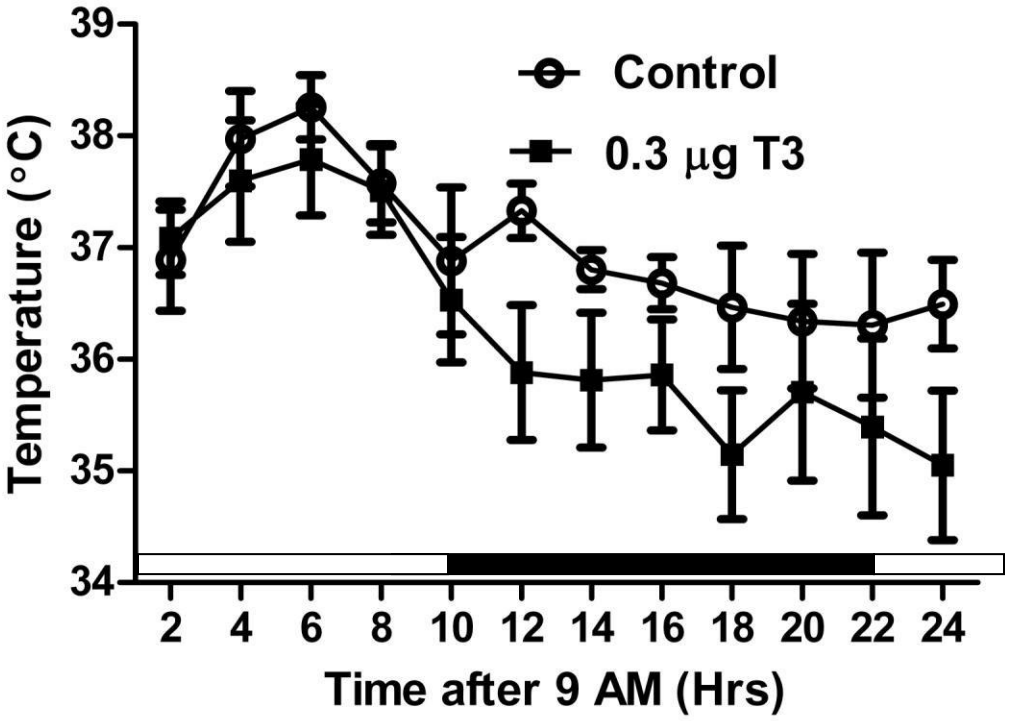


Figure 5A: Effect of microinjection of 0.3 g T_3 to the MePO on core body temperature. Four rats were given injections of PBS to the MePO (control; open circles). After twenty-four hours, rats were injected with 0.3 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent the mean body temperature during each two-hour interval and the error bars show SEM. Two-Way ANOVA did showed an extremely significant effect of hormone treatment ($P<0.0008$). There was an extremely significant effect of time ($P<0.0001$). However, there was no significant interaction between hormone treatment and time ($P<0.95$).

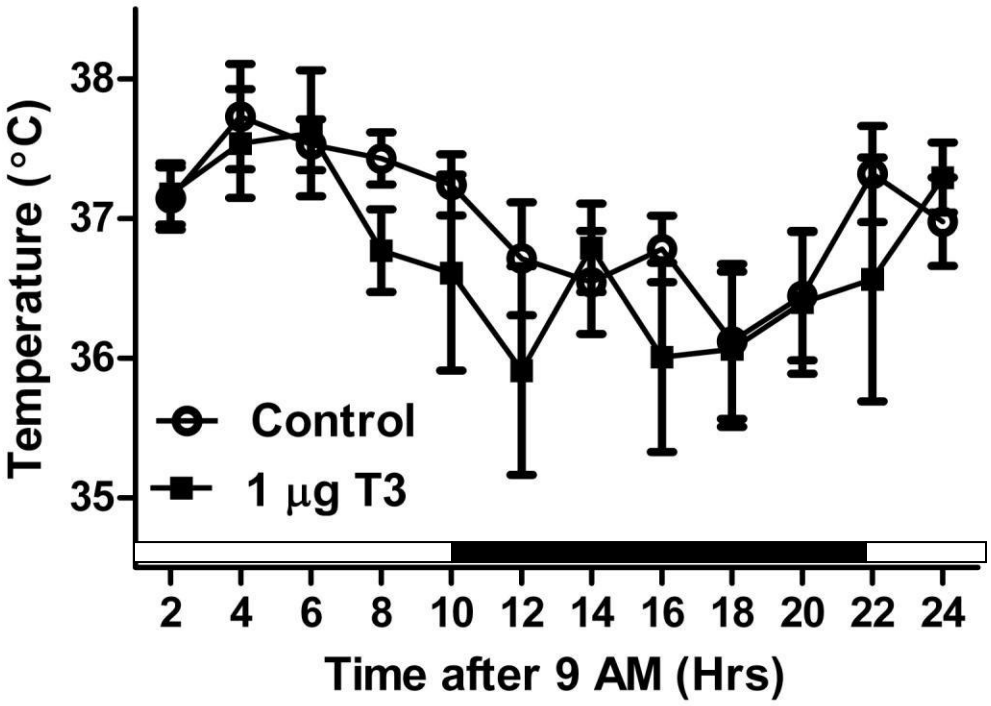


Figure 5B: Effect of microinjection of 1 g T_3 to the MePO on core body temperature. Five rats were given injections of PBS to the MePO (control; open circles). After twenty-four hours, rats were injected with 1 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent the mean body temperature during each two-hour interval and the error bars show SEM. Two-Way ANOVA did not indicate a significant effect of hormone treatment ($P<0.14$). There was a significant effect of time ($P<0.02$) but no significant interaction between hormone treatment and time ($P<0.93$).

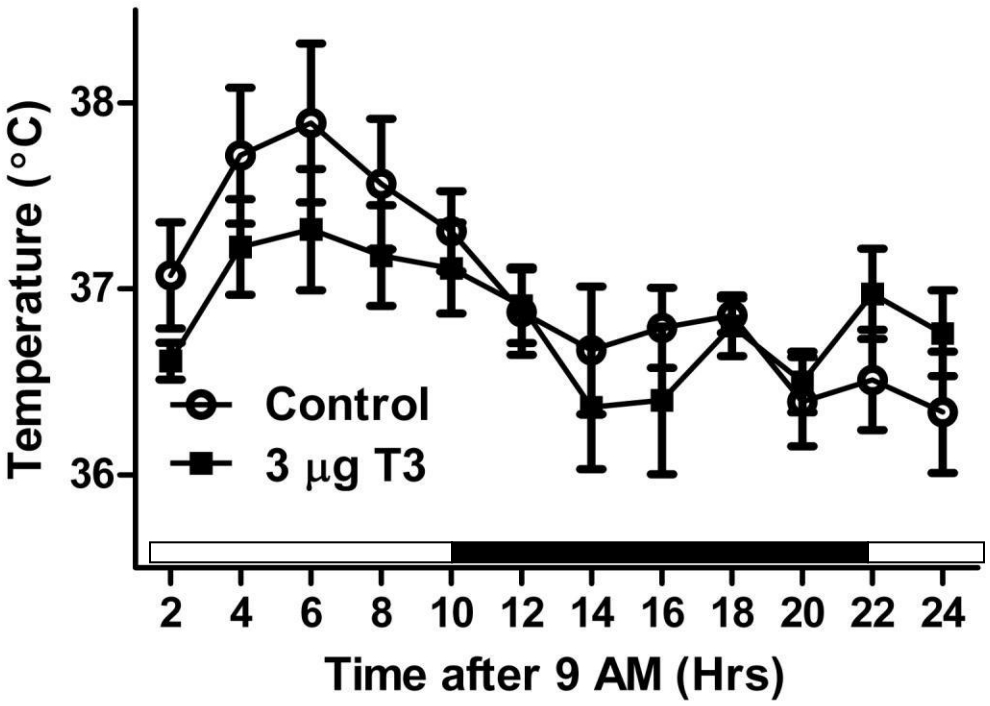


Figure 5C: Effect of microinjection of 3 g T_3 to the MePO on core body temperature. Six rats were given injections of PBS to the MePO (control; open circles). After twenty-four hours, rats were injected with 3 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent the mean body temperature during each two-hour interval and the error bars show SEM. Two-Way ANOVA did not show a significant effect of hormone treatment ($P<0.30$). There was an extremely significant effect of time ($P<0.0001$), but no significant interaction between hormone treatment and time ($P<0.64$).

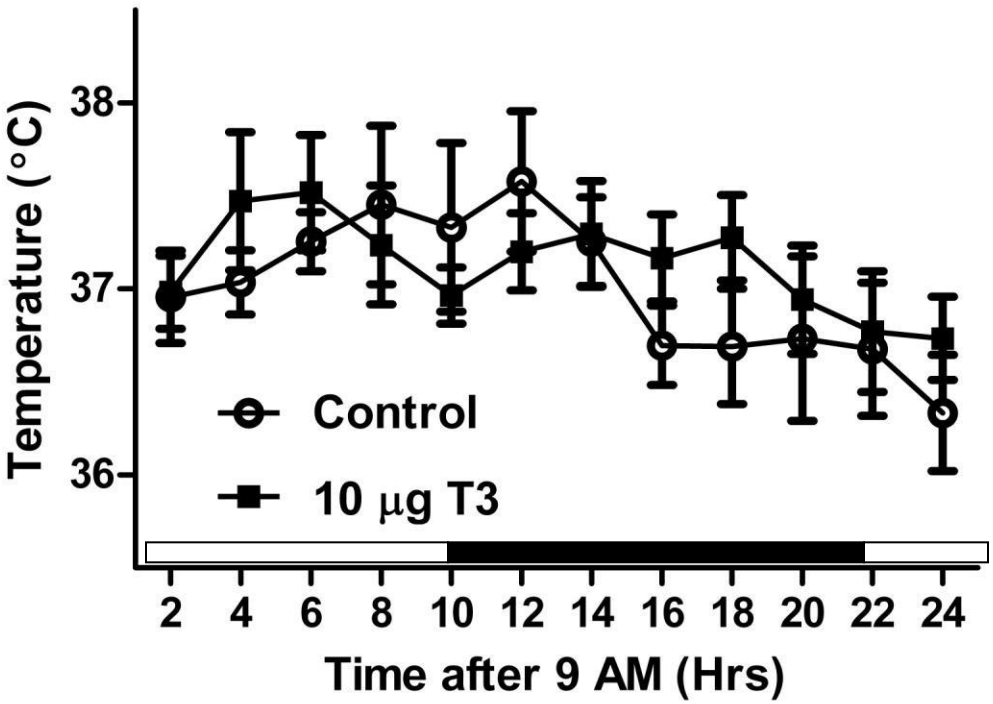
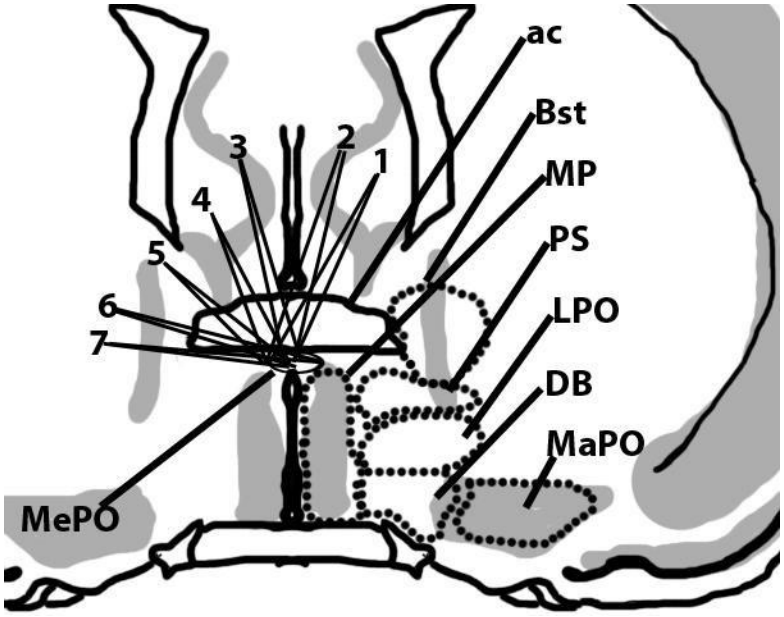


Figure 5D: Effect of microinjection of 10 g T_3 to the MePO on core body temperature. Six rats were given injections of PBS to the MePO (control; open circles). After twenty-four hours, rats were injected with 10 g T_3 dissolved in PBS (filled squares). The X-axis represents the time of day, beginning two hours into the light cycle. The points represent the mean body temperature during each two-hour interval and the error bars show SEM. Two-Way ANOVA did not show a significant effect of hormone treatment ($P<0.29$). There was not a significant effect of time ($P<0.07$), nor was there a significant interaction between hormone treatment and time ($P<0.80$).

Figure 6



Seven rats histologically confirmed with guide cannula placed in the MePO.

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