EFFECTS OF ACUTE MICROINJECTIONS OF THYROID HORMONE TO THE MEDIAN PREOPTIC NUCLEUS OF HYPOTHYROID ADULT MALE RATS ON SLEEP, MOTOR ACTIVITY, AND BODY TEMPERATURE

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ABSTRACT OF THE THESIS

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

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Thyroid hormone's role as a genomic regulator of basal metabolic rate does not explain neurological changes in patients with dysthyroidism. Hypothyroidism, characterized by depressive symptoms including somnolence, intellectual deterioration, and coma, and hyperthyroidism, illustrated by symptoms including insomnia, anxiety, and seizures, imply a mechanism of thyroid hormone action which influences behavior. In adults, thyroid hormone is taken up to discrete brain areas by organic anion and monocarboxylate transport proteins. One site of distribution, the median preoptic nucleus (MePO), contains clusters of sleep-active neurons and is considered a key sleep-regulatory structure. To test the acute effect of thyroid hormone at this locus, we stereotaxically implanted adult hypothyroid rats with microinjection guide cannulae at the MePO and installed electrodes and miniature transmitters for recording EEG, EMG, core body temperature, and locomotor activity. The rats were rendered hypothyroid by administration of 0.02% 6-n-propyl-2-thiouracil in drinking water until body weight

plateaued, indicating a hypothyroid condition. Rats were given a series of dosages (0.3 μg, 1 μg, 3 μg, or 10 μg in 0.25μL vehicle) of L-3,3´,5-triiodothyronine (T_3) in four weekly 48-hour tests. Each week, rats were bilaterally microinjected with a control solution. After 24 hours of recording, each rat was microinjected with one of the above doses of T_3 and recorded for an additional 24 hours. In seven rats with histologically-confirmed cannulae placements in the MePO, we observed significant effects on REM, non-REM, total sleep, core body temperature and motor activity analyzed by 2-way ANOVAs. For each sleep parameter the effect of dose of hormone was significant ($p < 0.0001$), as was the effect of time after injection ($p<0.0001$), except for in REM sleep. Temperature and activity also showed significant effects of dose ($p < 0.02$) and time ($p < 0.001$). There was no significant interaction between dose and time factors in any ANOVA. Since the effects of T_3 were seen within hours of acute injection, the data are most consistent with a non-genomic mechanism of action of thyroid hormones in the adult brain. The mechanism of thyroid hormone action may be due to inhibition of the $GABA_A$ receptor, by an unknown protein phosphorylation effect, or by its actions as an adrenergic system analogue.

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DEDICATION

I would like to dedicate this work to my family.

TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Thyroid hormone's role as part of a homeostatic mechanism to genomically regulate basal metabolism is not sufficient to explain primary clinical observations of patients with dysthyroidism, which is characterized by neurological signs and symptoms. Somnolence, intellectual deterioration, and coma characterize hypothyroidism, while insomnia and anxiety are associated with hyperthyroidism (Laurberg, 1990, Orgiazzi, 1990, Utiger, 1990). After the stimulation of O_2 consumption in the developing brain by thyroid hormone, little evidence of a general metabolic effect on the adult brain exists (Fazekas et al., 1951).

Thyroid hormone is, nonetheless, taken up into and distributed to discrete areas of the adult brain. Several organic anion transport proteins (including organic anion transport protein 2 (OATP2) and OATP14) lining brain capillary endothelial cells and choroid plexus are able to transport both T_3 and T_4 into brain tissue (Abe et al., 1998, Gao et al., 1999, Sugiyama, 2003). Monocarboxylate transporter 8 is responsible for the transport of T_3 both past the blood-brain barrier and directly into neurons (Friesema et al., 2003). Immunohistochemical (IHC) evidence showed that T_3 injected intravenously appeared in cell groups within the locus coeruleus and its projection sites (Rozanov and Dratman, 1996). Following injection of labeled T_4, radioactivity show the same distribution pattern, unless 5'-monodeiodination in brain was inhibited, implying that T_4 is deiodinated in neurons before distribution to nerve terminals (Dratman and Crutchfield, 1978). The enzyme responsible for the conversion of T_4 to T_3 in adult brain tissue is 5'-deiodinase II. It demonstrates a reliable circadian rhythm of action, rising during the dark

period and falling during the light (Campos-Barros et al., 1997).

Further, the synaptosomal concentration of iodocompounds rises at a greater rate than the rate of accumulation of other synaptosomal proteins in neonatal rats that were reared and weaned from mothers whose sources of iodine were ^{125}I-radiolabeled. This sharper rise in iodocompound accumulation lasted through the 45th postnatal day (Crutchfield and Dratman, 1983).

Homogenization and centrifugal fractionation of adult brain shows a disproportionately higher level of T_3 in the synaptosomal fraction than in other subcellular fractions following intravenous injection of T_3 (Dratman et al., 1976). This fraction, when isolated and exposed to a hypotonic environment, releases T_3, indicating that the T_3 is contained within the synaptic terminal *in vivo* rather than bound to the outside (Dratman and Crutchfield, 1978). At least two binding sites on synaptosomes and synaptic membranes *in vitro* have been demonstrated (Mashio et al., 1982, Bergmann et al., 1989, Sarkar and Ray, 1998). Uptake into the synaptic terminals in brain tissue follows a distinct and reproducible pattern of regional distribution. This uptake can be arrested by the administration of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine, a locus coeruleus-specific neurotoxin. These observations may be due to a mechanism of axoplasmic transport from the LC (Dratman et al., 1987, Gordon et al., 1999). The greater presence of T_3 in nerve terminals than other cellular areas could suggest a possible mechanism of synaptic release.

After inducing a hypothyroid state in adult rats with daily n-propylthiouracil

injections, Sarkar and Ray (1994) observed a 9.5-fold increase in the level of T_3 in synaptosomal fractions of brain homogenate. This effect was reversible with a single intraperitoneal injection of thyroid hormone. The uptake and distribution of T_3 into the synaptic terminal, as well as the homeostatic mechanism which increases T_3 in the synaptic terminal during the hypothyroid state, implies a possible neurotransmitter-like signaling or modulating mechanism. One potential site of activity is the $GABA_A$ receptor. T_3 inhibits GABA-induced Cl^- flux in highly-washed rat forebrain synaptoneurosomes at nanomolar concentrations (Martin et al., 1996). [^{35}S]t-butylbicyclophosphorothionate binds to the convulsant site of the $GABA_A$ receptor complex, and both T_3 and T_4 have a biphasic effect on its binding: a stimulatory effect at high nanomolar/low micromolar concentrations, and an inhibitory effect at high micromolar concentrations (Martin et al., 1996). These effects are reminiscent of effects of pregnenolone sulfate, a steroid similar in three-dimensional arrangement to thyroid hormone, which inhibits $GABA_A$ channels reversibly at micromolar concentrations *in vitro* (Majewska et al., 1988). Martin *et al* (1996) demonstrated inhibitory modulation of the $GABA_A$ receptor expressed on human embryonic kidney cells by T_3 and T_4 at high nanomolar and low micromolar concentrations. T_3 at low micromolar concentrations inhibited $GABA_A$ receptors expressed in *Xenopus* oocytes (Chapell et al., 1998). T_3 caused direct channel-gating on the $GABA_A$ receptor complex at mid and high micromolar concentrations, increasing Cl^- flux. Expression of the α1 subunit of the $GABA_A$ receptor is required for sensitivity to T_3 (Martin et al., 1996, Chapell et al., 1998).

T_3 and T_4 also have nongenomic effects distinct from those seen on the $GABA_A$

receptor complex. In HeLa and CV-1 cell lines, which have no nuclear thyroid hormone receptors, T_4 induces phosphorylation and nuclear activation of mitogen-activated protein kinase (Lin et al., 1999). T_3 and T_4 alter the phosphorylation of several proteins of varying size when incubated with lysed cerebral cortex synaptosomes containing no cell nuclei (Sarkar et al., 2006). Through Western blotting, T_3 is seen to stimulate phosphorylation at distinct residues on different proteins (Sarkar et al., 2006). All phosphorylation effects are observable within minutes (Sarkar and Ray, 1998, Lin et al., 1999, Sarkar et al., 2006).

Thyroid hormone and sleep physiology are reciprocally influential. Both hyperthyroid and hypothyroid patients often experience sleep disturbances (Watt et al., 2006). However, there is a lack of agreement among the results of sleep studies on the effects of thyroid hormones. Some hyperthyroid patients display an increase in sleep stages 3 and 4 according to electroencephalograms (Dunleavy et al., 1974, Eastman and Rechtschaffen, 1979), while hypothyroid patients experience a decrease in sleep stages 3 and 4 (Kales et al., 1967). Thyroxine administered to rats does not increase total sleep time (Eastman and Rechtschaffen, 1979). Conversely, Bergmann et al. and Everson and Crowley have consistently demonstrated a drop in thyroid hormone levels after severe chronic sleep deprivation (Bergmann et al., 1989, Everson and Crowley, 2004).

Pharmacological hypnotics and sedatives are thought to act mainly through the enhancement of the ionotropic $GABA_A$ receptor (Winsky-Sommerer, 2009). When GABA binds to the $GABA_A$ receptor complex, Cl^- permeability increases and negatively-charged chloride ions flow passively through a pore formed by five subunits in the

absence of an equilibrium. Pregnenolone sulfate increases REM sleep when administered intraperitonally or directly to sensitive brain areas (Darnaudery et al., 1999, Darbra et al., 2004). Pregnenolone sulfate has also been shown to inhibit non-REM sleep, while the corticosterone derivative 5α-pregnane-3α,21-diol-one has positive effects on the initiation of sleep (Majewska and Schwartz, 1987, Mendelson et al., 1987). Progesterone and its derivatives also have hypnotic effects (Lancel et al., 1996, Lancel et al., 1997). Those ligands which inhibit the effects of GABA are known as GABA-negative ligands, while those which enhance GABA's effect on the GABA receptor complex are known as GABA-positive ligands.

In sleep regulatory circuitry, the preoptic area is considered to be an important and behaviorally relevant region. C-Fos immunoreactive staining demonstrates that clusters of neurons in both the median preoptic nucleus (MePO) and the ventrolateral preoptic nucleus (VLPO) are highly active during times of sustained sleep. These areas have higher discharge rates during REM and non-REM sleep than during waking (Gong, 2004). MePO neurons also discharge at a faster rate just before the onset of sleep, suggesting a role in sleep initiation (Suntsova et al., 2002). Staining for glutamic acid decarboxylase indicates GABAergic neurotransmission in the MePO and VLPO areas, and some evidence suggests that they have connections with adrenergic systems that promote wakefulness (McGinty and Szymusiak, 2003, Gong, 2004). Injections of triazolam to the medial preoptic area increased sleep (Mendelson et al., 1989). A preliminary study by Zhang and Martin (2001) illustrated a decrease in sleep after injection of thyroid hormones to distinct regions of the preoptic area, with the most

significant decrease in sleep induced by injection to the MePO . Adenosine injected to the medial preoptic area increased sleep, and its effects were blocked by administration of flumazenil, a $GABA_A$ receptor benzodiazepine antagonist (Mendelson, 2000).

The current study characterizes the effects of the microinjection of thyroid hormone 3, 3' 5-triiodothyronine (T_3) into adult male rat MePO at varying doses on sleep, body temperature, and locomotor activity over a 24-hour period. The hypothyroid state has been shown to increase synaptosomal levels of T_3 9.5-fold (Sarkar and Ray, 1994), but these effects drop off sharply after 18 to 20 days (Kundu et al., 2006). The current study used rats rendered hypothyroid for at least 4 weeks and was thus long enough to deplete brain thyroid hormone. The hypothyroid condition would show an exaggerated response to the reintroduction of higher localized levels of thyroid hormone by an acute injection.

Materials and Methods

Subjects

Twelve male Sprague-Dawley albino rats ranging from 250-300 g, purchased from Hilltop Lab Animals (Scottsdale, PA), were housed individually in shoebox cages. After surgical implantation of cannulae along with electroencephalographic (EEG) and electromyographic (EMG) electrodes, the rats were handled daily and given at least one week to recover before the initiation of any experimentation. The rats were given 0.02% 6-n-propyl-2-thiouracil (PTU) in their drinking water until their body weights plateaued (~40 days), indicating an inhibition of thyroid function. They were housed for 24 hours

before an experiment in the chambers where they were to be tested. 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 they were off from 7:00 PM until 7:00 AM. The rats were handled daily 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.

Experimental Design and Procedure

Stereotaxic Surgery

A rat was set into an induction chamber primed with 5% gaseous isofluorane at a flow rate of 2 LPM for 5 minutes and then placed in a stereotaxic device on a heating platform maintained at 37ºC in order to prevent hypothermia. A midline scalp incision was made, and an injection of 0.45 ml Xylocaine was made at the wound site. The skull was cleared approximately 0.5 cm anterior of and 1 cm posterior of bregma. Using a 0.0125" drill, holes were made in the skull according to the medial-lateral (ML) and anterior-posterior (AP) coordinates of the desired locus with respect to bregma. Two 23-gauge stainless steel 1.2 cm cannulae suspended parallel to one another 1 mm apart were stereotaxically lowered below the surface of the skull according to the dorsal-ventral (DV) coordinates of the desired site of the cannulae tips and cemented in place with a small amount of dental acrylic. For EEG electrodes, a hole was partially drilled in each quadrant of the skull surface for placement of four 0-80 stainless steel screws. 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 other ends of these wires are stripped 0.1 mm and soldered to an Amphenol socket. The entire assembly of cannulae, electrodes and sockets were encased in dental acrylic and the injection guide cannulae were blocked with stylets. Bacitracin, an antibacterial ointment, was applied topically to the periphery of the wound. An E-Mitter Transponder (Mini-Mitter) was implanted through a small midventral incision in the abdominal wall, the muscle layer and the skin were closed with absorbable sutures and Bacitracin was applied topically to the wound. The rat was injected subcutaneously with 0.1 ml Metacam and intramuscularly with 0.15 ml Marcaine. The edges of the wound were checked daily for 7 days, at which time the rats were assessed to be healed adequately for inclusion in the study. Stereotaxic coordinates of the implanted cannulae in relation to bregma were as follows: bilaterally +0.5 mm ML, -0.2 mm AP, and -7.1 mm DV for the rats to be microinjected in the MePO, and unlilaterally +2.0 mm ML, -0.2 mm AP and -4.0 mm DV for the control group rats to be microinjected in the lateral ventricle (LV).

Microinjection Procedure

Each rat was tested four times for two consecutive days at weekly intervals in a randomly assigned order of treatment conditions. On Day 1 (baseline), 0.25 µl of a vehicle solution (30% 0.2 N NaOH diluted with 70% phosphate buffered saline [PBS]) was injected bilaterally for the MePO rat group and unilaterally for the LV rat group. On Day 2 (the test), either 0.3, 1, 3, or 10 µg of L-3,3’5-triiodothyronine (T_3) was injected in the same solvent immediately before use. The pH of the solvent was 7.8-8.5. While the

rat was quietly restrained, the stylet was replaced with a 26-gauge inner cannula extending precisely 1 mm past the end of each of the bilateral guide cannulae. Injections were made for the MePO rat group at a rate of 0.25 µL/min in each cannula for a total of two minutes (0.5 µL total volume). One injection of 0.5 µL was made for the LV rat group at a rate of 0.25 µL/min unilaterally. After the injection, the cannula was left in place for an additional 30 seconds.

Each weekly test was initiated at approximately 9 AM, near the beginning of the light (resting) phase of normal lighting condition. EEG, EMG and core body temperature were monitored for twenty-four hours following the injections. Immediately following the injections, each rat was brought to its isolation chamber. A cable was screwed onto the animal's headset, linking the implanted electrodes to a multichannel amplifier. The amplifier fed into a Keithley DAS-1800HC A/D board, and the signals were recorded on the hard disk of a Windows-based computer running TestPoint software for a 24 hour period. 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. The core body temperature broadcast from the Mini-Mitter transponder was recorded from signals picked up by a Mini-Mitter receiver placed underneath each rat's cage and fed into a Windows Pentium-based computer running VitalView software. Differences in signal strength over time was interpreted as locomotor data by VitalView. At a later date, the files were replayed in 21.2-second epochs for each rat, and a trained investigator who was unaware of the rat's treatment condition assigned a stage of consciousness (W, NREM and REM sleep) to each interval, according to standard criteria using TestPoint and

Spike2 Score software.

Histology

After experimentation, rats were sacrificed and perfused with formalin and the brain was extracted in one piece. Whole brains were mounted on a cryostat sliding microtome and cut into 40 μm-thick sections. Sections were then mounted on microscope slides. The locations of all probes and cannulae were verified by viewing sections obtained under a light microscope and comparing them with a photographic atlas of the rat brain (Kruger et al., 1995) (Figure 6).

Statistical Analysis

Statistical analysis using GraphPad Prism software was based on 2-way analysis of variance (ANOVA) for effects of microinjection of vehicle containing T_3 and time after injection. Separate ANOVAs were performed for examination of the effects of each dose of T_3 on stage of consciousness, total sleep, core body temperature, and locomotor activity. The hormone-treated condition was compared to control. All EEG data were expressed as the percent of the time spent in a particular state of consciousness in 2-hour intervals for 24 hours. Core body temperature and locomotor activity data were expressed as the mean value in each 2-hour period.

Results

Effects of T_3 administered to the lateral ventricle on total sleep

Injections of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 into the LV did not have a

significant effect on total sleep (Figure 1). Sleep varied significantly according to time of day for 0.3 μg, 1 μg, 3 μg and 10 μg T_3 injections. No significant interactions occurred between effects of injections of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 and the effect of the time of day for any dose or experimental condition throughout the study.

Effects of T_3 administered to the median preoptic nucleus on total sleep

The injection of 1 μg and 3 μg T_3 into the MePO influenced total sleep (Figure 2) by reducing total sleep for 18 hours following injection. The time of day had a significant effect on sleep, with more sleep apparent during the light phase than during the dark phase. No significant interactions were observable between the effects of injection of 1 μg and 3 μg T_3 and the effects of the time of day following injection.

The injections of 0.3 μg and 10 μg T_3 to the MePO did not significantly influence total sleep. However, a trend toward less sleep due to injection of 0.3 μg T_3 for the first 8 hours following injection as compared to injection of vehicle is apparent. Time of day significantly affected sleep, as seen in the ANOVAs for 0.3 μg and 10 μg T_3 injections.

Effects of T_3 administered to the lateral ventricle on non-REM sleep

Injections of 1 μg and 3 μg T_3 into the LV had small but statistically significant effects on non-REM sleep, whereas the effects of injections of 0.3 μg and 10 μg T_3 to the LV were not significantly different than controls (Figure 3). Time of day following injection to the LV significantly affected sleep for 0.3 μg, 1 μg, 3 μg and 10 μg T_3 doses.

Effects of T_3 administered to the median preoptic nucleus on non-REM sleep

Microinjections of 1 μg and 3 μg T_3 had significant effects on non-REM sleep (Figure 4). Less non-REM sleep was apparent in the first 18 hours after 1 μg and 3 μg T_3 injections compared to injections of the vehicle solution. The effect of time of day after injection was significant with the administration of 1 μg and 3 μg T_3, with less non-REM sleep apparent during the dark phase.

The injections of 0.3 μg and 10 μg T_3 did not have significant effects on non-REM sleep . A trend existed toward less non-REM sleep after the 10 μg T_3 injection from hours 4 to 12 than after injection of the vehicle solution. Time of day after injection significantly affected sleep with the administration of 0.3 μg and 10 μg T_3 , with less non-REM sleep apparent during the dark phase.

Effect of T_3 administered to the lateral ventricle on REM sleep

The effect of an injection of 0.3 μg T_3 to the MePO on REM sleep was statistically significant but the injections of 1μg, 3μg, and 10 μg T_3 had no significant effect on REM sleep (Figure 5). A significant effect of time of day on REM sleep was observable in the 3 μg T_3 dose condition. However, no significant effect on REM sleep occurred due to the time of day at the 0.3 μg, 1 μg and 10 μg doses of T_3.

Effects of T_3 administered to the median preoptic nucleus on REM sleep

The injection of 3 μg T_3 and 10 μg T_3 to the MePO had a significant effect on REM sleep (Figure 6). The effect of the time of day was significant after the 10 μg T_3 dose.

The effects of 0.3 μg and 1 μg T_3 injections to the MePO did not significantly affect REM sleep, but the data reflecting the injections of 0.3 μg and 3 μg T_3 showed a trend of lower amounts of REM sleep. The time of day did not have a significant effect on REM sleep following the 0.3 μg and 1 μg T_3 injections.

Effects of T_3 administered to the lateral ventricle on locomotor activity

The effects of the administration of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 to the LV on activity were not significant (Figure 7). Time of day significantly affected locomotor activity at the 0.3 μg, 1 μg, 3 μg, and 10 μg levels of T_3 injection, and no significant interactions between time of day and injection condition were found.

Effects of T_3 administered to the median preoptic nucleus on locomotor activity

The effects of 0.3 μg, 1 μg and 3 μg T_3 injections to the MePO on activity significantly raised the level of activity (Figure 8). The effect of time of day significantly affected locomotor activity. While no significant effect of 10 μg T_3 injection to the MePO on activity was found, time of day significantly affected activity.

Effects of T_3 administered to the lateral ventricle on core body temperature

The effects of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 injections to the LV on temperature did not significantly affect core body temperature (Figure 9). Significant effects due to the time of day existed for all injection levels of T_3.

Effects of T_3 administered to the median preoptic nucleus on core body temperature

A significant rise in body temperature due to the 0.3 μg T_3 injection to the MePO occurred, but no significant effect due to injections of 1 μg, 3 μg and 10 μg T_3 (Figure 10). The time of day did not significantly affect body temperature at the 0.3 μg, 1 μg, 3 μg and 10 μg levels of T_3 injections.

Discussion

Our results indicate that thyroid hormone acutely injected into the MePO of a hypothyroid adult rat decreased total and non-REM sleep and, at higher doses, increased REM sleep. The hormone, when injected to the MePO, also significantly increased locomotor activity and, at low doses, increased core body temperature.

These results are consistent with a growing body of evidence for nongenomic neurotransmitter-like effects of T_3 in adult brain. Thyroid hormone is taken up from the body and distributed to discrete brain areas where it is enzymatically converted to an active (Dratman et al., 1976, Dratman and Crutchfield, 1978, Crutchfield and Dratman, 1983, Dratman et al., 1987, Bergmann et al., 1989, Rozanov and Dratman, 1996, Campos-Barros et al., 1997, Abe et al., 1998, Sarkar and Ray, 1998, Gao et al., 1999, Friesema et al., 2003, Sugiyama, 2003). Many of the brain nuclei to which thyroid hormone is distributed are GABAergic (McGinty and Szymusiak, 2003). $GABA_A$ receptors have a known role in the regulation of sleep, as $GABA_A$ receptor-positive ligands are well-known to increase sleep. The preoptic area is a demonstrably important target region of the hypnotic benzodiazepine triazolam (Mendelson et al., 1989) and is has a high density of $GABA_A$ receptors (McGinty and Szymusiak, 2003). The mode of

action of benzodiazepines is to bind to a distinct site on the $GABA_A$ receptor complex and enhance the effect of GABA, increasing Cl- permeability. Thyroid hormone, when introduced to the $GABA_A$ receptor complex subunit arrangement common in the preoptic area, inhibits the flow of Cl-, and is thus described as GABA-negative (Martin et al., 1996, Chapell et al., 1998). In a behavioral context, the expected GABA-negative effect of thyroid hormone injection to the same brain region in which GABA-positive agents increase sleep would be a decrease in sleep, which is the primary observation of this experiment. The results of the experiment thus provide the first unequivocal demonstration of the acute effect of thyroid hormone in brain tissue on sleep behavior.

On a larger scale, the relationship between basal forebrain/ preoptic area sleep regulatory sites and brainstem monoaminergic control is expressed in the “sleep switch” model, put forth by Saper et al (2001, 2005). The sleep switch describes a bistable state transition mechanism between waking and the different stages of sleep. The switch is a mutually inhibitory model consisting of the wakefulness-promoting monoaminergic regions including the tuberomamillary nucleus (TMN), the locus coeruleus (LC), and the dorsal raphe nucleus (DR), versus the ventrolateral preoptic nucleus (VLPO) and the extended ventrolateral preoptic nucleus (eVLPO). During wakefulness, the TMN, LC, and DR fire rapidly (McGinty and Harper, 1976, Vanni-Mercier et al., 1984, Aston-Jones et al., 1991, Steininger et al., 1999). According to the model, the TMN projects to the VLPO and inhibits it with GABA, galanin, and/or endomorphin. The VLPO is unaffected by TMN's other neurotransmitter, histamine (Airaksinen et al., 1992). The VLPO inhibits the TMN with GABA and galanin (Sherin et al., 1998). The eVLPO's afferents are the

LC and DR, which are adrenergic and serotonergic, respectively, and are inhibitory to the eVLPO (Airaksinen et al., 1992, Gallopin et al., 2000, Chou et al., 2002). Further pressure in favor of wakefulness comes from the wake-active orexin neurons in the lateral hypothalamic area (LHA), which projects directly to the TMN, thereby stimulating it (Sherin et al., 1998). The LHA may indirectly inhibit the VLPO by enhancing the inhibitory adrenergic projections to it (Saper et al., 2001).

The MePO's efferents project to the VLPO and the eVLPO, the proximal and distal dendrites of the orexin neurons in the LHA, the LC, and the lateral division of the DR (Uschakov et al., 2007). The sleep-active nature of the MePO suggests that the monoaminergic and orexinergic regions are inhibited by the MePO and that the VLPO and eVLPO are stimulated during sleep. Unfortunately, the arousal system afferents to the MePO have not been completely elucidated. Recently, Suntsova et al (2007) found that muscimol, a GABA agonist, inhibits sleep when injected into the MePO. Injection of muscimol to the MePO also is accompanied by an increase in Fos activity in the orexin neurons in the LHA (Kumar et al., 2008). Injection of bicuculline, a $GABA_A$ receptor antagonist, to the MePO enhanced sleep (Suntsova et al., 2007). These data conflict with previous data related to the role of GABAergic systems in the MePO and are inconsistent with our interpretation of current data in terms of GABA-negative effects of thyroid hormone on sleep.

The possibility also exists that T_3 may act on and have similar actions to central noradrenergic systems. Immunohistochemical staining shows a coincident localization of T_3 with noradrenergic systems in brain dependent upon the presence and function of the

locus coeruleus, providing a morphological basis for a co-transmitter or neuromodulatory role of T_3 related to NE (Dratman et al., 1987). The MePO is a projection site for the noradrenergic system. Afferents from the adrenergic organum vasculosum of the lamina terminalis (OVLT), an important nucleus in the regulation of body fluid volume, increase the concentration of norepinephrine (NE) in the MePO when stimulated (Ushigome et al., 2004). Infusion of bicuculline, the $GABA_A$ antagonist, to the MePO also increases the MePO level of NE, while infusion with muscimol decreases it (Sakamaki et al., 2003). Kolaj et al (2001) demonstrated that NE applied to MePO cells caused an α2 receptor-mediated suppression of N-type calcium channels in high-voltage-activated (HVA) current channels, effectively modulating excitability in this region in a certain electrophysiological state. The possible action of T_3 in this area is, thus, somewhat ambiguous. The possibility remains that, if T_3 were to act as a GABA-negative ligand, its action would be to tonically disinhibit the region, priming the HVA cells to be further inactivated by NE and, if consistent with the data of Suntsova et al (2007), to decrease sleep.

Several lines of evidence point to differing roles that T_3 may have in sleep behavior. MePO cells are more active just before the onset of sleep, during non-REM, and during REM sleep, than during waking (Suntsova et al., 2002). Injection or infusion of GABA-positive and GABA-negative ligands show differing effects on sleep. Our results show a decrease in sleep due to injection of thyroid hormone to the MePO. The MePO has efferents to the majority of nuclei that make up the proposed sleep switch model. This nature of thyroid hormone's action could be explained by inhibition or

disinhibition of GABA receptors, or by another mechanism that is protein phosphorylation-based. The behavioral correlates of other studies imply that T_3 has an effect on a non-genomic membrane receptor, and suggests a role for T_3 in regulating sleep. As discussed earlier, the symptoms of the hypothyroid condition include disturbed sleep, but the experimental details do not form a physiological consensus. In the present study, the experimental conditions depleted background thyroid levels in blood serum and in brain in order to exaggerate any effects T_3 might have in brain following acute microinjection to the MePO. The results of this study, thus, provide the first demonstration of the acute effects of T_3 on the MePO and, more generally, on sleep.

Fig 1: Sleep over 24 hours after injection to LV (Control Rats)

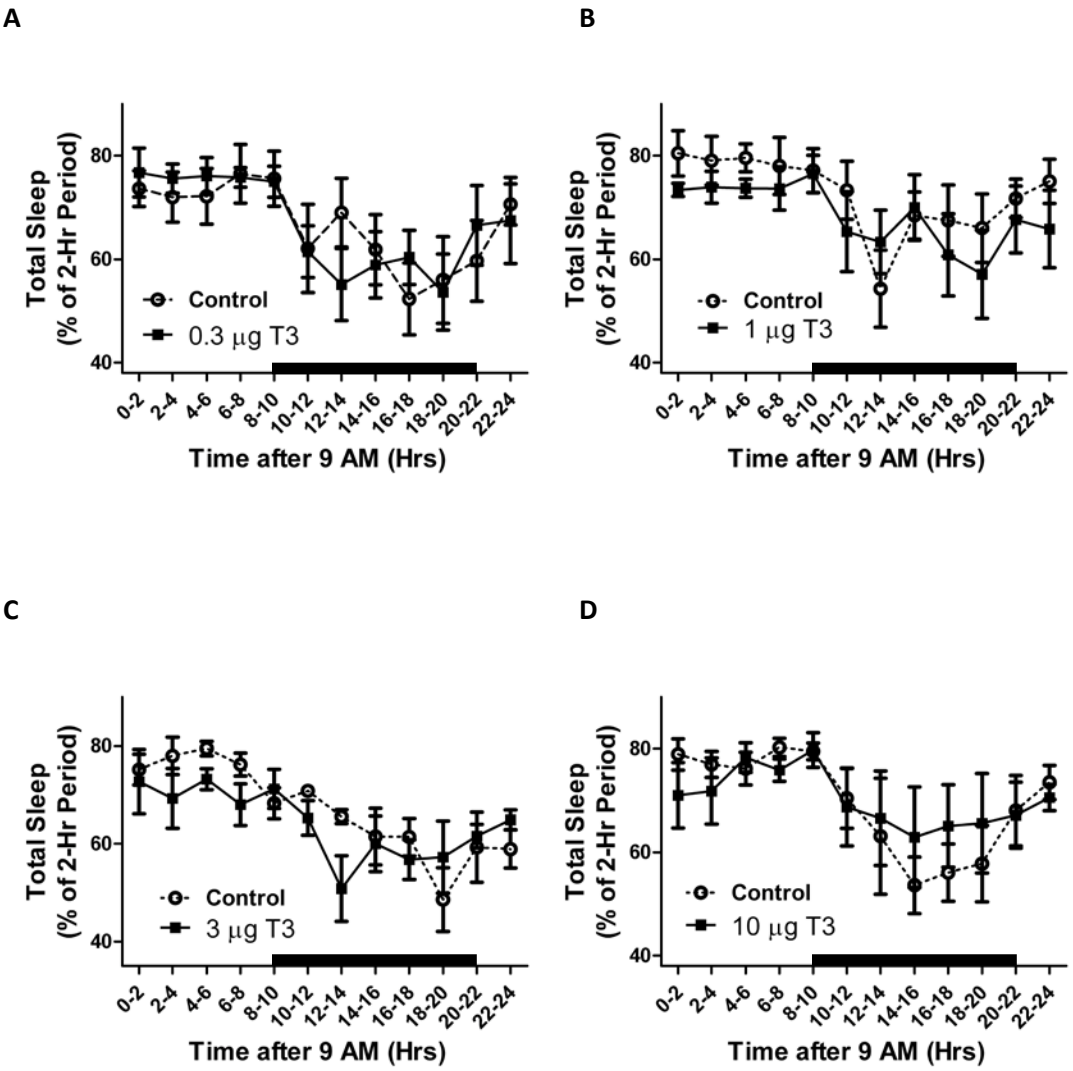


Figure 1 (A-D) for Total Sleep over 24 hours after injection to LV (Control Rats)

Total sleep was expressed as the percent of time spent in that state over a 2-hour period. The graphs show the sleep behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. Injections of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 into the lateral ventricle did not significantly affect total sleep ($F_{1,96} = .002$, $p<0.97$; $F_{1,96} = 3.27$, $p<0.08$; $F_{1,96} = 2.07$, $p<0.16$; $F_{1,96} = .09$, $p<0.77$ respectively). There was a significant effect by time of day on total sleep in the rats receiving doses 0.3 μg, 1 μg, 3 μg and 10 μg T_3 injections ($F_{11,96} = 3.50$, $p<0.0005$; $F_{11,96} = 2.60$, $p<0.007$; $F_{11,96} = 5.63$, $p<0.0001$; $F_{11,96} = 2.83$, $p<0.004$

respectively).

Fig 2: Total Sleep over 24 hours after injection to MePO (Experimental Rats)

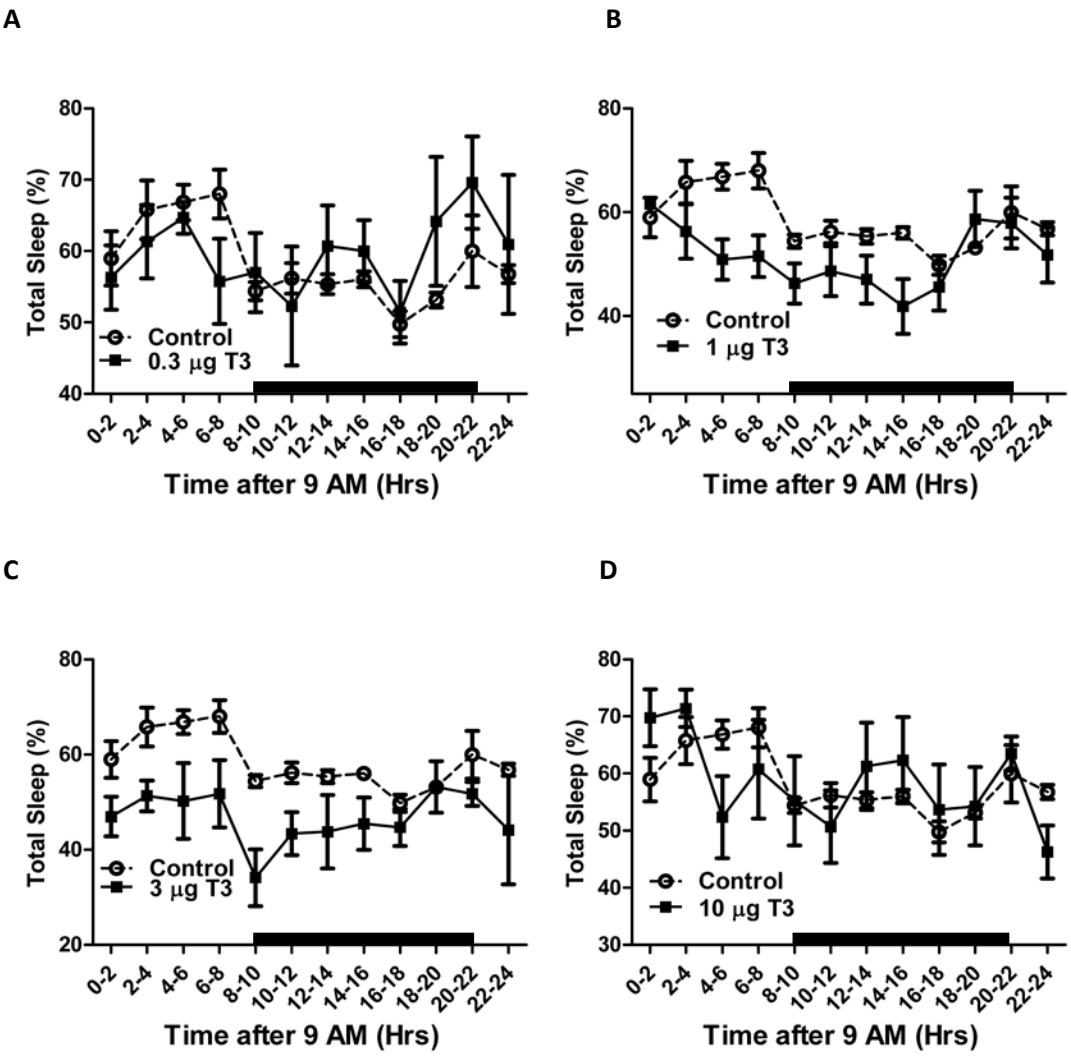


Figure 2 (A-D) for Total Sleep over 24 hours after injection to MePO (Experimental Rats)

Total sleep was expressed as the percent of time spent in that state over a 2-hour period. The graphs show the sleep behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. The effects of the injection of 1 μg and 3 μg T_3 into the MePO on total sleep were significant ($F_{1, 144} = 20.44$, $p<0.0001$; $F_{1, 126} = 42.27$, $p<0.0001$ respectively). The data corresponding to the administration of 1 μg and 3 μg T_3 reflected a significant effect due to time of day ($F_{11, 144} = 3.24$ $p<0.0006$; $F_{11, 126} = 2.55$, $p<0.007$ respectively).

The effects of 0.3 μg and 10 μg T_3 injections into the MePO on were not significant on total sleep ($F_{1, 111} = 0.41$, $p<0.53$; $F_{1, 132} = 0.0002$, $p<0.99$ respectively). There was a

significant effect due to time of day for these doses ($F_{11, 111} = 2.34$; $p<0.02$; $F_{11, 132} = 2.72$, $p<0.04$ respectively).

Fig 3: Non-REM sleep over first 24 hours after injection to LV (Control Rats)

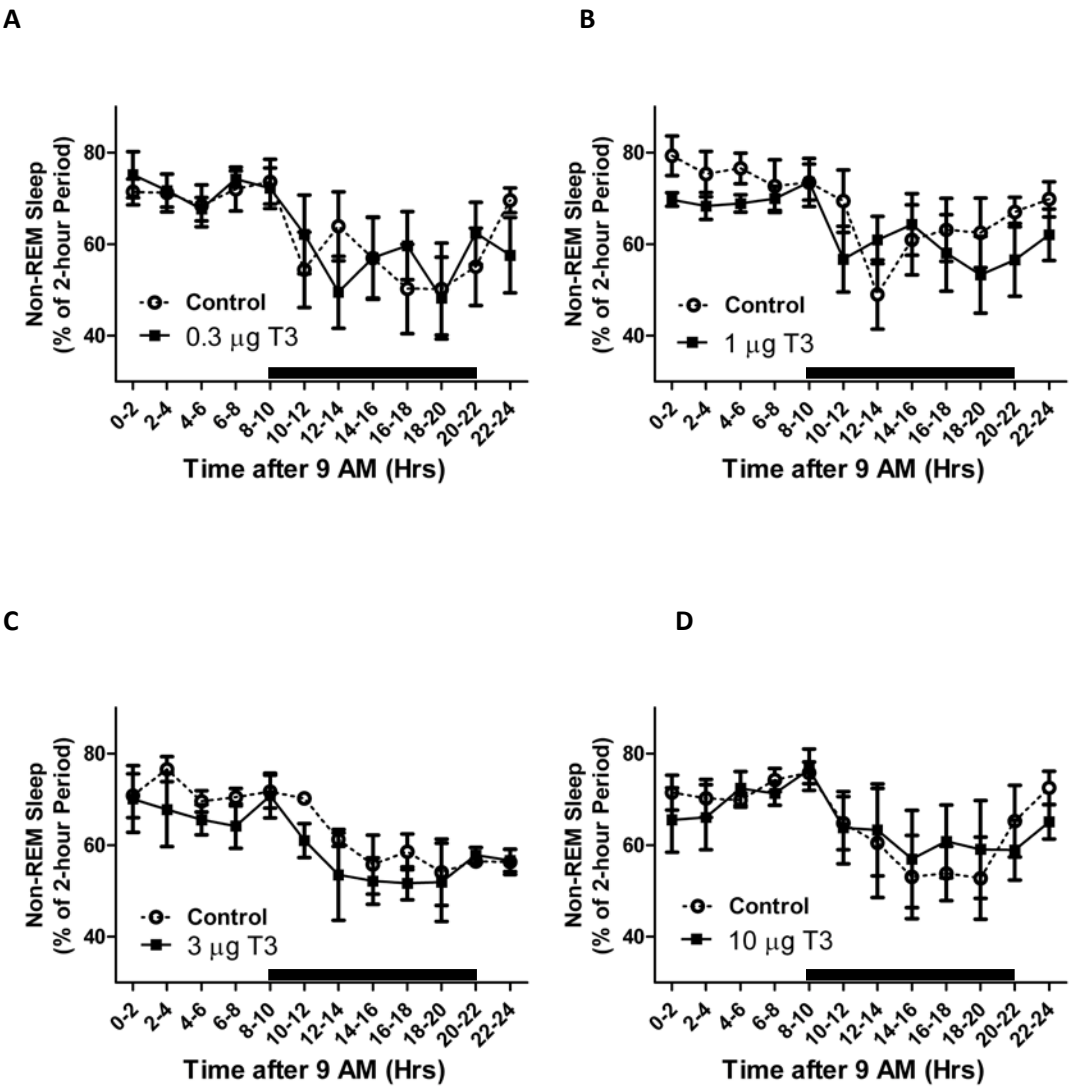


Figure 3 (A-B) for Non-REM sleep over first 24 hours after injection to LV (Control Rats)

Non-REM sleep was expressed as the percent of time spent in that state over a 2-hour period. The graphs show the sleep behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. Both the injections of 1 μg and 3 μg T_3 had small but statistically significant effects ($p= 0.05$) on non-REM sleep ($F_{1,96} = 3.97$; $F_{1,96} = 4.07$ respectively), whereas the injections of 0.3 μg and 10 μg T_3 had no significant effect ($F_{1,96} = .0004$, $p<0.99$; $F_{1,96} = .002$; $p<0.90$ respectively). Time of day significantly affected non-REM sleep for 0.3 μg,

1 μg, 3 μg and 10 μg T_3 injections ($F_{11, 96} = 3.06$, $p<0.002$; $F_{11, 96} = 2.67$, $p<0.006$; $F_{11,96} = 4.50$, $p<0.0001$; $F_{11,96} = 1.98$, $p<0.04$ respectively).

Fig 4: Non-REM sleep over first 24 hours after injection to MePO (Experimental Rats)

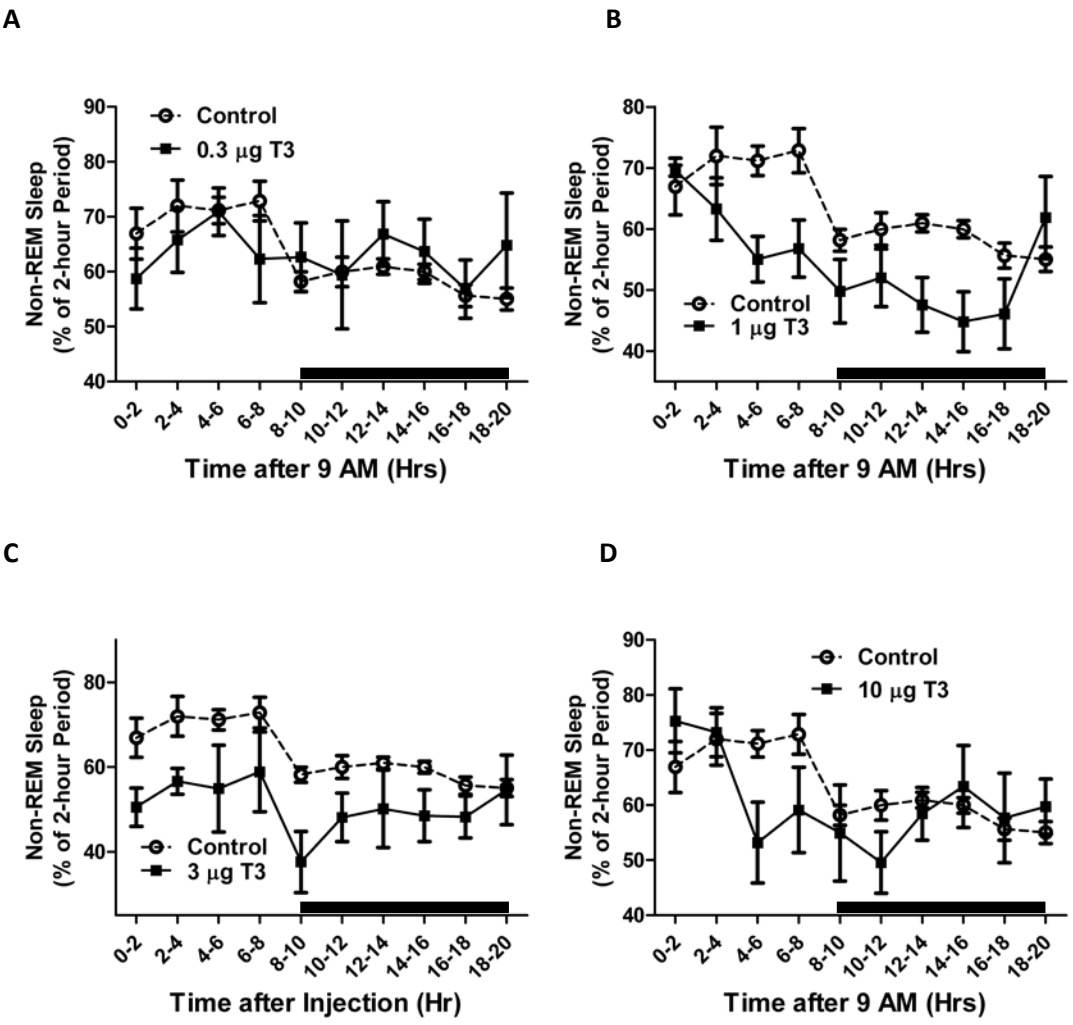


Figure 4 (A-B) for Non-REM sleep over first 24 hours after injection to MePO (Experimental Rats)

Non-REM sleep was expressed as the percent of time spent in that state over a 2-hour period. The graphs show the sleep behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. The injections of 1 μg and 3 μg T_3 had significant effects on non-REM sleep ($F_{1, 120} = 22.98$, $p<0.0001$; $F_{1, 109} = 29.13$, $p<0.0001$ respectively). The effect of time of day significantly affected non-REM sleep in rats receiving doses of 1 μg and 3 μg T_3 ($F_{9, 120} = 5.24$, $p<0.0001$; $F_{9, 109} = 2.56$, $p<0.02$ respectively).

The injections of 0.3 μg and 10 μg T_3 to the MePO did not have significant effects on non-REM sleep ($F_{1, 95}$ = .0008, $p<0.98$; $F_{1, 110}$ = 1.65, $p<0.21$ respectively). There was a trend indicating less non-REM sleep for the 10μg T_3 injection from 4 to 12 hours compared to the vehicle solution. There was a significant effect of time on non-REM sleep with the administration of 0.3 μg and 10 μg T_3 to the MePO ($F_{9, 95}$ = 2.06, $p<0.05$; $F_{9, 110}$ = 3.25 $p<0.002$ respectively).

Fig 5: REM sleep over first 24 hours after injection to LV (Control Rats)

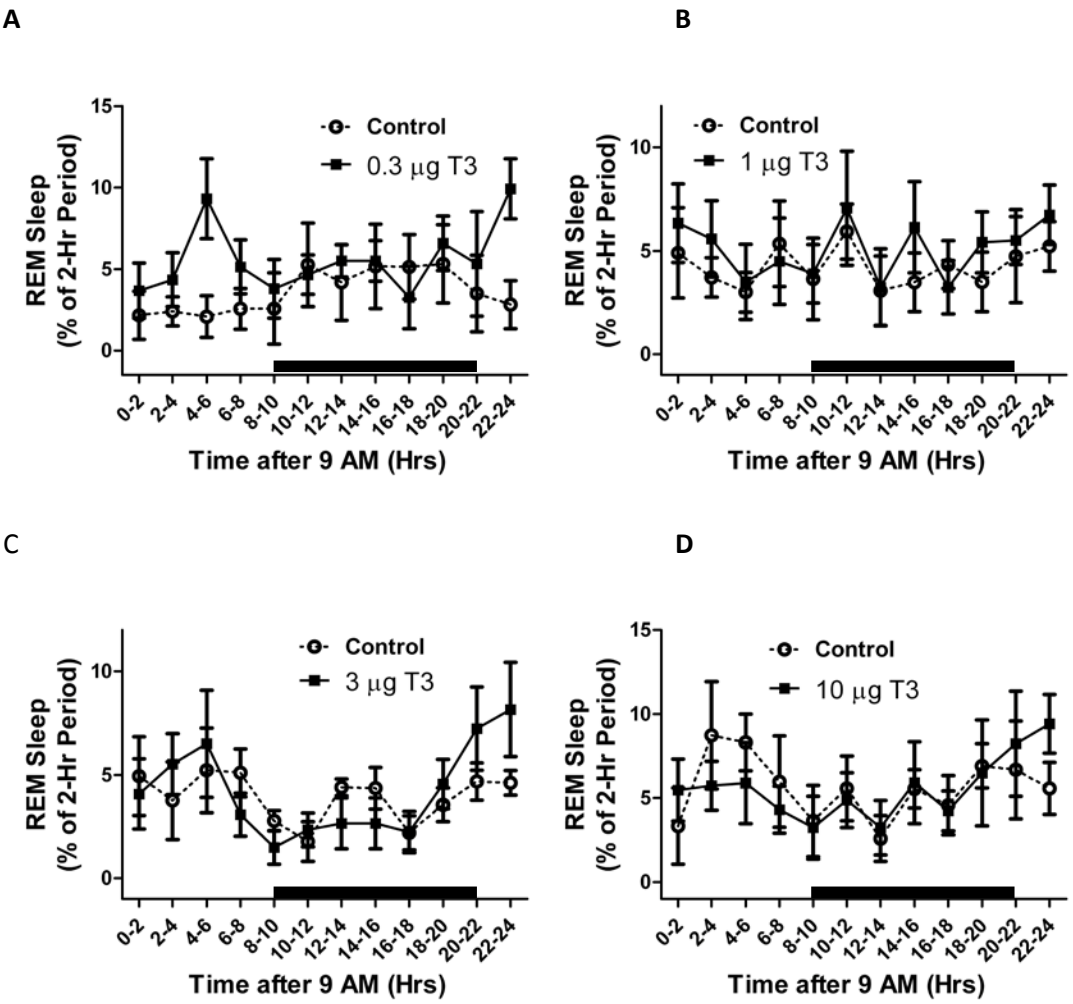


Figure 5 (A-D) for REM sleep over first 24 hours after injection to LV (Control Rats)

REM sleep was expressed as the percent of time spent in that state over a 2-hour period. The graphs show the sleep behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. The injection of 0.3 μg T_3 to the MePO on REMS was significant ($F_{1, 96} = 6.41$, p= 0.02 level), but the injections of 1μg, 3μg, and 10 μg T_3 were not significant on REM sleep ($F_{1, 96} = 1.48$, p<0.23; $F_{1, 96} = .21$, p<0.65; $F_{1, 96} = .001$, p<0.97 respectively). There was a significant effect of time on REM sleep for the 3 μg T_3 injection ($F_{11, 96} = 2.3$, p<0.02). However, there was no significant effect due to time of day on REM sleep at 0.3 μg, 1 μg and 10 μg T_3 injections ($F_{11, 96} = 0.69$, p<0.75; $F_{11, 96} = .75$, p<0.70; $F_{11, 96} = 1.18$, p<0.32 respectively).

Fig 6: REM sleep over first 24 hours after injection to MePO (Experimental Rats)

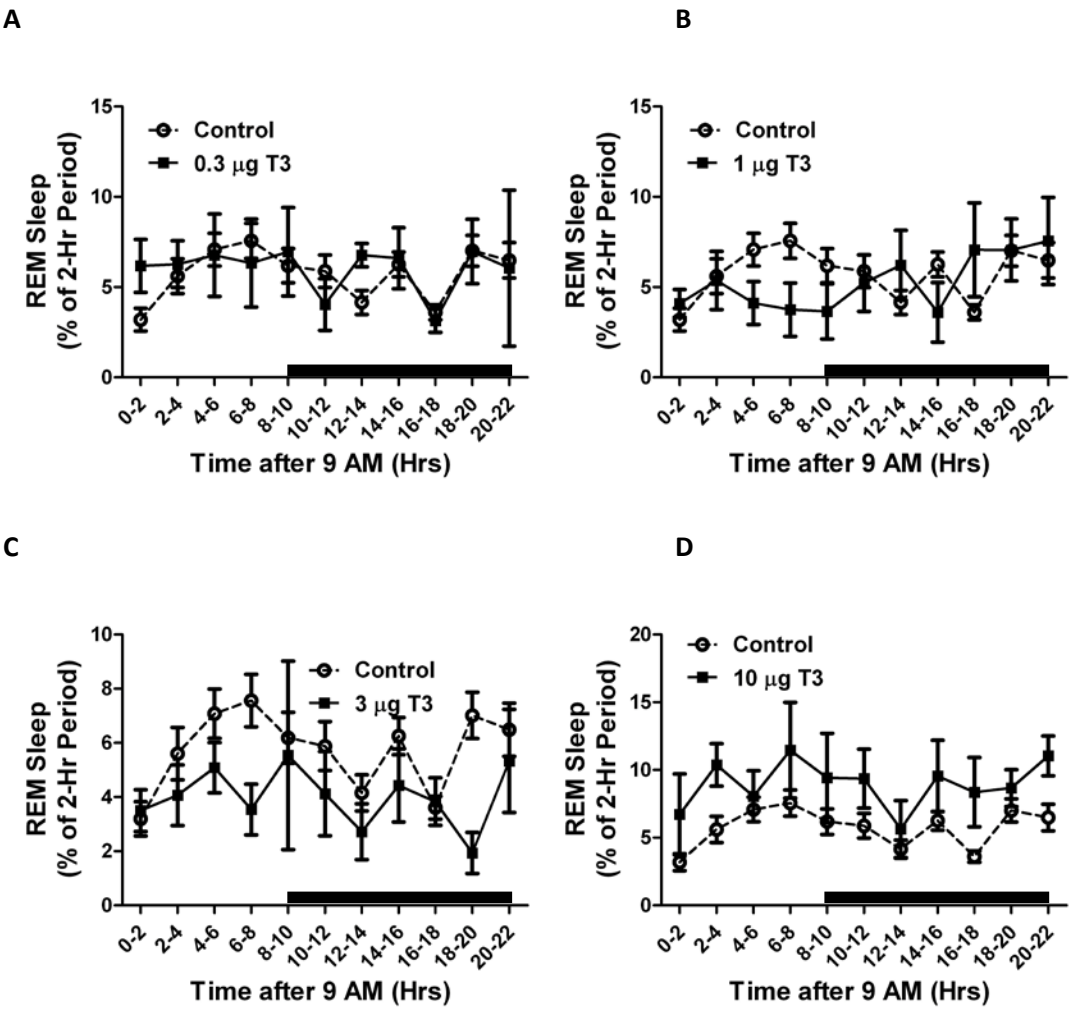


Figure 6 (A-D) for REM sleep over first 24 hours after injection to MePO (Experimental Rats)

REM sleep was expressed as the percent of time spent in that state over a 2-hour period. The graphs show the sleep behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. The effect of 3 μg T_3 injection to the MePO on REM sleep was significant ($F_{1, 326}$ = 8.532, $p<0.0038$).The effect of 10 μg T_3 injection to the MePO on REM sleep was also significant ($F_{1, 330}$ = 27.00, $p<0.0001$), with the T_3 dose yielding higher amounts of REM sleep than the vehicle dose. The effect of time was significant ($F_{10, 330}$ = 2.016, $p<0.0313$).

The effects of injection of 0.3 μg and 1 μg T_3 to the MePO were not significant on REM

sleep ($F_{1, 312} = 0.1725$, $p<0.6783$; $F_{1, 340} = 0.7547$, $p<0.3857$, respectively). There was not a significant effect of time for 0.3 μg, 1 μg and 3 μg T_3 injections ($F_{10, 312} = 1.065$, $p<0.3892$; $F_{10, 340} = 1.036$, $p<0.4125$; $F_{10, 326} = 1.097$, $p<0.3641$ respectively).

Fig 7: Activity over first 24 hours after injection to LV (Control Rats)

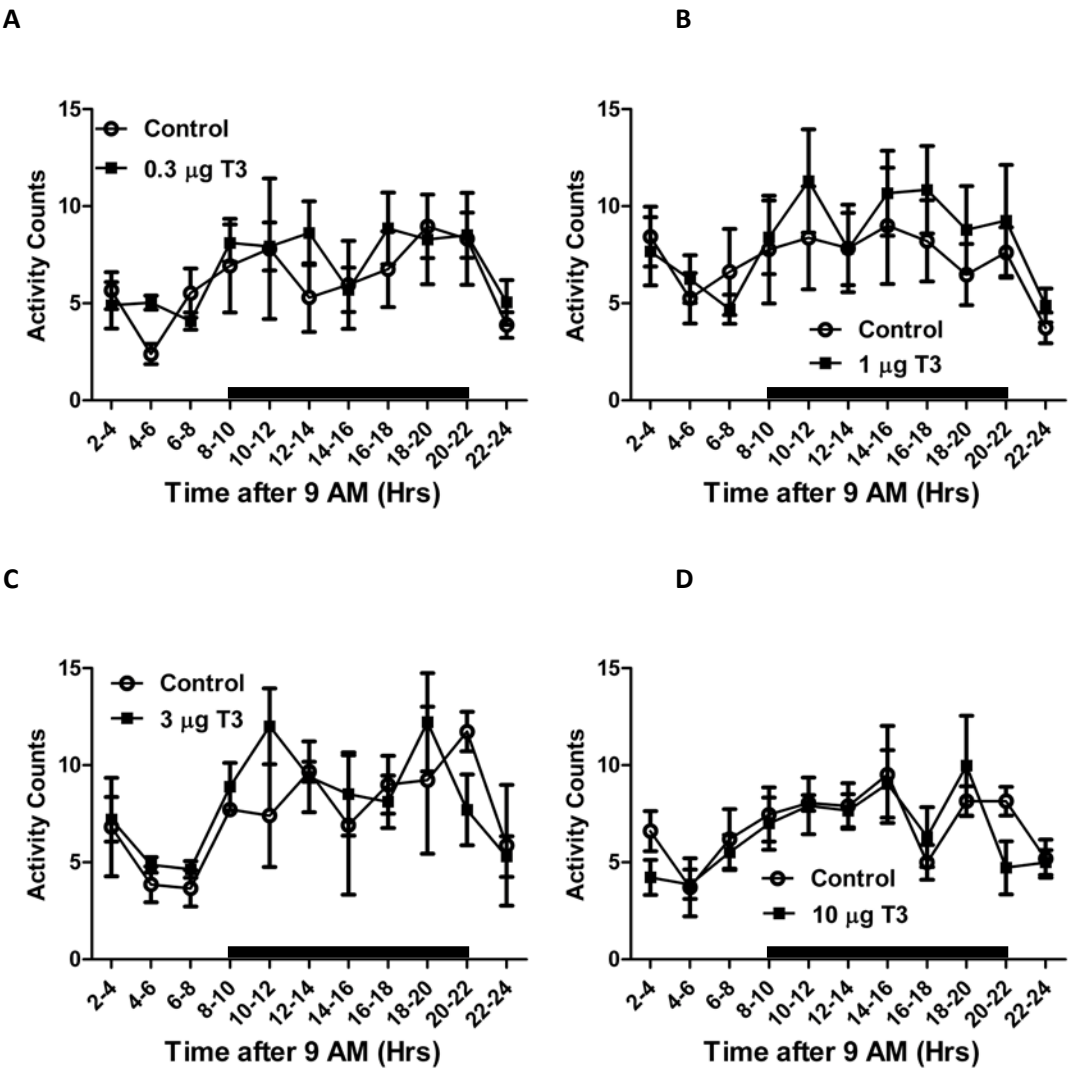


Figure 7 (A-D) for Activity over first 24 hours after injection to LV (Control Rats)

Activity was expressed as the number of activity counts over a 2-hour period. The graphs show the locomotor behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. Administration of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 to the LV were not significant on locomotor activity ($F_{1, 88} = 0.93$, $p<0.34$; $F_{1, 88} = 1.41$, $p<0.24$; $F_{1, 88} = 0.92$, $p<0.48$; $F_{1, 88} = 3.64$, $p<0.06$ respectively). The effect due to time of day was significant at 0.3 μg, 1 μg, 3 μg and 10 μg injections of T_3 ($F_{10, 88} = 2.11$, $p<0.04$; $F_{10, 88} = 2.25$, $p<0.03$; $F_{10, 88} = 2.39$,

$p<0.02$; $F_{10, 88} = 3.72$, $p<0.0004$ respectively).

Fig 8: Activity over first 24 hours after injection to MePO (Experimental Rats)

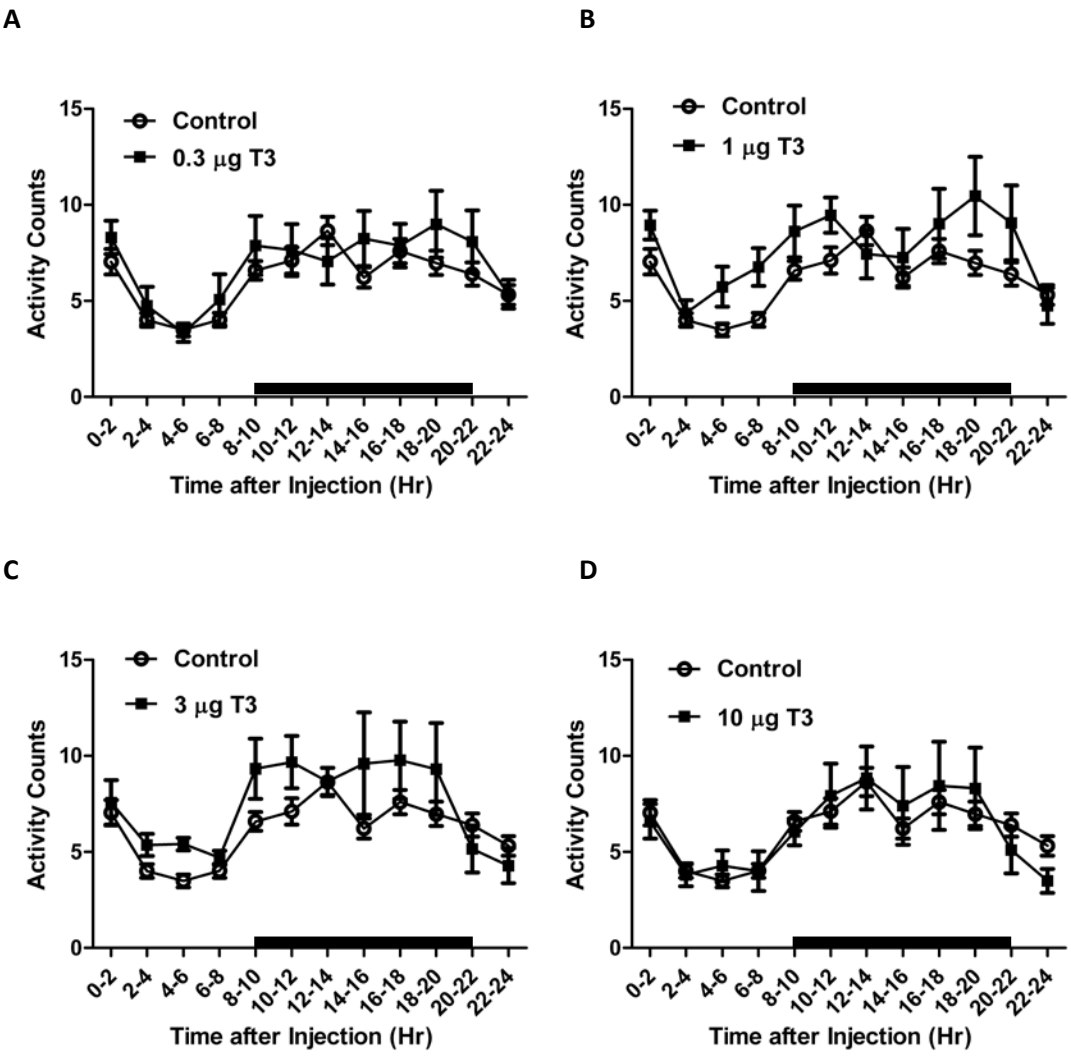


Figure 8. Activity over first 24 hours after injection to MePO (Experimental Rats)

Activity was expressed as the number of activity counts over a 2-hour period. The graphs show the locomotor behavior of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. The effects of 0.3 μg, 1 μg and 3 μg T_3 injections to the MePO on activity were significant ($F_{1, 348} = 4.60$, $p<0.04$; $F_{1, 348} = 17.90$, $p<0.0001$; $F_{1, 324} = 10.23$, $p<0.002$ respectively). The effect due to time of day was significant ($F_{11, 348} = 6.81$, $p<0.0001$; $F_{11, 348} = 6.72$, $p<0.0001$; $F_{1, 324} = 6.60$, $p<0.0001$ respectively).

There was no significant effect by the 10 μg T_3 injection to the MePO ($F_{1, 324} = 0.03$, $p<0.87$) on activity. The effect of injection was extremely significant effect of time ($F_{1,}$

$_{324}$ = 0.51, p<0.0001).

Fig 9: Temperature over first 24 hours after injection to LV (Control Rats)

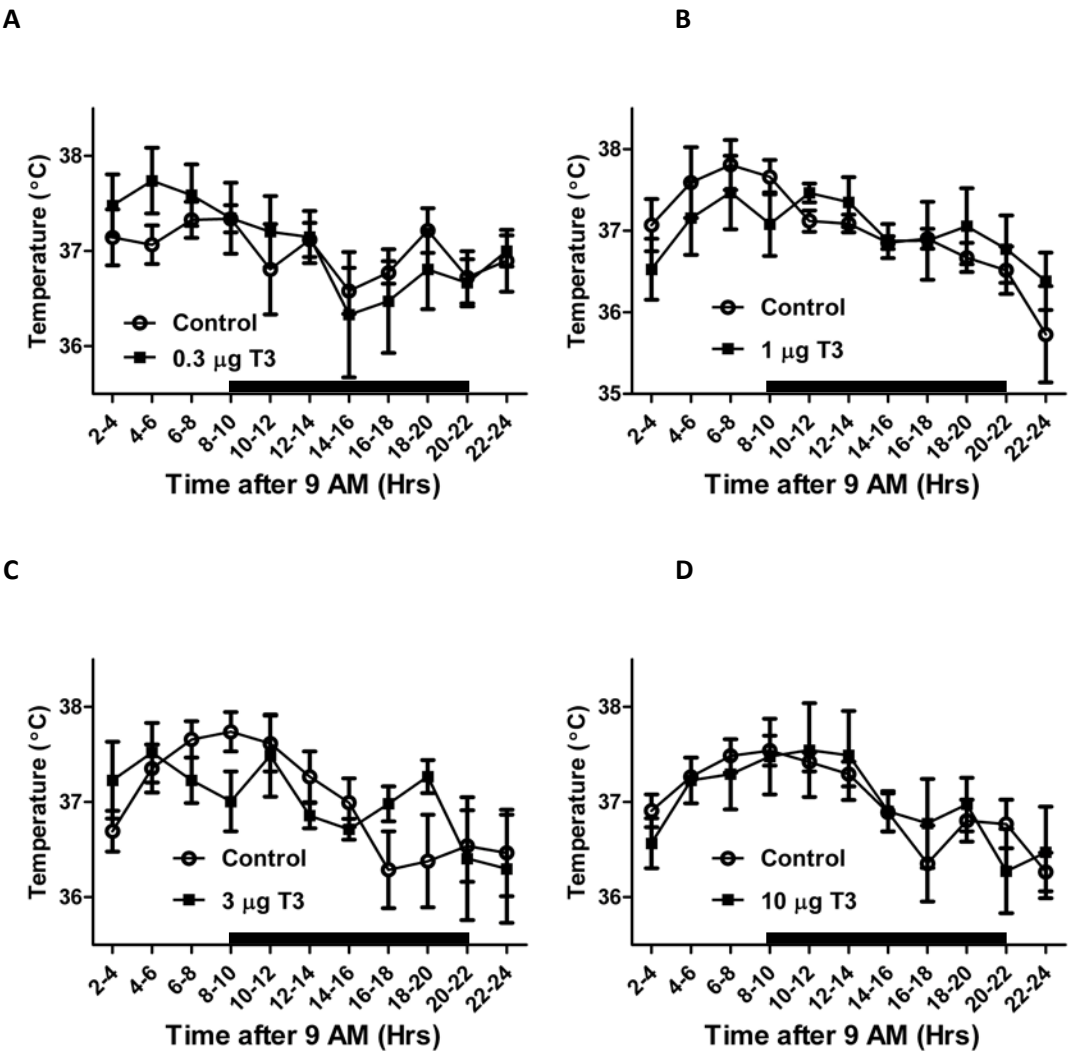


Figure 9. Temperature over first 24 hours after injection to LV (Control Rats)

Core body temperature was expressed as the average body temperature of the rats over a 2-hour period. The graphs show the average temperature of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. The effects of 0.3 μg, 1 μg, 3 μg and 10 μg T_3 injections to the LV were not significant on core body temperature ($F_{1, 88} = 0.25$, $p<0.62$; $F_{1, 88} = .0000001$, $p=1$; $F_{1, 88} = .000002$, $p<1$; $F_{1,88} = 0.00009$, $p<0.96$ respectively). There were

significant effects due to time for all injections of T_3 ($F_{10, 88} = 2.03$, $p<0.04$; $F_{10, 88} = 3.34$, $p<0.002$; $F_{10, 88} = 2.85$, $p<0.005$; $F_{10,88} = 3.67$, $p<0.0005$ respectively).

Fig 10: Temperature over first 24 hours after injection to MePO (Experimental Rats)

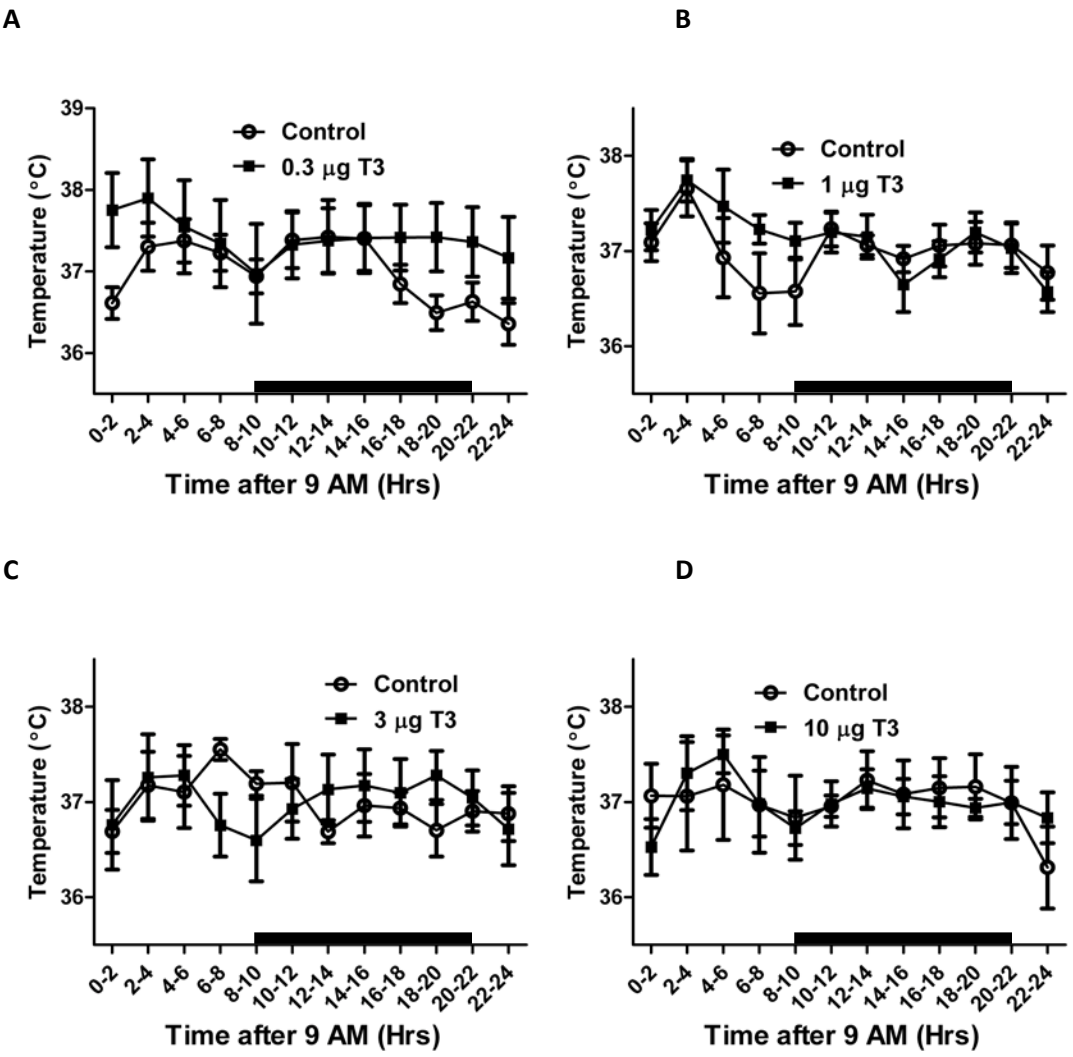
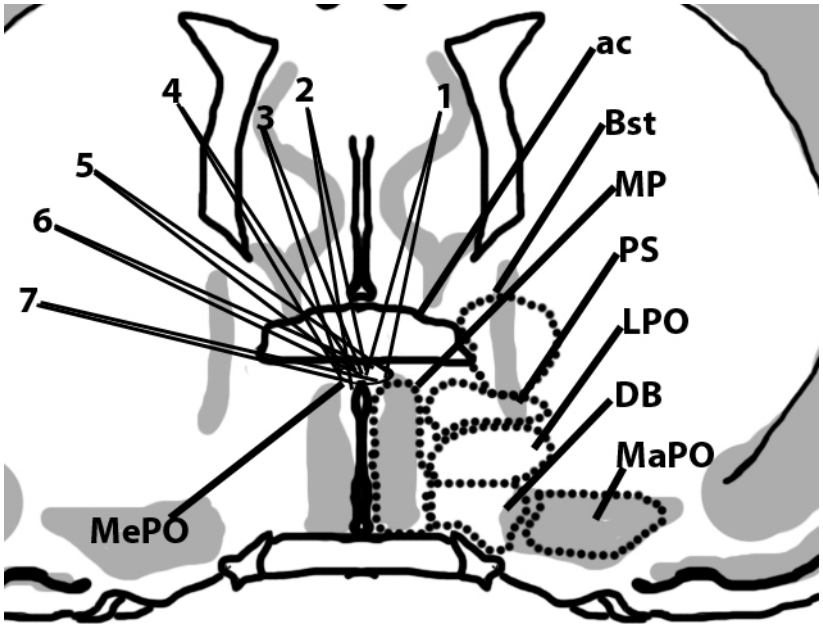


Figure 10. Temperature over first 24 hours after injection to MePO (Experimental Rats)

Core body temperature was expressed as the average body temperature of the rats over a 2-hour period. The graphs show the average temperature of rats receiving the vehicle solution (dashed lines) and those receiving a given dose of T3 (solid lines). The black bars indicate the dark period. There was a significant effect of 0.3 μg T_3 injection on temperature to the MePO ($F_{1, 144} = 6.76$, p= 0.02 level). However, there were no significant effects due to injections of 1 μg, 3 μg and 10 μg T_3 ($F_{1, 144} = 1.39$, $P<0.25$; $F_{1, 144} = 0.00003$, $p<0.99$; $F_{1, 144} = 0$, P=1 respectively).

Fig 11: Histological confirmation of cannulae placement



Seven rats histologically confirmed to have cannulae reaching the MePO. Each number points to the two cannulae terminae. MePO is labeled.

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