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Examining a Hypothyroid Model of Depressive Symptoms in Mice: Tests of Behavioral Measures

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Abstract

There is abundant evidence suggesting that Major Depressive Disorder (MDD) is closely related to thyroid hormone (TH) function, but the exact nature of this relationship is poorly defined in the literature. The present study examined whether hypothyroidism could viably model symptoms of MDD in mice using established behavioral paradigms. It was expected that hypothyroidism would produce anhedonia-like behavior in the saccharin preference test, anxiety-like behavior in the elevated plus maze, and spatial memory impairment in the object placement task. C57BL/6J mice were randomly assigned to one of three groups, which received either a control diet, diet infused with 6-propyl-2-thiouracil (hypothyroid group), or a combination of 6-propyl-2-tiouracil and thyroxine (hyperthyroid group). Each group had *ad libitum* access to food and water for 4 weeks prior to behavioral assessment. Contrary to our hypothesis, hypothyroid mice did not exhibit more anhedonia or greater spatial memory impairment than controls. However, they did spend a significantly lower percentage of time in the open arms of the elevated plus-maze compared to both the control and hyperthyroid groups. Additionally, hyperthyroidism was associated with increased preference for sweetened water over tap water in the saccharin preference test. These findings raise interesting questions about how TH could regulate specific components of the depressive phenotype, which will be discussed at length. This project also lays the groundwork for a larger investigation of glutamate neurotransmission in hypothyroidism-induced depression. As a future extension of this research, AMPA receptor binding will be examined in the obtained cortical and hippocampal tissue so that relationships between depressive behaviors, thyroid status, and glutamatergic activity can be explored in a truly integrated fashion.

Thesis Signature Page

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Examining a hypothyroid model of depressive symptoms in mice: Tests of behavioral measures

by

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Examining a Hypothyroid Model of Depressive Symptoms in Mice: Tests of Behavioral Measures

Major depressive disorder (MDD) affects over 300 million people worldwide and is the leading cause of disability across the globe (World Health Organization, 2018). This psychiatric disorder takes a major toll at both the individual and societal levels, leading to severe public health and socioeconomic burdens. The cost of the disorder – through both treatment expense and loss of productivity –has been estimated to be $210 billion per year worldwide (Aleksandrova, Phillips, & Wang, 2017) and $36.6 billion in the United States alone (Kessler et al., 2006). In a sample of 9,282 adults in the US workforce, major depression was associated with an annual average of 27.2 lost work days and $4,426 lost capital per capita. These losses do not fully reflect the social, emotional, and cognitive turmoil that accompany the disorder. Mortality rates are high with an estimated 800,000 deaths by suicide per year, and the World Health Organization lists suicide as the second leading cause of death in the 15- to 29-year-old demographic (2018).

Despite the high number of antidepressants available on the market and the general perception that modern antidepressant treatments are effective, these treatments have important limitations that must be addressed. The percentage of patients who clinically benefit from antidepressant treatment is shockingly small. In one of the largest and studies evaluating antidepressant treatments (STAR*D), it was reported that only about one-third of patients experienced remission after 4 months of treatment with standard SSRIs while another 30% were only partially responsive (Rush et al., 2006). Other large-scale studies have yielded similar concerning findings (e.g. Thase et al., 2005), with one reporting only 28% remission in a sample of over 2,800 patients (Trivedi

et al., 2006). Furthermore, standard antidepressants notably take an average of 6-8 weeks to show any clinical benefit in patients who do respond. This delay in efficacy is especially problematic given that suicide attempts are at increased risk during the first month of treatment (Murrough, 2012). It is thus widely agreed upon in the literature that although antidepressant drugs are generally thought to be effective, the low response rate and slow efficacy onset featured by these treatments represent important unmet needs for MDD patients that must be addressed.

One major challenge to this advancing pharmacological treatments for MDD is that the despite decades of research, the pathophysiology underlying MDD remains relatively unclear. Heterogeneity of symptoms, lack of consistent biomarkers, and interaction with socio-environmental variables all likely contribute to the poor understanding of its mechanisms (Duman & Aghajanian, 2012).

In the present study, the investigation of thyroid hormone (TH) effects on depressive symptoms was conducted in order to elucidate the disorder's underlying etiological processes. There is abundant evidence suggesting that depression is closely related to TH function; for example, hypothyroidism is significantly more prevalent among depressed patients than the general population, with one study reporting hypothyroidism in 52% of treatment-resistant depressed patients compared to 5% of the non-depressed controls (Wu, Chien, Lin, Chou, & Chou, 2013). The prevalence of depression among hypothyroid patients has additionally been reported to be as high as 63% (e.g. Chaudhary, Chabra, Singla, Mishra, & Sharma, 2014; Bathla, Singh, & Relan, 2016), which suggests that low TH levels are associated with depressive symptoms. However, the precise nature of the relationship between hypothyroidism and specific

MDD symptoms remains poorly defined in the literature – likely due to the great complexity of both disorders and methodological differences across studies. It was our hope that further clarity about TH's specific role in depressive behaviors would, in turn, provide further clarity about the processes behind the depressive behaviors themselves.

Recent work in the depression literature has highlighted the importance of intracellular signaling cascades, neuroplasticity, and synaptogenesis-induced activity in the neuropathology of MDD (e.g. Machado-Vieira et al., 2009; Duman & Aghajanian, 2012). Thyroid hormones, which regulate metabolism, growth, and development in virtually every cell in the body, are highly likely to be implicated in the dysfunction of these processes. Another notable line of research has identified the glutamatergic system as an important target for antidepressant action; a system which, importantly, has been found to be regulated by TH activity as well (e.g. Mendes-de-Aguiar et al., 2008; Zarif, Petit-Paitel, Heurteaux, Chabry, & Guyon, 2016). Support for the role of glutamate in MDD comes from reports of robust antidepressant properties of ketamine, which is an antagonist of the glutamate receptor N-methyl-D-aspartate (NDMA). In a groundbreaking and widely replicated clinical study, Berman et al. (2000) demonstrated that a single low dose of ketamine significantly reduced symptoms in treatment-resistant MDD patients in only 72 hours. Ketamine itself is associated with dissociative and psychotomimetic side effects that make it an inappropriate treatment for MDD (Aleksandrova et al., 2017), but examining the mechanisms behind its rapid effects can aid in the identification of new drug targets and more effective treatments. Subsequent studies have used ketamine to investigate potential roles of the glutamate receptor AMPA, the inhibitory neurotransmitter GABA, NMDA-mediated long-term potentiation (LTP) processes, and

factors important for cell growth like brain-derived neurotrophic factor (BDNF). The following review outlines how each of these factors relate not only to the pathology of depression, but also to the various components of thyroid function.

Literature Review

Relationships between thyroid conditions and psychiatric disorders have long been observed, and records of these observations can be traced back as early as 1786 (Bahls & Carvalho, 2004). In 1949, Asher published descriptions of 14 clinical cases where hypothyroidism accompanied severe melancholic and psychotic states – a condition that he termed "myxedema madness." Importantly, treatment of the hypothyroidism with thyroid hormones (THs) alleviated the psychiatric symptoms as well (Asher, 1949). Several lines of empirical research since then have similarly examined the hypothyroid-mood relationship. Many of the resulting findings – such as antidepressant-like properties of THs, abnormal TH levels among depressed patients, and depressive symptoms associated with hypothyroidism – support our hypothesis that induced hypothyroidism can model MDD symptoms in animal studies. Since the present research is being done in the context of finding more effective antidepressant treatments, research that explores the underlying mechanisms THs and MDD have in common is particularly relevant. The following review examines some of the work that forms the basis of this project.

Overview of the Hypothalamic-Pituitary-Thyroid (HPT) Axis

First, it is crucial to have a basic understanding of THs and their production, secretion, and function throughout the body. THs are regulated through a complex interplay of hormones in the hypothalamic-pituitary-thyroid axis (Figure 1). Briefly,

neurons in the hypothalamus produce thyrotropin-releasing hormone (TRH) which is released to thyroroph cells in the anterior pituitary. This stimulates the synthesis and release of thyroid stimulating hormone (TSH); which, in turn, stimulates TH production in the thyroid gland. High levels of thyroid hormone in the bloodstream reduces the sensitivity of pituitary thyrotropes to TRH, which decreases the rate at which TSH is secreted. In this way, THs regulate TSH synthesis through a negative feedback loop (Rao et al., 1996; Nussey & Whitehead, 2001). Abnormal levels of any one of these hormones – as well as irregularities in any of their many receptors, enzymes, and protein transporters – can therefore threaten the careful maintenance of this system (Hage & Azar, 2012). Indeed, as this review will address, there are several different ways by which TH function is reportedly altered in depressed patients.

The two major THs produced in the thyroid gland are triiodothyronine (T3) and thyroxine (T4). Production starts with iodide ions being actively transported from the plasma to follicular cells in the thyroid, where they are oxidized by the enzyme thyroperoxidase (TPO) to form atomic iodine molecules (Nussey & Whitehead, 2001). TPO is also responsible for using these molecules to iodinate the tyrosine residues of thyroglobulin, which is a glycoprotein synthesized in the thyroid follicles. Once iodinated, adjacent tyrosine residues combine with one another (a process also initiated by TPO) to form the thyroid hormones. Combinations containing three iodides result in the hormone T3, while combinations containing four iodides result in T4 (Nussey & Whitehead, 2001). With regard to the HPT axis, TSH influences TH levels by stimulating various processes essential for TH synthesis such iodine uptake, production of the enzyme TPO, and the release of TH into the bloodstream.

TH mechanisms of action. Once THs are released by the thyroid into the bloodstream, their actions continue to be dependent on a number of factors. One such factor is the conversion of T4 into T3. Although T4 makes up about 90% of the TH secreted by the thyroid gland, T3 is the more biologically active hormone (10 times more so than T4) and has a higher affinity for nuclear receptors. T4 is more likely to be bound to proteins in the plasma like thyroxine-binding globulin (resulting in less "free" T4) – and when it does enter cells, it is immediately converted to the active hormone T3 through deiodination (Nussey & Whitehead, 2001). Deiodinase enzymes, which alter TH activity by removing iodine molecules, are therefore crucial components of TH action. Deiodinases types 1 and 2 (D1, D2) convert T4 into the more active T3, while deiodinase type 3 (D3) converts T3 into the biologically inactive reverse T3 (rT3) (Nussey & Whitehead, 2001; Leach & Gould, 2015). D2 activity in glial cells, particularly astrocytes, provides most of the T3 needed for neuronal function (Leach & Gould, 2015).

Thyroid hormone receptors (TRs) in the nuclei of target cells also play an important role in thyroid function. THs primarily exert their action through the binding of T3 to TRs, which bind to specific DNA sequences and initiate gene transcription. Recent work has found that thyroid hormone receptors can additionally act without THs present, mainly through inhibitory mechanisms that suppress gene expression (Buras, Battle, Landers, Nguyen, & Vasudevan, 2013; Venero et al., 2005).

Finally, thyroid hormone transporters – such as the monocarboxylate transporters 8 and 10 (MCT8, MCT10) and the organic anion-transporting polypeptides (OATP) – are proteins that allow THs to cross the membranes into target cells (Raymaekers & Darras, 2017). MCT8 is especially important for transport of TH through the blood-brain barrier

and CSF; in fact, MCT8 mutations in humans are associated with severe developmental delays and neurological damage (Bernal, 2005). One study found that D2 and MCT8 are both crucial for normal activity in the mouse cortex (cited in Leach & Gould, 2015), which highlights how these various enzymes and transporters are just as important to proper thyroid function as the THs themselves.

TH dysfunction. The importance of thyroid hormones can be demonstrated through the detrimental effects that result from their abnormal functioning. Too much TH synthesis and secretion leads to hyperthyroidism, characterized by symptoms like weight loss, fatigue, increased heart rate, and tremors. Too little leads to symptoms associated with hypothyroidism like weight gain, dry skin, hair loss, fatigue, low energy levels, and overall weakness (Nussey & Whitehead, 2001).

Proper TH function is particularly essential during fetal and postnatal development, and deficiencies during critical time windows can cause permanent neuronal damage. For example, thyroid hormone dysfunction in pregnancy has been found to impact crucial processes like neurogenesis, cell migration, and axonal myelination in rats (Bernal, 2015). In humans, maternal iodine deficiency – which results in decreased TH production to the fetus – is associated with impairments like ADHD, decreased intelligence, and impaired mental and motor functioning (Morreale de Escobar, Obregon, & Escobar del Rey, 2004). Recent work suggests that THs influence development and neurogenesis in the adult brain as well, making adult-onset thyroid disorders of serious concern (Remaud, Gothie, Moryan-Dubois, & Demeneix, 2014; Leach & Gould, 2015).

As described above, proper thyroid function involves multiple components. The stimulatory and inhibitory effects that TRH, TSH, T4, and T3 have on one another emphasizes how complex this system is, and abnormalities within the system could therefore have various underlying causes. This study focuses on hypothyroidism as a potential model of depression, but it should be noted that there are three main forms of the disorder. Primary, or overt hypothyroidism, involves having low concentrations of TH with an increased level of TSH. Thyroid tissue is thus stimulated by TSH, but complications in the thyroid gland (as well as antibodies against crucial elements of TH synthesis like TPO and thyroglobulin) result in fewer THs being synthesized and secreted (Nussey & Whitehead, 2001). Central hypothyroidism is characterized by low TH and TSH levels, wherein the gland is insufficiently stimulated due to defects in the pituitary (secondary hypothyroidism) or hypothalamus (tertiary hypothyroidism). Subclinical hypothyroidism (SCH) is considered to be milder, where TSH levels are elevated but serum T3 and T4 appear normal (Forman-Hoffman & Philibert, 2006).

Thyroid dysfunction is a significant health problem worldwide, with one review estimating its prevalence in 10% of the total population (Leach & Gould, 2015). A report on its epidemiology found specific populations and regions to be differentially affected by thyroid disorders, with economic advancement and iodine nutrition being key determining factors (Taylor et al., 2018). Prevalence of overt hypothyroidism ranges from .2% to 5.3% in Europe, and 0.3% and 3.7% in the United States according to most large-scale studies. This rate increases among individuals from 85 and 89 years of age, is approximately 10 times more prevalent in females, and does not include subclinical or other non-overt cases. Additionally, about one-third of the global population lives in

iodine-deficient areas where incidence of thyroid dysfunction is expected to be significantly higher (Taylor et al., 2018).

Associations between TH Function and MDD

Like hypothyroidism, MDD is a multifaceted disorder involving multiple subtypes, symptomologies, and degrees of severity. According to the DSM-V, individuals must display at least one of the two main diagnostic criteria – depressed mood and decreased interest in once-enjoyable activities – in ways that are both persistent and detrimental to normal functioning. The remaining criteria include a broad range of symptoms, such as changes in sleep, appetite, and energy; recurrent negative thoughts or thoughts of suicide; and deficits relating to psychomotor and/or cognitive functions (American Psychiatric Association, 2013). Specific symptoms can thus differ greatly from patient to patient, as can the socio-environmental and genetic factors that influence them.

The great complexity of both conditions is important to keep in mind while reviewing studies that examine their association, and it should also be noted that studies may differ in their operationalization of each. For example, TH concentrations can be measured in the plasma or CSF, and researchers can look at free or total levels. Depression can be measured via self-report scales (of which there are many), clinical interviews, or the mere existence of a past diagnosis. Studies in this literature also differ in when and where they were conducted; participant age, type, and sample size; and overall methodology and research design.

Depressive symptoms in hypothyroid patients. The disturbances in mental health that accompany hypothyroidism, particularly depressive symptoms, are well

known and widely accepted in the literature (Dayan & Panicker, 2013; Bathla, Singh, & Relan, 2016). A review by Boswell et al. (1997) found the prevalence of depression in hypothyroid patients to be near 50%, and Jain (1972) reported clinical depression in 40% in another hypothyroid sample. More current literature similarly reports high frequencies of depressive symptoms in hypothyroid patients, and the results are remarkably consistent. One study by Bathla et al. (2016) found that 60% and 63% of 100 hypothyroid patients reported some degree of depression or anxiety, respectively. Another by Chaudhary et al. (2014) reported that 63% of patients in their 100-patient sample had signs of depression as assessed by scores on the Hamilton Depression Rating Scale (HDRS). Demartini, Masu, Scarone, Pontiroli, and Gambini (2010) found an identical depression prevalence of 63% compared to 28% of euthyroid controls. Depressive symptoms were also found to be significantly more severe in patients with overt thyroid disorders compared to controls (measured with the HDRS) in a study by Gulseren et al. (2006), which followed 140 participants with overt or subclinical dysfunction. Their additional finding of significantly lower quality of life (QOL) scores among patients was not surprising, given that patient QOL scores were compared with those of healthy participants. Still, the authors found it telling that depression and QOL scores both improved significantly over the course of treatment despite the fact that thyroid treatment did not include antidepressant drugs. These data suggest that restoration of TH levels can improve concomitant psychological symptoms.

Some authors question the association between thyroid function and depression based on large-scale studies with negative findings. One study of 30,589 participants in Norway, for example, failed to find a definitive association between thyroid dysfunction

and scores on the Hospital Anxiety and Depression Scale (Engum, Bjoro, Mykletun, & Dahl, 2002). In fact, they reported that a subset of subjects with overt hypothyroidism, as determined by blood tests of T4 and TSH, had significantly lower risks of both depression and anxiety. Although higher depression rates were found in subjects with a self-reported thyroid disorder, the authors found that the majority of these subjects actually had normal levels of TH. They suggested that patients with self-knowledge of thyroid dysfunction might be more inclined to report symptoms of depression in a type of "labeling effect." Another study that followed 606 elderly patients at risk for cardiovascular disease (aged 70-82) found no relationships between thyroid status and self-reported depressive symptoms, either at baseline or 3 years later (Blum et al., 2016).

It is possible that factors like age affect relationships between mood and thyroid dysfunction, which is supported by work from Zhao, Chen, Zhao, and Shan (2018). Their meta-analysis of 14 studies examining associations between subclinical hypothyroidism and depression failed to find significant connections between the two, but there was a significant correlation when only studies with younger subjects were analyzed (mean subject age < 60). This finding could potentially help explain the negative results by Blum et al. (2016) in their large geriatric population.

The prevalence of depression among hypothyroid patients is thus inconsistent across studies and seems to depend on a number of factors. Still, patterns in the literature do support their association. The following section examines the opposite end of this relationship – thyroid dysfunction among patients with MDD.

Thyroid dysfunction in MDD. There is widespread evidence that depressed patients – especially those who are resistant to traditional antidepressant treatment – have

a higher prevalence of thyroid dysfunction than the general population. One meta-analysis reported that 52% of patients with treatment-refractory depression exhibited subclinical hypothyroidism across 6 studies, compared with 8-17% of patients with nonrefractory depression and 5% of healthy controls (Sintzel, Mallaret, & Bougerol, 2004). An analysis of 1,000,000 random subjects from a National Health Research Institute database also found a higher incidence of primary or secondary hypothyroidism among MDD patients than the general population (0.4% compared to 0.13%), and a higher incidence of hyperthyroidism as well (0.72% compared to 0.32%; Wu, Chien, Lin, Chou, & Chou, 2013). Studies have additionally found abnormal TH concentrations in MDD patients, with the most consistent findings being that depressed patients have decreased TSH response to TRH as well as increased levels of T4, rT3, and TRH (reviewed in Nemeroff & Evans, 1989; Bahls & Carvalho, 2004; Hage & Azar, 2012).

TSH. TH response to TRH is normally measured with the TRH stimulation test, where TRH is administered intravenously and blood samples are taken at several time points to measure the ensuing TSH levels. Recall that TRH will normally stimulate release of TSH from the anterior pituitary, which subsequently stimulates TH production and release. An early study by Prange et al. found that 25% of MDD patients showed a decreased TSH response despite having normal baseline TSH and TH levels, and a comprehensive review of 45 studies confirmed this finding (Loosen, Garbutt, & Prange, 1987). Other groups have come to the opposite conclusion, however. One review reported increased TSH response in 10-17% of depressed patients (Nemeroff, 1989), and another study found the increase in an astounding 60% of cases (Gold, Pottash, & Extein, 1982).

Results of studies that simply measure TSH concentrations in the bloodstream (without administering TRH beforehand) are similarly mixed. Cleare, McGregor, and O'Kearne (1995), for example, found that hypothyroid patients with clinical depression had significantly higher TSH levels than those who were non-depressed. These concentrations correlated positively with self-reported depression scores. In contrast, depressed inpatients in a study by Rao et al. (1996) had lower plasmatic TSH than controls. Saxena, Singh, Srivastava, and Siddiqui (2000) reported lower TSH levels in mildly depressed patients and higher TSH levels in severely depressed patients when compared to a control group, although all values were considered to be within the normal range. It is thus possible that TSH expression in MDD varies among patients with different levels or subtypes of the disorder.

Another interesting line of research found that MDD patients lack the nightly surge in serum TSH levels that occurs in healthy subjects (Bahls & Carvalho, 2004). This nightly TSH increase has been found to return to normal once patients have recovered. Hormone concentration differences relating to the time of day they are measured could be another potential explanation for inconsistent findings.

T4. Depression is also commonly associated with increased total and plasma T4 levels, a finding that reportedly applies to 20-30% of MDD patients (Bahls & Carvalho, 2004). Correlations have been found between increased T4 and depression severity, and T4 levels have been found to decrease as the disorder remits. One study found significantly elevated T4 in the CSF of 12 depressed patients, which normalized after successful electroconvulsive treatment (Kirkegaard & Farber, 1991). Another found that both total and free T4 levels significantly decreased after 4 weeks of antidepressant

treatment in a 21-patient sample (Rao et al., 1996). Berent, Zboralski, Orzzechowska, and Galecki (2013) similarly found significant positive relationships between free T4 concentrations and depression severity in 44 inpatients (as measured by the HDRS upon admission). Interestingly, both Rao et al. (1996) and Berent et al. (2013) also found that higher baseline plasma T3 and T4 levels were associated with greater reported clinical improvement on depression rating scales. Rao et al. (1996) suggested that higher TH levels might compensate for the decrease in THs elicited by antidepressant treatment. Berent et al. (2013) speculated that higher T4 levels could indirectly improve symptoms by being a source for more T3.

One theory explaining the increased T4 levels in depression is that the increase results from inflated TSH secretion (and therefore increased stimulation of the thyroid and TH synthesis). This idea was suggested by Kirkegaard, Korner, and Faber (1990) in a turnover study using radiolabeled T4, where a small sample of depressed patients showed significantly increased production of T4 compared to controls. One would expect TSH levels to then be decreased in these depressed patients due to the negative feedback loop, but serum TSH levels in both groups were the same. Furthermore, depressed patients had higher serum TSH levels when compared to a separate group with identical levels of T4 production (these were nondepressed patients taking thyroxine for a hypothyroid condition). This supports the hypothesis that inflated TSH concentrations in depression could precede (and thus result in) the increased levels of T4.

Another possibility is that increased cortisol, which is heavily implicated in the disorder, could lead to inhibition of the D2 enzyme that converts T4 to T3. Recall that T3 is the biologically active thyroid hormone that binds to nuclear receptors and carries out

specific functions. In the cerebral cortex, 80% of T3 used is not secreted by the thyroid gland directly, but rather produced through local deiodination of T4 (Hage & Azar, 2012). Inhibition of D2 would lead T4 to be primarily converted to rT3, the hormone's inactive form, through the D3 enzyme instead. This idea is supported by the increased levels of rT3 commonly found in the CSF of depressed patients (Hage & Azar, 2012). Depressed patients were also found to have decreased CSF concentrations of transthyretin, a protein important for T4 transport to the brain. Levels were lowest among patients with greater symptom severity (Sintzel, Mallaret, & Bougerol, 2004). This suggests that even if T4 concentrations are found to be normal or increased in the plasma, there could be less TH available in the brain for neuronal use.

TRH. Several groups have also reported increased TRH levels in the CSF of depressed patients, with one study reporting a nearly threefold increase in MDD patients compared to controls (Nemeroff & Evans, 1989). Hage and Azar (2012) suggested that the T4 increase could result from increased activation of TRH-producing neurons in the hypothalamus, based on the finding that tricyclic antidepressants and SSRI's have both been found to reduce TRH concentration in rats in a dose-dependent manner. Antidepressants also reduced TRH response to glucocorticoid stimulation, which suggests that the rise in cortisol associated with depression could result in increased stimulation of TRH. However, studies looking for increased TRH in the CSF of depressed patients have yielded negative findings as well (Roy, Wolkowitz, Bissette, & Nemeroff, 1994).

Summary. In sum, although there are some inconclusive findings in the literature, there is also overwhelming evidence associating depression with some form of thyroid

dysfunction. It should be noted that "thyroid dysfunction" in this context does not necessarily refer to formal diagnoses of thyroid disorders, as even subtle changes in TH levels can have profound impacts on mood (Bathla, Singh, & Relan, 2016). In many of the studies described below, for instance, depressed patients showed hormone levels that are significantly different from controls but still within the "normal" euthyroid range (e.g. Rao et al., 1996; Saxena, Singh, Srivastava, and Siddiqui, 2000; Hage & Azar, 2012). Authors have discussed the possibility of "brain hypothyroidism in the setting of systemic euthyroidism" (Hage & Azar, 2012), wherein lower TH availability in the brain might to unnoticed if hormone levels in the peripheral organs are normal. Findings of increased CSF levels of rT3 and decreased CSF levels of the T4 transporter transthyretin, discussed above, both provide support for this idea. A brain-specific TH deficiency would also explain why some larger-scale population studies fail to find a definitive link between hypothyroidism and depression diagnoses (e.g. Engum et al., 2002; Blum et al., 2016).

Forman-Hoffman and Philibert (2006) conducted a large-scale study that was particularly interesting in that none of the 6,869 participants they included had any previously documented thyroid disorders. Depressive symptoms were assessed with self-reports and clinical interviews, and serum T4 and TSH levels were measured. In men, current depressive symptoms in these euthyroid subjects were significantly associated with lower TSH. T4 was also strongly associated with depressive symptoms in both males and females, which is supported by evidence reviewed above. Interestingly, TH abnormality was associated with current depressive symptoms but not lifetime history of the disorder. This is consistent with numerous reports that TH levels normalize after successful antidepressant treatment.

This study, like many other reported findings in this literature, demonstrates how TH abnormalities can exist in depression without being attributed to an overt thyroid disease. This also underscores the important point that although TH disorders and MDD share many common mechanisms, the two conditions remain qualitatively different. The DSM-V accordingly states that a diagnosis of depression may be inappropriate if changes in mood are thought to be a result of medical conditions like hypothyroidism (American Psychiatric Association, 2013). Along these lines, TH treatment – further reviewed in the following section – has been found to show antidepressant-like effects on MDD patients who had no diagnosed thyroid-related illness.

Antidepressant properties of thyroid hormones. One review reported that about 30% of depressed patients fail to respond to antidepressant treatment, and 67% of these treatment-resistant patients benefit from the addition of thyroid hormones (Henley & Koehnle, 1997). These authors argued that because traditional antidepressants take weeks to show clinical benefits, they likely act on second messenger systems that trigger the intracellular signals responsible for the treatment effects. Thyroid hormones act on a synaptic level and directly influence gene expression, so their addition could theoretically strengthen and speed up the process. Clinical studies have supported this idea, such as an early study by Goodwin, Prange, Post, Muscettola, and Lipton (1981). A sample of 12 MDD patients with no signs of TH dysfunction showed no response to tricyclic antidepressants (TCAs) after a 4-week trial, so T3 was added to their medication treatment. Depression ratings were assessed twice a day on the Bunney-Hamburg scale. Nine of the 12 patients showed significant symptom reductions – an effect that was detected within the first three days of T3 treatment. Another meta-analysis found that in

five out of six clinical studies, addition of T3 to TCAs was more effective than addition of placebo with a weighted effect size of 0.58 (Altshuler et al., 2001).

Significant effects of T3 augmentation have not always been found, however. A meta-analysis of randomized, double-blind trials by Papakostas et al. (2009) found no added benefit of T3 to SSRIs when compared to SSRI treatment alone. Another by Cooper-Kazaz and Lerer (2008) reviewed three different studies where T3 was added with SSRIs before the start of treatment. One study found T3 to have a strong and positive effect on treatment, one found no benefit, and one found a slight trend that was ultimately insignificant. Additionally, a STAR*D report (part of a larger collaborative antidepressant study) did find that addition of T3 was more effective than lithium in patients resistant to the SSRI citalopram (25% vs. 16% achieved remission), but this was also an insignificant trend (Nierenberg et al., 2006). It has been suggested that combination of T3 with antidepressants – SSRIs in particular – is most effective for a specific subgroup of patients with atypical depression. The meta-analyses mentioned above are limited in that the studies reviewed differ in terms of their specific patient population. In addition, the T3-SSRI combination has been found effective in patients with a D1 enzyme polymorphism that limits T4 deiodination to T3 in the bloodstream (Papakostas et al., 2009).

Studies investigating treatment of depression with thyroxine (T4) have yielded similarly mixed results. One group found addition of T4 to antidepressant treatment significantly reduced HRSD scores in MDD patients who had been treatment-resistant to at least two chemically different medications (Bauer, Hellweg, Graf, & Baumgartner, 1998). However, thyroxine was found ineffective as monotherapy for comorbid SCH and

MDD patients (i.e. without additional antidepressant treatment) which suggests that T4 cannot substitute for traditional antidepressants (Kalra & Balhara, 2014). In their pooled analysis of 6 papers and 266 subjects, Loh, Lim, Yee, and Loh (2019) also found that in patients with both SCH and depression, treatment of SCH with T4 was not associated with improvement in depressive symptoms. In general, T4 is not thought to be as effective as T3 for treatment in MDD patients (Bahls & Carvalho, 2004; Zhao, Chen, Zhao, & Shan, 2018). This is consistent with the hypothesis that impaired conversion of T4 to T3 could be a driving mechanism behind depressive symptoms.

Antidepressant effects of thyroid hormones have also been studied in animal models, and treatment has often successfully reduced hypothyroid-induced symptoms of depression. Further literature on this subject is reviewed below.

Depressive symptoms and thyroid function in animal models. Given that the current study examined depressive symptoms in hypothyroid mice, past experiments in animal models are especially relevant. There are several studies that support the idea of using hypothyroid animals as an MDD model based on the behavioral effects observed. In one, subclinical hypothyroidism was induced in rats by surgically removing one lobe of the thyroid gland. These rats showed increased depression-like behavior in two common behavioral measures with predictive validity for antidepressant-like drug effects: the tail suspension test and forced swim paradigm (Ge, Peng, Qi, Chen, & Zhou, 2013). Interestingly, induced hypothyroidism did not affect behavior in the sucrose preference test – the measure used for depression in the current study.

Reductions in sucrose preference have been reported in other hypothyroid models, however, including a study by Rivlin, Osnos, Rosenthal, and Henkin (1997) that rendered

rats hypothyroid through administration of radioactive iodine. Control rats consistently preferred water sweetened with sucrose to regular water, but sucrose preference in hypothyroid rats was significantly lower (below 50%). This is commonly used as a measure of anhedonia, a characteristic symptom of major depression. Hypothyroid rats additionally had an increased preference for salty and bitter water (water with NaCl and quinine sulfate, respectively); but the hypothyroid preference for quinine sulfate was successfully reversed after treatment with thyroxine.

Further evidence supporting depressive-like effects of hypothyroidism comes from Ge, Xu, Qin, Cheng, & Chen (2016), who induced subclinical hypothyroidism in rats through hemithyroid electocauterization. Untreated hypothyroid rats had significantly lower sucrose preference, significantly more anxiety-like behavior in an open field test (decreased locomotion and rearing activity), and significantly more depressive behavior in the forced swim paradigm (increased immobility). Separate groups of rats were treated with either thyroxine or resveratrol, which has been found to induce antidepressant-like effects in chronic stress models. Both treatments reversed all of the depressed and anxious behaviors, as well as the increased plasma TSH levels induced by subclinical hypothyroidism.

Surgically thyroidectomized rats also displayed more immobility in the forced swim test in a study by Kulikov, Torresani, and Jeanningros (1997). Thyroidectomized rats on a low-iodine diet – referred to as having severe hypothyroidism – had 90% decreased immobility compared to controls. Rats with mild hypothyroidism (intact thyroids but low-iodine diets) also displayed significantly more immobility, with an increase of 60%.

While the studies above involved lesion models of hypothyroidism – including surgical, electrical, and radioactive removals of the thyroid – other groups have utilized genetic models. Rodents in these studies are genetically bred to lack the thyroid receptor alpha 1, which binds to T3 and is vital for TH expression. One study found that mice lacking this receptor exhibited significantly less exploratory behavior and more freezing than control mice in an open field test (Guadano-Ferraz et al., 2003). Another found that mutant hypothyroid mice displayed significantly more depressed behavior in a learned helplessness paradigm, as well as significantly more anxiety in startle response and light-dark box tests (Pillhatsch et al., 2010). These effects were reduced for hypothyroid mice treated with T3, demonstrating that the thyroid hormone could have antidepressant properties in animal models. When control mice were treated with T3, however, they displayed more depressive behavior and reduced locomotion. The authors suggested that T3 treatment in the absence of hypothyroidism could have resulted in a hyperthyroid state, explaining the depressive-like effects.

In another study, Wilcoxon, Nadolski, Samarut, Chassande, and Redei (2007) put mutant and control mice on hypothyroid, hyperthyroid, or euthyroid diets. As a group, mice lacking thyroid receptor alpha exhibited more depressive behavior than controls in the forced swim test. The hypothyroid diet increased this effect on mutant mice, but it had the opposite effect on controls. TH receptor deletion was also associated with spatial learning and memory deficits in the Morris Water Maze – an effect that was also exaggerated by the hypothyroid diet. The associations between hypothyroidism and related cognitive deficits are further reviewed in a separate section below.

The current study used a chemical hypothyroid model by adding propylthiouracil (PTU) to the animal diets. This compound induces hypothyroidism by blocking the effects of TPO and inhibiting TH synthesis. Yu, Tang, Feng, and Cheng (2014) found that rats given this treatment had decreased body weight, decreased locomotor and rearing activity in an open field test, and decreased sucrose preference to regular drinking water. The degree of sucrose preference reduction additionally correlated with reduced glucose metabolism in the caudate putamen and nucleus accumbens.

Interestingly, groups have also reported findings that are nearly opposite to those reported above. For instance, Yu et al. (2015) created hypo- and hyperthyroid models of rats using radioactive iodine or levothyroxine as part of their daily diet. When tested in the forced swim paradigm, hypo- and hyperthyroid rats both showed significantly less depressive behavior than controls. Hypothyroid rats were also significantly less depressed than the hyperthyroid group. In addition, hypothyroid rats showed an increased preference for sucrose – a response indicating less depression – and decreased anxiety compared to both hyperthyroid and normal rats. Treating hypothyroid animals with T4 before testing actually increased depression- and anxiety-like behaviors, which further suggests that hypothyroidism had an antidepressant-like effect in this particular study.

Mechanisms Implicated in both MDD and TH Dysfunction

The significant limitations of current antidepressant drugs, such as low remission rates and delayed onset of action, have inspired a line of research devoted to uncovering the "next generation" of antidepressant treatment (e.g. Machado-Vieira et al., 2008; Murrough, 2012; van Calker, Serchov, Normann, & Biber, 2018). The mechanisms behind current antidepressants are largely based on the monoamine hypothesis, which

proposes that depression stems from depleted concentrations of monoamines like serotonin, norepinephrine, and dopamine (Hillhouse & Porter, 2015). In the late 1950s, the drug iproniazid – originally developed for treating tuberculosis – was found to also significantly reduce depressive symptoms after several weeks of treatment. Iproniazid is an inhibitor of the enzyme monoamine oxidase (MAO), which is responsible for the breakdown of monoamines in the presynaptic terminal. The classes of antidepressants developed since then (e.g. tricyclic antidepressants [TCAs], selective serotonin reuptake inhibitors [SSRIs], and serotonin norepinephrine reuptake inhibitors [SNRIs]) similarly work by increasing concentrations of serotonin and/or norepinephrine in the synaptic cleft though differing mechanisms of action. However, the late onset of therapeutic effects suggests that monoamines might work indirectly by modulating additional intracellular signaling pathways that are more directly responsible for alleviating symptoms (Hillhouse & Porter, 2015).

Although most drug treatments have remained contingent on this 50-year-old hypothesis, research on the underpinnings of the disorder has actually come quite far. Notable work that has been done on MDD includes identifying specific brain areas implicated in its pathology, studying how MDD impacts normal neurogenesis, and investigating the role of glutamatergic neurotransmission. Furthermore, work examining the rapid antidepressant effects of ketamine has revealed other critical components like AMPA and NMDA receptors, glial astrocytes, and synaptic plasticity. Achieving a "next generation" of antidepressants will require an advanced understanding of the biological mechanisms behind MDD – none of which occur in isolation. Given the well-documented relationships between depression and thyroid function, as well as the critical

role of TH on broad aspects of cell metabolism, it is of great relevance to examine the roles of TH in various MDD-related processes. The review below will demonstrate how the thyroid system and MDD share many biological processes, ultimately supporting the use of hypothyroidism in an MDD model.

Monoamines. The hypothesis that depression stems from deficiencies in the serotonin (5-HT), norepinephrine (NE), and/or dopamine (DA) neurotransmitter systems is one of the most well-known theories of the disorder. Despite significant limitations and delayed treatment effects, drugs that increase these neurotransmitter levels have undoubtedly helped MDD patients achieve remission for over 50 years. It is thus widely acknowledged that while monoaminergic systems are likely not the primary target of antidepressant action, they nonetheless likely play a major role in depression's pathology (Maeng & Zarate Jr., 2007). As supporting evidence, changes in serotonergic neurotransmission are associated with response to antidepressants; unmedicated MDD patients have reduced 5-HT metabolite levels in the CSF; and MDD patients show reduced 5-HT transporter availability in imaging studies (Bauer, Heinz, & Whybrow, 2002). Thus, close relationships found between thyroid hormones and monoamine activity supports the use of hypothyroidism in a depression model.

Cleare, McGregor, and O'Kearne (1995) found that compared to controls, hypothyroid patients had reduced levels of central serotonin (indexed by measuring serotonin-mediated responses of cortisol to dexfenfluramine). Reduced 5-HT responsivity was also associated with higher self-rated depression scores as well as higher levels of TSH. The majority of research in this area similarly reports reduced 5-HT response in hypothyroid patients that reverses with TH treatment, and this finding has been heavily

replicated and reviewed (Bauer, Heinz, & Whybrow, 2002; Bahls & Carvalho, 2004; Correa Santos et al., 2012).

In rats, neonatal hypothyroidism has been associated with reduced concentrations of NE, DA, and 5-HT – as well as decreased activity of their rate-limiting enzymes, tyrosine and tryptophan hydroxylase (Singhal, Rastogi, & Hrdina, 1975). This study also found the thyroid-monoamine relationship to be time-dependent, as changes in monoamine metabolism were reduced when hypothyroidism was induced later in life (20 days after birth). Treatment of hypothyroidism with T3 was also more effective in reversing its effects when it was introduced earlier. In a similar study, induced hypothyroidism at birth inhibited the normal developmental increases of NE and its synthesizing enzyme (activity was delayed by 34% and 31%, respectively). Rats treated with T3 early in life showed reduced neurochemical changes, while treatment later in life did not reverse the effects significantly (Rastogi & Singhal, 1974). The authors postulated that thyroid hormones might have to be present during a critical period for normal patterns of NE development. Studies of adult hypothyroid rats found reduced cortical 5-HT concentrations and reduced whole-brain concentrations of the serotonin precursor 5-HTP (Bauer, Heinz, & Whybrow, 2002).

Reverse effects have been found in hyperthyroid models. For example, one study found that experimentally-induced hyperthyroid rats had nearly 3 times more blood serotonin levels than controls, which decreased after a 3-week recovery period (Noll, Goke, Willemer, Richter, & Arnold, 1998). Another study similarly reported increased blood 5-HT levels in human hyperthyroid patients compared to controls, which were mitigated after three months of thyroid treatment (Upadhyaya, Argrawal, Dubey, &

Udepa, 1992). This study also reported strong correlations between serotonin levels and T3 concentrations, providing further evidence of their relationship.

In addition, components of the HPT axis have been found to be affected by traditional antidepressant treatment. One study with rats found that after 2 weeks on the tricyclic antidepressant desipramine, D2 increased in various brain areas commonly implicated in MDD including the frontal cortex and amygdala (Campos-Barros et al., 1994). Increased D2 results in more deiodination of T4 and, consequently, more T3 available for neuronal use. Serum levels of T4 were also reduced after treatment, which is consistent with decreased T4 found in antidepressant-treated patients (e.g. Brandy & Anton, 1989). Another study (Baumgartner, Dubeyko, Campos-Barros, Eravci, & Meinhold, 1994) found similar D2 enhancement after 2 weeks of fluoxetine administration in rats. They also found decreased D3 activity in several limbic and cortical regions, which would lead to a rise in T3 concentrations.

Hypothyroid status therefore can decrease monoaminergic activity; and, likewise, increasing monoamine neurotransmission through traditional antidepressant treatment can enhance TH function. The following sections will review how thyroid status and mechanisms of depression could overlap in their relationship to the glutamatergic system.

Glutamatergic receptors and the antidepressant effects of ketamine

Overview of glutamatergic receptors. The finding that ketamine induced long-lasting antidepressant effects in treatment-resistant MDD patients in a matter of hours (Berman et al., 2000) has been one of the most significant advances in modern antidepressant research. Because ketamine is a noncompetitive antagonist of the glutamate receptor N-methyl-D-asparate (NMDA), glutamate neurotransmission has

taken on a central role in models of MDD pathology. Glutamate is regarded as the primary excitatory neurotransmitter in the nervous system, with significant roles in learning, cognitive function, and cellular plasticity. It acts by binding to various receptor types on pre- and post-synaptic neurons; as well as astrocytes, which convert glutamate to glutamine, which pre-synaptic cells use for glutamate synthesis (Hillhouse & Porter, 2015). NMDA and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are the two most implicated in ketamine's antidepressant-like actions.

NMDA and AMPA are both ionotropic glutamate receptors. As ion channels, they depolarize the membrane potential of neurons which consequently activates signaling cascades with the cell (Machado-Vieira et al., 2009). The mechanical differences between NMDA and AMPA receptors reflect their differing roles and functions within the glutamatergic system. NMDA receptors are made of several types of NR subunits – specifically GluN1, GluN2A-D, and GluN3A-B. The amino acid glycine has binding sites on NR1, while glutamate binds to the various NR2 subunit types. In order for NMDA receptors to function, glycine and glutamate must both be bound to their respective subunits as co-agonists. NMDA action also requires that magnesium ions, which block NMDA channels, are removed through depolarization of the membrane by influx of sodium ions. AMPA receptors can achieve this depolarization to remove the magnesium ion blockade, making NMDA receptor action dependent on AMPA receptor stimulation (Maeng & Zarate, 2007; Hillhouse & Porter, 2015). AMPA receptors, on the other hand, are comprised of the subunits GluR1 – GluR4. Because they do not require co-agonist action or blockade removal, AMPA receptors are primarily responsible for the rapid and early excitatory synaptic response to glutamate (Machado-Vieira et al., 2009).

NMDA receptors are associated with longer-term synaptic changes, such as long-term potentiation (LTP) implicated in processes of learning and memory (Zarif, Petit-Paitel, Heurteaux, Chabry, & Guyon, 2016). Unlike AMPA channels, NMDA channels also allow for the influx of calcium ions which are crucial for initiating various signaling cascades (Hillhouse & Porter, 2015).

Ketamine studies, as well as the ensuing lines of research they inspired, have demonstrated that these glutamatergic receptors are closely related to symptoms of depression and may be implicated in its pathology. Associations have also been consistently found between AMPA receptors, NMDA receptors, and thyroid function. Evidence of these relationships raises the exciting possibility that TH could mediate depressive actions through its role in the glutamatergic system.

Antidepressant actions of ketamine. The definitive mechanisms behind ketamine's rapid antidepressant effects are still unclear. Despite being a noncompetitive NMDA receptor antagonist, ketamine administration is associated with increased glutamatergic activity; for instance, its administration was found to increase overall glutamatergic neurotransmission in the prefrontal cortex in healthy volunteers (Aleksandrova et al., 2017). One popular hypothesis is that ketamine could thus preferentially act on NMDARs located on inhibitory GABAergic interneurons. Blocking inhibition of excitatory neurons would result in a surge of glutamate transmission, leading to rapid synaptogenesis (Machado-Vieira et al., 2010; Duman & Aghajanian, 2015; Aleksandrova et al., 2017). As support for this hypothesis, ketamine has been found to have a higher affinity for the NR2D subunits which are particularly concentrated in inhibitory interneurons in the forebrain (Zanos & Gould, 2018).

On the contrary, evidence also suggests that NMDA antagonism might not be the primary mechanism of antidepressant action. Other NMDA antagonists such as dizocilpine (MK-801) have demonstrated antidepressant-like effects similar to that of ketamine (Machado-Vieira et al., 2010), but some researchers argue that their weaker and shorter-lasting results suggest that other factors could primarily modulate the therapeutic effects (Aleksandrova et al., 2017). Ketamine notably increases the presynaptic release of glutamate (Moghaddam, Adams, Verma, & Daly, 1997); and because NMDA receptors are blocked before cell activation, the glutamate preferentially favors AMPA. Some have suggested that this increased glutamatergic activity of AMPA relative to NMDA receptors could be an underlying mechanism behind the ketamine's effects (Maeng et al., 2008; Machado-Vieira et al., 2009).

In this regard, there is accumulating evidence that AMPA receptors could play a primary role in the antidepressant actions of ketamine. Multiple research groups have found that ketamine significantly reduced behavioral symptoms of depression in rodents; but when animals were first treated with the AMPAR antagonist 6-Nitro-2,3-dioxo-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), it was no longer effective as an antidepressant (e.g. Maeng et al, 2008; Li et al., 2010; Koike, Iijima, & Chaki, 2011). Zhou et al. (2014) similarly found that inhibiting AMPA activity with NBQX weakened ketamine's effects, but enhancing AMPA activity with an agonist increased them. Chronic treatment with TCA and SSRI antidepressants was associated with increased synaptic expression of AMPA subunits (Martinez-Turrillas, Frechilla, & Del Rio, 2002; Du et al., 2004), and injections of AMPA have even been found to elicit significant antidepressant-like effects on their own (Akinfiresoye & Tizabi, 2013).

A notable study by Li et al. (2010) comprehensively illustrated how various signaling pathways and synaptic plasticity are implicated in the actions of ketamine. They demonstrated that the mammalian target of rapamycin (mTOR) pathway – which is critical in rapid cell signaling and synthesizing proteins for synaptic function – was activated by ketamine in the prefrontal cortex (PFC) of rats. Ketamine also induced a rapid increase in extracellular signal-regulated kinases (ERK) and protein kinase B (Akt) expression, both of which are involved in pathways critical in mTOR activation. Additional effects of ketamine included increased expression of postsynaptic proteins, increased spine density in the medial PFC, increased electrophysiological response to 5-HT, and decreased depressive- and anxiety- like behaviors in three behavioral paradigms. Importantly, the mTOR pathway, ERK and Akt pathways, and activation of AMPA were all vital to the antidepressant response, as inhibition of any of them blocked all of these ketamine-induced effects.

Ketamine studies have thus demonstrated the critical role of glutamate receptors AMPA and NMDA on mood, showing that NMDA inhibition and AMPA enhancement are both associated with reduced depressive behaviors. Findings that THs mediate glutamate receptor activity, as well as expression of their subunits, therefore support the hypothesis that thyroid function could be involved in the MDD pathology. Importantly, they also play important roles in synaptic plasticity and intracellular signaling cascades – which, as Li et al. (2010) exemplified, are needed for antidepressant effects to occur.

Relationship between TH and glutamate receptors. Several studies have demonstrated the effects of TH on glutamate receptor activity as well as effects of glutamate receptors on TH levels. For example, Losi, Garzon, and Puia (2008) used

electrophysiological recordings to measure the effect of TH on NMDA-dependent currents in cultured rat hippocampal neurons. Both T3 and T4 administration resulted in decreased NMDA activity, illustrated through the dose-dependent inhibition of NMDA-evoked currents. This is consistent with findings of ketamine-induced NMDA receptor downregulation, and suggests that THs could have a ketamine-like effect on NMDA receptor activity.

Another interesting study by Alva-Sanchez, Becerril, Anguiano, Aceves, and Pacheo-Rosado (2008) demonstrated that hippocampal damage in hypothyroid rats could "depend" on NMDA channel activation. Rats were administered either tap water (euthyroid) or water with methimazole (hypothyroid), and two additional groups of hypothyroid rats received daily injections of either ketamine or MK-801 (both of which are noncompetitive NMDA receptor antagonists). After four weeks of treatment, the hypothyroid group had significantly more neuronal damage and cell death in CA1, CA2, CA3, and CA4 regions compared to controls. Interestingly, administration of both ketamine and MK-801 prevented this hippocampal damage. NMDA receptor inhibitors thus had a neuroprotective effect on hippocampal cells; and although depressive-like behavior was not assessed in this study, there is abundant evidence linking depression to hippocampal atrophy (Duman, 2004). It is plausible given these findings that NMDA overstimulation could be associated with a depressive phenotype. One weakness of this design, however, is that both ketamine and MK-801 have produced antidepressant-like effects. It is thus unclear whether they prevented cell damage through NMDA antagonism or through another shared therapeutic mechanism.

On the other hand, multiple reports have associated hypothyroidism with reduced expression and transcription of NMDA receptor subunits (primarily in the hippocampus). For example, Cortes et al. (2012) found that in hypothyroid mice, postsynaptic densities of hippocampal neurons showed reduced expression of NR1 and NR2A/B subunits. Lee, Brady, and Koenig (2003) similarly found reduced mRNA expression of the NR1 subunit in the hippocampal region of thyroidectomized rats. Both groups thought this might reflect an association between TH deficiency and reduced NMDA receptors in the hippocampus. These findings appear to contradict that of the ketamine literature, but they do support the widespread reports of learning and memory impairment associated with hypothyroidism (Lee, Brady, & Koenig, 2003) which depend on glutamatergic and NMDA-mediated activity. Additionally, they could provide support for the growing contention that NMDA receptor antagonism does not necessarily underlie the antidepressant effects of ketamine (e.g. Maeng et al., 2008; Machado-Vieira et al., 2009). Of note, Lee, Brady, and Koenig (2003) found reduced NR1 mRNA in the hippocampus only, with no difference to controls in the hypothalamus, cortex, or amygdala. They also found no effects of hypothyroidism on AMPA subunit mRNA.

There are also findings suggesting that thyroid hormones are regulated by the glutamatergic system, which adds further complexity to their relationship. Arufe, Duran, Perez-Vences, and Alfonso (2002) investigated the effects of various glutamate receptor agonists and antagonists on levels of TH when administered into the third ventricle. Administration of MK-801 and CNQX – which are NMDA and AMPA receptor antagonists, respectively – lead to decreased TSH and TH levels in the serum. The AMPA agonists kainic and domoic acid lead to increased serum concentrations of TSH

while CNQX blocked these effects. These results suggest that TH production and release could be upregulated by glutamate, particularly through AMPA receptors. Similarly, another study demonstrated that administration glutamate (L-glu), NMDA and the AMPA agonists kainic and domoic acid all increased serum levels of T3, T4, and TSH (Alfonso, Duran, & Arufe, 2000). The increase in TSH occurred within 5 minutes, while the TH level increase took about 30 minutes to take effect. The authors suggested that glutamatergic receptors could play a role in stimulating TH secretion, and the timeline seems to confirm a direct effect on TSH followed by consequent TH synthesis.

Long-term potentiation. Further support for a role of TH in glutamate neurotransmission comes from their reported involvement in LTP. LTP refers to the process of strengthening synaptic activity in response to external stimuli which leads to long-term structural changes in neuronal connections. In the literature, these long-term changes are widely accepted as cellular indicators of learning and memory (Gerges & Aldakhi, 2004; Alzoubi, Gerges, Aleisa, & Aldakhi, 2009). Synaptic responses are notably mediated by NMDA and AMPA receptors (e.g. Zarif et al., 2016), so TH involvement in this process supports the contention that thyroid function could regulate these glutamatergic receptors.

Associations between TH and LTP are reported particularly in hippocampal regions. As a brief overview, there are two major hippocampal pathways implicated in studies of LTP. Action potentials are transmitted from the entorhinal cortex to the dentate gyrus via the performant pathway. Pyramidal neurons in the CA3 hippocampal region then transmit signals received from the dentate to cells in the CA1 region via Schaffer collateral synapses (Gerges & Aldakhi, 2004). LTP can be experimentally induced by

applying high-frequency electrical stimulation to electrodes implanted in hippocampal tissue. Activity can then be operationalized by excitatory postsynaptic potentials (EPSPs) recorded from the postsynaptic (receiving) cells (Gerges & Aldakhi, 2004).

Reviews of this subject convey a rich literature concluding that TH function is important for these processes (Leach & Gould, 2015). In one study, rats made hypothyroid through PTU administration at one week of age had significantly reduced LTP in CA1 compared to control rats – an effect seen after only 2 weeks of PTU treatment (Niemi, Slivinski, Audi, Rej, & Carpenter, 1996). Another found that thyroidectomized rats had significantly impaired LTP in the dentate gyrus which could not be reversed with 20 days of TH treatment (Fernandez-Lamo et al., 2009). In contrast, Gerges and Aldakhi (2004) reported no differences in LTP between thyroidectomized and control rats in the dentate gyrus. However, LTP in the CA1 hippocampal region of hypothyroid rats was found to be completely abolished.

This experiment by Gerges and Aldakhi (2004) is particularly noteworthy in that they also reported reduced levels of phosphorylated ERK1 and ERK2 in the CA1 region of hypothyroid rats. These protein kinases are important regulators of cell growth and activate various functions through phosphorylation. Recall that Li et al. (2010) found that ketamine, in addition to inducing antidepressant-like effects in behavioral paradigms, enhanced ERK signaling in the prefrontal cortex. ERK inhibitors abolished ketamine's antidepressant effects; as well as its activation of the mTOR pathway, which they found to be critical for ketamine's actions. The fact that Gerges and Aldakhi (2004) found these reductions in the CA1 region, where LTP was found to be impaired – and not the dentate gyrus, where hypothyroidism did not affect LTP – indicates that these kinases could be

implicated in the mechanisms by which TH regulate synaptic plasticity. Taken together with Li et al.'s (2010) findings, it is possible that they could also be implicated in TH regulation of depressive and anxiety-like behaviors. This contention is reasonable given that ERK's function by inducing rapid signaling cascades within the cell.

Further support for the role of cellular signaling molecules in the relationship between LTP and thyroid function comes from a later study out of the same lab (Alzoubi, Gerges, Aleisa, & Alkadhi, 2009). This study assessed learning and memory performance, LTP in area CA1, and immunoblot analyses of signaling molecules in three groups of rats: control rats, thyroidectomized rats, and thyroidectomized rats treated with T4. The radial arm water maze task consisted of learning, short-term memory, and long-term memory phases. Hypothyroid rats were significantly impaired for all three measures, and T4 treatment prevented the impairment. Hypothyroidism similarly impaired L-LTP in CA1, which T4 treatment normalized as well. Finally, the team reported reduced levels of total and phosphorylated cyclic-AMP response element binding protein (CREB) in CA1 neurons – which promotes gene expression for many plasticity-related proteins – as well as reduced phosphorylated ERK1 and ERK2. Rats treated with T4 did not show either these reductions, suggesting that TH can restore these molecules to normal levels. Because the ratio of total to phosphorylated ERK1/2 was reduced in hypothyroid tissue, the authors suggested a specific effect of TH on phosphorylation processes.

LTP has also been investigated in regard to MDD, and chronic stress models have shown LTP impairments similar to that of hypothyroidism. One review cited several early studies wherein stress-induced depression in rats was associated with reduced LTP

in hippocampal area CA1 (Popoli, Gennarelli, & Racagni, 2002). In one study, Shors, Seib, Levine, and Thompson (1989) found that rats in an "escapable shock" group – that is, rats who were administered electric shocks with the option of escaping into another compartment – showed reduced LTP in CA1 compared to control rats. Rats in an "inescapable shock" group who were unable to avoid shock administration showed reductions that were even more significantly more pronounced. Thus, it is possible that different levels of depression or stress can affect the degree of synaptic impairment. Antidepressant treatment has been found to reverse these effects, implying that enhanced glutamatergic activity and synaptic strengthen is involved in antidepressant mechanisms. For example, Stewart and Reid (2000) found increased LTP in the dentate gyrus of rats following treatment with two common antidepressant treatments – the SSRI fluoxetine and electroconvulsive stimulation (ECS). While fluoxetine and ECS showed comparable effects on transmission in the dentate, only ECS was associated with significant impairments in a spatial memory task. These findings led them to argue that their shared effects on hippocampal plasticity were likely related to processes of mood rather than cognition.

Glutamate uptake. Involvement of the thyroid system on glutamatergic neurotransmission can be further demonstrated through its role in glutamate uptake. Excessive stimulation of glutamate receptors can result in neurotoxic levels of extracellular glutamate, which is regulated by astrocytes through Excitatory Amino Acid Transporter 1 (EAAT1) in humans, GLT-1 and GLAST transporters in rodents (Mendes-de-Aguiar et al., 2008). In rat cortical neuron cultures, TH administration was found to enhance cell viability in glutamate- and NMDA-induced toxicity experiments. High

levels of glutamate decreased cell survival by 64% in control cultures, while TH-treated cultures had no significant loss (Losi, Garzon, & Puia, 2008). This neuroprotective response was demonstrated in T4, T3, and even rT3. In another study, Mendes-de-Aguiar (2008) cultured newborn rat neurons with either T3-treated or control astrocytes. Cultures with T3 treated astrocytes showed significantly increased glutamate uptake, significantly increased mRNA and protein expression of GLAST and GLT-1, and stronger neuronal resistance against glutamate-induced toxicity. In combination with their involvement in important signaling cascades, these findings support the notion that THs could have important roles in cell protection, growth, and function. Furthermore, multiple studies have additionally reported increased extracellular glutamate in response to stressors in animal depression models (Sanacora, Treccani, & Popoli, 2013). One could hypothesize that decreasing toxic levels of glutamate might be one way in which THs protect against a depressive response.

Neurogenesis. Many groups are now looking at depression primarily as a disruption of synaptic plasticity and growth. There are exciting lines of research that support a "neurogenetic hypothesis of depression" (Remaud, Gothie, Morvan-Dubois, & Demeneix, 2014), but the links between mood disorders, cognitive deficits, and neurogenesis are just starting to be fully uncovered. In adult brains, new neurons continue to be generated in the subventricular zone (SVZ) and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. Briefly, neural stem cells in these areas divide to form progenitors, which generate neuroblasts (Remaud, et al., 2014). Literature suggests that the neuroblasts migrate into existing neural circuitry, where they eventually become mature, functioning neurons (Montero-Pedrazuela et al., 2006). While the exact

functional purpose of neurogenesis is unclear, many hypothesize that it contributes to hippocampal function (Remaud et al., 2014) and there is evidence that it is implicated in learning, memory, and mood (Desouza et al., 2005).

Studies have consistently shown impaired hippocampal neurogenesis in animal depression models, as well as restored neurogenesis after antidepressant treatment (Duman, 2004). Additionally, blocking hippocampal neurogenesis with irradiation exposure makes antidepressants ineffective – suggesting that this neuronal growth is necessary for antidepressant efficacy (Santarelli et al., 2003). There is ample evidence that neurogenesis is affected by thyroid status as well (e.g. Remaud et al., 2014), which underscores the thyroid's contribution to synaptic plasticity and growth. Desouza et al. (2005) was one of the first groups to demonstrate a clear role of TH function in neurogenesis in rats and found that induced hypothyroidism significantly decreased cell survival and neuronal differentiation in the SGZ. Hypothyroid rats that were treated with daily injections of T3/T4 did not show these reductions, suggesting that increased TH prevented hypothyroid-induced damage.

A study by Montero-Pedrazuela et al. (2006) demonstrated not only that THs are essential for normal neurogenesis in the SGZ, but also the that the hypothyroidism-induced impairments were related to depressive symptoms among individual animals. Their experiment compared control rats, rats made hypothyroid through surgery and low-iodine diets, and recovery rats who received a T3/T4 treatment after surgery. The number of proliferating cells in the dentate was reduced by 30% in hypothyroid animals, along with significant reductions in newborn neuroblasts and dendritic tree growth. Hypothyroidism did not affect behavior in an object recognition task; but it significantly

increased depressive behavior in a forced-swim paradigm, with higher depression related to reduced proliferating cells. TH treatment reversed all behavioral and neurological impairments, and recovery rats even had significantly more proliferation than euthyroid controls. It is unclear whether this increase was due to higher TH levels from the treatment, or whether it reflected a type of compensatory mechanism to recover from the thyroidectomy.

Hippocampal atrophy. Research on hypothyroidism and depression has consistently found the hippocampus to be implicated in both conditions. The hippocampus is recognized for its involvement in memory encoding, consolidation, and storage, which all rely heavily on processes of synaptic strengthening (Taskin et al., 2011). LTP and neurogenesis are also primarily mediated by the hippocampus, as reviewed above, which both have strong associations with thyroid function and MDD. With the goal of elucidating the mechanisms of depression as they relate to TH function, it is worth exploring their common implications in this area.

Notably, the density of TH receptors is particularly high throughout the hippocampus. This indicates that the hippocampus an important target region for TH action in the brain, which is supported by TH involvement in neurogenesis, LTP, cognition, and other hippocampal-related processes (Cooke, Mullally, Correia, O'Mara, & Gibney, 2014). There are many documented effects of hypothyroidism on hippocampal cells; for example, hypothyroidism in adulthood is associated with decreased HC volume and weight, reduced differentiation and survival of granule cells in the dentate gyrus, and increased expression of apoptotic signaling molecules in CA3 neurons (Lee, Brady, & Koenig, 2003; Taskin et al., 2011; Cortes et al., 2012; Raymaekers & Darras, 2017). In

addition, behavioral and cognitive impairments associated with TH deficits reportedly correlate with reduced synaptic plasticity in the hippocampus (Losi, Garzon, & Puia, 2008). An early study by Madeira et al. (1992) found significant hippocampal volume reduction after hypothyroid treatment in rats – an effect found whether the treatment was induced at birth or adulthood, and whether its duration was acute or chronic. Cell volume was significantly reduced in the CA3 hippocampal region, while the CA1 region showed both reduced volume and cell number. The degree of CA1 atrophy was increased when hypothyroidism was chronic. Another study found that offspring of rats made hypothyroid during pregnancy had reduced hippocampal volume and a thinner granule cell layer in adulthood (Gilbert, Goodman, Gomez, Johnstone, and Ramos, 2017), suggesting that these effects can be irreversible in some cases.

There is also a rich literature linking depression to cell loss and atrophy in the hippocampus (Duman, 2004). A variety of brain imaging studies, for example, have reported reduced hippocampal volume in MDD patients. It is possible that decreased volume could be an indicator of reduced neurogenesis, but there are likely other factors – like cell death, glia loss, and damage to existing neurons – that contribute to this finding (Duman, 2004). An MRI study found a significant negative correlation of -0.6 between the length of time that patients had been depressed and bilateral gray matter volume in the hippocampus (Sheline, Sanghavi, Mintun, & Gado, 1999). Like the results of Madeira et al. (1992) which found greater hippocampal volume reduction in chronic vs. acute hypothyroidism, these data suggest that neuronal loss is greater for patients with longer durations of the depressed state.

Much of the ketamine literature is additionally focused on hippocampal regions. For example, Tizabi, Bhatti, Manaye, Das, and Akinfiresoye (2012) found that genetically depressed rats given a chronic dose of ketamine exhibited an approximate 25% increase in hippocampal AMPA receptor density, which was associated with significant reductions in depressive-like behavior. Zhou et al. (2013) yielded similar results with an AMPA receptor potentiator, and additionally found increased mTOR and brain-derived neurotrophic factor (BDNF) in both the hippocampus and PFC. Furthermore, traditional antidepressants like desipramine and fluoxetine lead to increased mRNA expression of AMPA receptor subunits in hippocampal regions (Du et al., 2006).

It has been suggested that associations between hippocampal structure and depression could be related to the stress response. Elevated cortisol resulting from stress can have neurotoxic effects on hippocampal neurons, which could potentially lead to decreased neurogenesis, synapse formation, and cell survival (Liu, Liu, Wang, Zhang, & Li, 2017). Impairments in the hypothalamic-pituitary-adrenal (HPA) axis have been noted in depressed patients, resulting in increased cortisol and glucocorticoid levels. Experimental glucocorticoid administration has been found to reduce hippocampal neurogenesis in adult animals, which suggests that elevated glucocorticoid levels in depression could similarly have damaging effects (Duman, 2004). Thyroid function has also been associated with stress in the literature, and studies have reported lower TH concentrations in rodents using multiple chronic stress paradigms (Guo et al., 2014). Close associations between stress, TH activity, depressed mood, and neuronal growth could be another way by which hypothyroidism and MDD are connected to one another.

The Present Research

Although the relationship between depression and hypothyroidism is not clearly defined, it is apparent from the literature that they share a number of underlying processes. This study sought to better understand their connection by examining the effects of hypothyroidism on three different depression-related constructs. The behavioral measures that were utilized – the saccharin preference test, elevated plus maze, and object placement test – allowed for the examination of a broad range of symptoms within the hypothyroid model. Importantly, each of these measures has been found to successfully predict antidepressant efficacy in previous literature.

Behavioral measures.

Saccharin preference test. This behavioral test is a measure of anhedonia, which is a well-known symptom of MDD. The DSM-V describes anhedonia as decreased enjoyment toward stimuli or activities that were once found pleasurable (American Psychiatric Association, 2013), which has been operationalized in rodents as decreased preference for sweetened water. In this paradigm, animals are briefly deprived of water and then exposed to two water bottles: one with regular tap water, and one with sweetened water. Rodents have been found to consume significantly less sweetened water after depression is induced – an effect shown to be reversed after chronic antidepressant treatment (e.g. Willner, Towell, Sampson, Sophokleous, & Muscat, 1987).

Elevated plus maze (EPM). The EPM is commonly used in animal models as a validated measure of anxiety. This test has been able to successfully evaluate the anxiogenic and anxiolytic properties of specific compounds, as well as examine the brain areas and mechanisms related to anxiety (summarized in Walf & Frye, 2007). Assessing

anxiety-like behaviors is vital for the validation of a depression model, considering the close association between the two constructs. In humans, anxiety is extremely prevalent among patients with MDD and there is a high level of comorbidity between the two diagnoses (Preisig, Merikangas, & Angst, 2001). Some researchers have even proposed that a more dimensional model of psychiatric disorders, using depression and anxiety as axes, would represent symptomology more accurately than the categorical diagnoses in the DSM (Goldberg, 1996).

To this end, researchers commonly include the EPM in tests of animal depression models; and correlations between validated depression- and anxiety-related behavioral measures have consistently been found (e.g. Ducottet & Belzung, 2005; Hinojosa et al., 2006; Lee et al., 2007; Chiba et al., 2012; Mendez-David et al., 2017). This particular test operationalizes anxiety as the ratio of time spent in open versus closed sections of a maze. Mice are placed onto an elevated maze shaped like a plus sign, where two "arms" of the plus sign have walls (and are thus "closed") while the other two are open. Rodents are thought to be conflicted between two innate characteristics: a fear of heights and open/unprotected spaces, and a desire to explore novel environments. Greater amounts of time spent inside the protected, closed arms relative to the open arms reflect higher levels of anxiety (Walf & Frye, 2007). In addition to correlating with depressive-like behaviors, scores on the EPM are also correlated with biological markers of depression like reduced glucocorticoid receptor expression in the prefrontal cortex (Chiba et al., 2012) and decreased serotonin expression in the hippocampus (Lee et al., 2007). Performance has also found to improve after treatment with certain antidepressants such as chronic

administration of fluoxetine (Silva et al., 1999) and the AMPA receptor positive allosteric modulator S47445 (Mendez-David et al., 2017).

Object placement test (OP). The OP is a measure of spatial memory, which has been found to be significantly impaired in depressed patients (e.g. Porter, Gallagher, Thompson, & Young, 2003; Duman, 2004). Many studies have also reported decreased hippocampal volume in depressed patients compared to healthy controls, which is a brain area strongly implicated in spatial memory function (Sheline et al., 1999; Videbech & Ravnkilde, 2004). Briefly, rodents are first familiarized with two identical objects and one of them is moved to a new location in a subsequent testing session. Due to their natural preference for novel objects over familiar ones, normal animals should spend more time exploring the object in the new location. Contrary behavior is thought to reflect spatial memory impairment. Novel object preference in this test has been found to be significantly decreased in chronic stress models of depression (e.g. Bowman, 2005) while treatment with antidepressants, such as vortioxetine, can significantly reverse the effects (Li, Sanchez, & Gulinello, 2017).

Hypotheses. It was hypothesized that mice made hypothyroid through administration of 6-propyl-2-thiouracil (PTU) would display more depressive-like behavior than euthyroid control mice on each of the behavioral tasks. They were specifically expected to show a reduced preference for saccharin in the saccharin preference task, a lower percentage of time spent in the open arms of the EPM, and a decreased preference for exploring novel objects in the OP test. Additionally, adding thyroxine (T4) to the PTU treatment was expected to reverse any hypothyroidism-induced effects by restoring TH concentrations to a normal level.

Methods

Ethical Approval

All experimental procedures were approved by Montclair State University's Institutional Animal Care and Use Committee (approval number 2018-047) and were consistent with the ethical guidelines set in the National Institute of Health's *Guide to the Care and Use of Laboratory Animals* (2011).

Materials and Measures

Animals. This study used male C57BL/6J mice for the hypothyroid model. Mice are commonly used in medical research and have been documented to be able to successfully perform cognitive and behavioral tasks. Rodents have also been found to resemble humans in terms of their genetic and behavioral characteristics, and symptoms of various human conditions have been successfully replicated in mouse models (Melina, 2010). Male mice were used exclusively to avoid potential hormonal confounds related to the female estrous cycle (Beery & Zucker, 2010).

Forty-five male C57BL/6J mice arrived at the university vivarium at 5 weeks of age (The Jackson Laboratory, Farmington, CT). Mice were housed 5 to a cage and maintained at a room temperature ranging from 65-75°F with 30-70% humidity. They were kept under a standard 12-hour light/dark cycle, lights on at 7:00 am, and given water *ad libitum* from a central water source. In accordance with IACUC guidelines, mice were left undisturbed for a one-week period to acclimate to the environment with free access to standard rodent chow (Laboratory Autoclavable Rodent Diet 5010; LabDiet, St. Louis, MO). During the following two weeks, mice were weighed and

handled twice a week to habituate researcher interaction. They were also switched onto the study control diet (see below) until experimental treatments began.

Experimental treatments.

6-propyl-2-thiouracil (PTU): PTU is an antithyroid compound that works by inhibiting function of thyroid peroxidase (TPO), a crucial enzyme implicated in thyroid hormone synthesis. Specifically, the drug blocks the oxidation of iodide ions and the iodination of tyrosine induced by TPO (Taurog, 1976), which are necessary for the production of T3 and T4. PTU is commonly administered to human patients to help lower the severely high thyroid hormone concentrations associated with hyperthyroidism and Graves' disease (U.S. Food and Drug Administration, 2015). Administration of PTU has also successfully induced hypothyroidism in mice and rats in published experimental studies (e.g. Silva & Giusti-Paiva, 2014; Yu, Tang, Feng, & Chen, 2014; Armada-Dias, Carvalho, Breitenbach, Franci, & Moura, 2001).

In the present study, PTU (Sigma-Aldrich, St. Louis, MO) was administered with food at a concentration of 0.5g per kg of rodent chow (0.05% of food weight). Table 1 estimates the dosages of PTU based on the amount of food consumed.

(2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propanoic acid (thyroxine): In humans, thyroxine is used to treat hypothyroidism and other thyroid hormone deficiencies (Vaidya, 2008). It is also secreted naturally by the thyroid gland along with its metabolite T3, and both induce metabolic effects by binding to thyroid hormone receptors in cells throughout the body. Experimental administration of thyroxine has been found to increase serum thyroid hormone levels in rodents (e.g. Kulikov & Zubkov, 2007; Zubkov, Kulikov, Naumenko, & Popova, 2009) as well as

successfully reverse the behavioral and biological effects of induced hypothyroid states (e.g. Kulikov, Torresani, & Jeanningros, 1997; Armada-Dias et al., 2001). It was therefore expected that in the current study, administering thyroxine would help to normalize the thyroid hormone production inhibited by PTU.

Thyroxine (Sigma-Aldrich, St. Louis, MO) was administered through food at a concentration of 0.5 g/kg of chow (0.05% of food weight; see Table 1 for the estimated dosages). It was only administered with PTU, as the combination treatment was meant to provide a comparison group of euthyroid controls. In the present study, however, thyroxine administration did not produce this desired euthyroid effect. Mice in this condition began to show signs of hyperthyroidism around the third week of treatment, suggesting that the thyroxine dose used was higher than anticipated. The results section describes some of the unexpected effects resulting from thyroxine treatment, such as weight loss and shedding.

Rodent chow. Standard rodent chow was purchased from Research Diets Inc. (New Brunswick, NJ). The manufacturer infused the PTU diet with 0.5g PTU per kilogram of chow, and the PTU/thyroxine diet with 0.5g PTU plus 0.5g thyroxine per kg of chow. Thus, three separate types of chow were received: PTU-infused, PTU/thyroxine infused, and control chow.

Food was weighed and refilled for each cage at the start of every week. The amount of food (g) eaten each week was recorded in order to estimate drug dosages, as well as track any differences in food consumption between groups.

Behavioral measures.

Saccharin preference test. The procedure used for the saccharin preference test was based on methods described by Sadler and Bailey (2016). Both sucrose and saccharin have been utilized as sweeteners in this test, but the present study used saccharin to avoid the potential confound of caloric value. Approximately 4 weeks after the start of treatment, mice were transferred to cages containing one bottle of tap water and one bottle filled with a 0.03% saccharin solution. This concentration of saccharin was determined based on the results of previous pilot testing (see Appendix A). Mice were habituated to this solution with their cage mates for approximately 12 hours, after which they returned to their regular cages with their normal drinking water.

Saccharin preference testing began 3 days after the habituation period. Mice were housed in individual cages for approximately 12 hours (overnight) with a bottle of tap water and a bottle of saccharin solution. Cage dimensions were approximately 30.8 cm x 30.8 cm x 14.3 cm. Bottles of saccharin were randomly placed on either the left or right side of the cage. The bottles were weighed before and after the testing period to determine the volume of liquid consumed. Because there were not enough cages available to individually house each mouse, half were randomly assigned to test the following night. A “preference score” was calculated for each mouse by dividing the amount of saccharin solution consumed by the total amount of consumed solution. Lower preference scores for saccharin therefore reflected higher levels of anhedonia. As a secondary variable, we also recorded to total amount of solution consumed.

It should also be noted that as the saccharin preference tests were being conducted, we noticed that the tap and saccharin solutions tended to drip out of the bottles unintentionally. This seemed to be partially due to the cage design (Thoren

Caging Systems, Inc. Hazelton, PA), as bottles needed to be inverted to fit into position. Liquid also tended to spill when the cages were being arranged on the storage shelves, which seemed to reflect a flaw in the bottles and/or lids themselves. This spillage was especially problematic given that our measures directly depended on the bottle weights. The values we report do not account for such spillage, however, as spillage amounts were found to be normally distributed and therefore equally present in each treatment group. Appendix B reviews the empirical investigation conducted to address this concern.

EPM. The EPM was conducted approximately 5 weeks after the start of experimental treatment, and began after saccharin preference testing was complete. The maze (Maze Engineers, Boston, MA) consisted of 4 arms, each 35 inches long and 5 cm wide. The two closed arms contained walls on either side that were 40 cm in height. The arms and walls were constructed with blue Plexiglass, which allowed the mice to stand out on the SMART video system used to track mouse movement (described under "software"). The four arms extended out from a small central area of 5 square cm, and the entire maze was elevated at a height of 61cm above the ground by four metal legs (see Appendix B for a scaled drawing of the apparatus).

Each mouse was placed by the experimenter at the central area of the maze facing one of the open arms and allowed to explore freely for 3 minutes. Behavior was monitored by both a trained observer and a digital video tracking system. The maze was wiped down with a prepared disinfectant solution between each mouse trial, containing 21.4% potassium peroxymonosulfate and 1.5% sodium chloride. The primary variable of interest was the amount of time spent in the open versus the closed arms. Each mouse was given a "percent open" score by dividing the amount of time spent in the open arms

by the total time spent in the maze. Higher scores reflect lower levels of anxiety, and mice in the hypothyroid condition were expected to yield significantly lower scores. Total distance traveled was measured as a secondary variable to see whether PTU affected general locomotion.

OP. The OP was conducted in a 40x40x30 cm open field apparatus (Maze Engineers, Boston, MA) constructed from blue Plexiglass (see Appendix B for a scaled drawing). Black and white laminated shapes were taped to the walls to provide spatial cues. Small, colorful ceramic flamingo figurines were used as stimuli, which were found to encourage exploration in pilot tests (Appendix A). Between each training and testing session, both the arena and the figurines were thoroughly cleaned with prepared disinfectant.

Mice first explored the arena during a 5-minute information trial. A researcher placed each mouse at the center of the open field. Two ceramic flamingos were set up several inches apart in their initial locations. The mice were then put back in their cages for a 1-hour inter-trial interval, after which they re-entered the field for a 5-minute retention trial. Before the retention trial began, one of the flamingos was moved to its pre-established "novel" location closer to the opposite wall. Care was taken to ensure that during this novel condition, each flamingo was approximately the same distance away from a corner. Figure 2 describes how the objects were positioned during information and retention trials. For a scaled drawing of the open field apparatus, see Appendix B.

All trials were digitally recorded via the SMART video tracking system (see "Software"). A trained data coder went through each video file and used a stopwatch application to manually record how much time was spent exploring each object. Behavior

was considered to be exploratory if the mouse's head was clearly oriented toward the object. Behavior was not counted if the head orientation was unclear from the video, or if the mouse was sitting directly on top of an object. Each video file was labeled with a randomly assigned number to ensure that the data coder was blind to experimental condition.

The primary dependent variable was the amount of time spent exploring the object in the new location relative to the object in the familiar location. This was calculated by dividing the total time spent with the object in the new location by the total amount of exploration time. Lower scores therefore reflected more spatial memory impairment. As a secondary variable, we also measured the total amount of time spent investigating the objects.

Software. Digital video tracking software was used during the EPM and OP tests to record behavioral data. The SMART video tracking system by Harvard Apparatus used a digital camera to track mouse movement in real time. The system was calibrated to a stationary image of the Plexiglass such that when the mice were added, movement of their darker pixels could be easily followed. Behaviors were additionally monitored by trained observers to ensure accuracy.

Procedure and Design

This study used a between-subjects experimental design. When mice reached 8 weeks of age, they were randomly assigned to either a PTU, PTU/thyroxine (henceforth PTU/thy), or control group (n = 15 per group). PTU mice were referred to as the "hypothyroid" group, while PTU/thy mice were meant to be "euthyroid." However, because PTU/thy mice showed unanticipated signs of hyperthyroidism, it is clearer to

refer to each group by their experimental treatment rather than their thyroid status. See Table 2 for an additional overview of the study design.

Mice of the same group were housed together in order to simplify the feeding process. There were thus three cages of each experimental group with five mice in each cage. On the first day of treatment, mice underwent a noninvasive ear notching procedure so that each mouse could be individually identified. The experimental treatment period lasted for 4 weeks. Mice were weighed and observed 2-3 times a week, and food was weighed and refilled at the start of each week. Mice had free access to water and were maintained in accordance to IACUC standards.

Behavioral tests took place 4 weeks after the start of treatment at 12 weeks of age. Mice remained on their regular experimental diets during this testing period. Saccharin habituation was completed first, followed by the saccharin preference test and EPM. Due to concerns of observed weight loss, all PTU/thy mice were sacrificed before OP testing occurred. Thus, there is no spatial memory data available for PTU/thy mice. The observed weight loss in PTU/thy mice will be addressed further in the results and discussion sections. The remaining PTU and control mice were sacrificed after the OPT was complete. Figure 3 provides a timeline of experimental procedures.

Tissue collection. After behavioral tests were completed, mice were anesthetized with CO_2 and sacrificed by decapitation. Brains were quickly separated from the skull and placed on a thin sheet of plastic situated on a bed of crushed dry ice. When the brains were completely frozen after about 15 minutes, they were transferred to small glass tubes and stored at -20°C. Tissue from the prefrontal cortex and hippocampus were sliced 10 micrometers thick with a cryostat and mounted onto glass slides for future binding

experiments. Appendix D describes the procedures used for tissue preparation, and Appendix E gives a proposed methodology for examining AMPA receptor binding using autoradiography. Although binding was not assessed in the present study, we believe it to be a valuable extension of this research.

Statistical Analyses

Attrition. Two mice in the PTU/thy group were excluded from the behavioral analyses. One was found deceased shortly before the start of behavioral testing, and one was sacrificed the same day after a 20% decrease from maximum body weight was observed. This degree of weight loss requires animals to be humanely euthanized according to ethical guidelines. These two mice were also excluded from analyses involving body weight, as they were missing data from the 12-week-old time point.

Behavioral data. Analyses were conducted to compare variables of interest among the three conditions (PTU, PTU/thy, and control). For the behavioral tests, the primary variables of interest were the following: preference score (saccharin preference test), time spent in the open arms of the maze (EPM), and spatial memory impairment (OPT). Formulas used to calculate each score are provided below. Secondary analyses were conducted for additional variables of interest: total amount of solution consumed in the saccharin preference test, total distance traveled in the EPM, and total exploration time during the OP. P-values were not adjusted for these secondary analyses. All behavioral data was analyzed with R Studio using the following procedure.

First, a Lilliefors test determined whether the data was normally distributed. The test was run on the residual values (as opposed to the raw values) by first subtracting each

value by the group mean. This step was taken remove the possibility that group differences stemming from treatment effects could affect the normality analysis.

If the data proved normal, they were screened for outliers using Pierce's criterion. Outliers were excluded from analysis but reported in the supplemental materials (see Appendix E). A one-way, between-subjects ANOVA was then calculated for each variable and followed by Tukey-Kramer post-hoc tests if necessary. Independent samples t-tests were used for OP-related dependent measures, because there was only data from two experimental groups available in this task. This change was required because unforeseen events required euthanasia of mice in one treatment group prior to starting this test (see discussion below in the OP/Spatial Memory section)

Non-normal data was analyzed with the Kruskal-Wallis H test, followed by a Mann-Whitney U post-hoc. This nonparametric test was used because it does not assume normality. Additionally, no outliers were eliminated from non-normal data because Pierce's criterion detects outliers at an unacceptably high rate in non-normal data. Effect sizes are reported in terms of eta^2 for normal behavioral data, but these values could not be calculated for non-normally distributed data.

Behavioral score formulas. For primary variables: Saccharin preference (%) was defined as the total saccharin water intake (in grams) divided by the total amount of solution consumed (saccharin intake + tap intake) * 100. Percentage of time spent in the open arms of the EPM was similarly defined as the time (in seconds) spent in the open arms divided by the sum of time spent in the open and closed arms. Novel object preference, or spatial memory, was expressed by the amount of time spent exploring the novel object (in seconds) divided by the total amount of exploration time. For secondary

variables: Total consumption in the saccharin preference test was obtained by adding the amount of tap water consumed with the amount of saccharin water consumed. Total distance traveled in the EPM was the sum of distances traveled in the closed arms and the open arms. Total exploration time on the OP was the sum of time the mice spent exploring objects in the both the familiar and novel locations.

Food consumption and body weight. Body weight and food consumption data were analyzed to examine potential treatment-related effects. For each week beginning with the first week of treatment, average body weight was calculated for each mouse from the 2-3 times they had been weighed. Food consumption was estimated by determining the difference in cage food weight between the start and end of each week, and then dividing the obtained value by the number of mice in the cage. A mixed between-within subjects ANOVA was performed in SPSS to examine the effects of time and group on each measure. Pairwise comparisons were adjusted with a Bonferroni correction, and values are expressed as mean ± SEM.

Results

Behavioral Measures

Saccharin preference/anhedonia. A Lilliefors test revealed a non-normal distribution among saccharin preference scores ($D = 0.15$, $p = 0.02$). A nonparametric Kruskal-Wallis test found significant differences between groups (χ^2 (2, $n = 42$) = 15.86, $p < 0.001$). Further analysis with a Mann-Whitney U post-hoc found that PTU/thyroxine mice had significantly higher preference scores than both the hypothyroid and control groups ($U = 151$, $p = 0.002$; $U = 161$, $p < 0.001$, respectively). Contrary to our

hypothesis, however, PTU mice did not differ in preference with controls. These data are summarized in Figure 4.

As a secondary variable, we also looked at the total amount of water consumed (both saccharin and tap). A one-way ANOVA showed a significant effect of treatment group total consumption ($F(2,38) = 9.1$, $p < 0.001$, $eta^2 = 0.32$), and a Tukey HSD post-hoc revealed that PTU/thyroxine mice drank significantly more than the other two groups ($M = 18.49$, $SD = 3.47$). Although PTU mice consumed less than controls, this difference was not significant ($M = 14.69$, $SD = 0.98$; $M = 16.31$, $SD = 1.91$, respectively).

Elevated Plus-Maze/anxiety. Statistical results from the EPM are represented in Figure 5. The percentage of time spent in the open arms of the maze – with lower percentages reflecting higher anxiety – was significantly different between treatment groups ($F(2,37) = 7.403$, $p = 0.002$, $eta^2 = 0.286$). As predicted, hypothyroid mice spent significantly less time in the open arms compared to the other groups ($M = 17.67\%$, $SD = 0.12$ for control mice; $M = 5.38\%$, $SD = 0.06$ for PTU mice; $M = 22.54\%$, $SD = 0.14$ for PTU/thyroxine mice). Secondary analyses revealed that the total distance traveled (cm) also differed between groups ($F(2,39) = 6.30$, $p = 0.004$), with significantly lower travel distance for PTU mice ($M = 518.47$, $SD = 54$) than PTU/thyroxine mice ($M = 622.79$, $SD = 96.4$). However, neither group differed significantly from controls ($M = 581.18$, $SD = 80$).

Object placement/spatial memory. There was no difference in either novel object exploration time ($t(24.1) = -0.39$, $p = 0.7$) or total exploration time ($t(24.1) = 0.067$, $p = 0.95$) between the PTU and control groups. Degrees of freedom were adjusted in order to control for heterogeneity of variance. These data are shown in Figure 6.

Treatment Effects

General observations. During the first week of experimental treatment, researchers noted that PTU/thy mice appeared to be more aggressive than mice in the other groups; specifically, they were noticeably more physical with their cage-mates and more difficult for the experimenters to handle. This observation persisted over the next few weeks. During the third week of treatment, it was also observed that PTU/thy mice had a noticeable decrease in fur quality. Researchers reported clumps of fur throughout the cages and described a "shaggy" and "ragged" appearance. It was also during this time that researchers noticed that PTU/thy mice were smaller than the others.

At the end of the fourth week of treatment (right before the onset of behavioral testing), one of the PTU/thy mice was found deceased. Another was found to be 20% below their maximum body weight and was therefore euthanized according to ethical guidelines. Several other PTU/thyroxine mice had dropped 10% below their maximum weight, and it seemed evident that weights would continue to decrease. The remaining PTU/thy mice were able to perform the saccharin preference and EPM tests, but researchers and supervisors decided to euthanize the group before putting them through the lengthier OPT. It should therefore be noted that PTU/thy mice were euthanized approximately a week before the rest of the mice.

Body weight. Figure 7 shows average body weight (g) during experimental treatment. A mixed between-within subjects ANOVA found significant main effects for age ($F(2.85, 114.1) = 55.08$, $p < 0.001$, partial $eta^2 = 0.58$) and group ($F(2,40) = 5.2$, $p = 0.01$, partial $eta^2 = 0.21$), as well as a significant interaction between the two ($F(5.7, 114.1) = 83.99$, $p < 0.001$, partial $eta^2 = 0.81$.). Bonferroni-adjusted pairwise

comparisons found that on average, PTU/thy mice weighed significantly less than PTU mice (M = 22.25g, SD = 1.54 compared to M = 23.62g, SD = 1.37) and controls (M = 23.84g, SD = 2.08, p = 0.014).

Food consumption. Figure 8 shows the average food consumption (g) per mouse, estimated from per cage consumption, in each treatment group over the course of experimental treatment. A two factor mixed between-within subjects ANOVA revealed that treatment group ($F(2,6) = 105.90$, $p < 0.001$, partial $eta^2 = 0.97$) and an age/group interaction ($F(2.4, 7.2) = 10.60$, $p = 0.006$, partial $eta^2 = 0.78$) both had significant effects on the amount of food consumed (degrees of freedom were adjusted for heterogeneity of variance). Mouse age did not have a main effect on consumption alone. Bonferroni-adjusted pairwise comparisons showed that on average, PTU/thy mice consumed significantly more than PTU mice (M = 28.12g, SD = 2.89 compared to M = 18.07g, SD = 1.27, $p < 0.001$) and controls (M = 19.89g, SD = 1.16, $p < 0.001$). Food consumption differences between the PTU and PTU/thy groups are especially important to note, as this indicates a difference in estimated drug dosages as well. Table 1 describes the estimated dosages of treatments for each group.

Discussion

We hypothesized that inducing a hypothyroid state in mice through administration of PTU would induce reductions in saccharin preference, increased anxiety-like behavior in the EPM, and impaired performance of the object placement task in comparison to controls. Furthermore, we hypothesized that restoring thyroid function through thyroxine treatment would attenuate these differences. Our results found that treatment with PTU failed to induce differences in saccharin preference or object placement performance

compared to control mice. However, PTU-treated animals did exhibit a decrease in the percentage of time spent in the open arms of the EPM relative to controls, which can be interpreted as an increase in anxiety-like behavior. In addition, mice given the PTU/thyroxine treatment had a significantly higher preference for saccharin, as well as significantly higher levels of water consumption, compared to control and hypothyroid mice. Furthermore, the PTU/thyroxine group performed at equal levels to controls in the EPM, suggesting that increased thyroid function did, indeed, attenuate the anxiogenic effects of PTU treatment.

Interpretation of our results is complicated by findings from the PTU/thyroxine group. TH-treated groups of hypothyroid animals are typically included in these types of designs to test whether restoring TH function can block potential hypothyroidism-related effects. In this regard, this study's addition of T4 to experimental treatment seemed to normalize the anxiety-like behaviors associated with PTU in the EPM task. However, observations of increased food take, significant body weight reduction, and poor fur quality indicated that mice within the PTU/thyroxine group exhibited some hyperthyroid-like symptoms. Although exact serum measurements of TH levels are unavailable at this time, we inferred from these observations that the PTU/thyroxine dose used here likely raised TH concentrations above normal levels. A hyperthyroid state therefore restored responses to baseline levels during the EPM, but raised responses above baseline levels in the saccharin preference test.

Taken together, these data suggest that our hypotheses were only partially supported and hint at a complicated relationship between thyroid status and our three measures of depression-like symptoms. The findings suggest that TH deficiency could

result in anxiety-like behaviors, but has little effect on measures of spatial memory and anhedonia. TH overstimulation, on the other hand, could lead to increased preference for sweet solutions – as well as prevent hypothyroidism-induced anxiety without necessarily affecting anxiety in itself. Such results raise broader questions about how individual facets of MDD could have different types of relationships with TH function. These questions are considered below in greater detail as they relate to each behavioral measure.

Saccharin Preference/Anhedonia

Primary measure: Saccharin preference. In the saccharin preference test, decreased preference for sweetened versus tap water is thought to reflect a depression-like reduction of enjoyment to pleasurable stimuli (Willner et al., 1987). This study did not find the expected relationship between hypothyroidism and anhedonia, but it did find an increased preference for saccharin in mice treated with a PTU/thyroxine combination. The negative findings in the PTU group are inconsistent with several studies that reported decreased sucrose or saccharin preference in hypothyroid mice (e.g. Rivlin et al., 1997; Yu et al., 2014; Ge et al., 2016). However, the majority of studies demonstrating depressive-like behavior in hypothyroid models have done so using fundamentally different behavioral paradigms – such as the forced swim test (FST) and tail suspension test (TST) – which measure depression in terms of immobility time (e.g. Wilcoxon et al., 2007; Pilhatsch et al., 2010; Ge et al., 2013; Ge et al., 2016; Bocco et al., 2016). A particular study by Ge et al. (2013) found that hypothyroid mice exhibited significant depressive-like behavior in the FST and TST but showed no behavioral changes in tests of sucrose preference. They additionally reported decreased hippocampal T3 concentrations in hypothyroid rats, which correlated with immobility time in the FST and

TST but not with levels of anhedonia. Taken together, suggest that reduced TH levels could be more strongly associated with measures of behavioral despair than with anhedonia-specific measures of depression.

Alternatively, preference for sweetened water could be an inappropriate way to operationalize anhedonia in hypothyroid models. There is some evidence that THs influence taste perception in both humans (McConnell, Menendez, Smith, Henkin, & Rivlin, 1975) and animals (Rivlin et al., 1977), which raises the possibility that differences in saccharin preference could be attributed to fondness for different tastes rather than a reflection of anhedonia. In one experiment, Rivlin et al. (1977) found that hypothyroid rats had a significantly lower preference for sweetened water but a higher preference for water made bitter with quinine sulfate. Treatment with TH normalized the preferences for both bitter and sweetened water back to control levels, suggesting that TH concentrations could affect inclinations toward particular flavors. If increased TH levels are in fact associated with a fondness for sweet flavors, this could reasonably explain why PTU/thyroxine mice had a higher saccharin preference than the other groups in the present study. It would also make sense, in this case, for thyroxine to elicit higher saccharin preference despite the absence of PTU-induced anhedonia. Furthermore, it would help explain why the PTU/thyroxine combination raised saccharin preference above control levels but did not show a similarly increased effect on the EPM.

There are several other ways in which these saccharin preference findings can be interpreted. One possibility is that the present dose of PTU was too low to detect hypothyroidism-induced anhedonia, and a higher dose would have revealed the expected effects. It is also possible that processes underlying the anxiety- and anhedonia-related

behaviors assessed in the current study have qualitatively different relationships with TH function. EPM-related processes, for example, could have been more sensitive to the TH deficiency than the specific mechanisms utilized in the saccharin preference test. In support of this idea, Pilhatsch et al. (2010) found that treating euthyroid mice with T3 had significant effects on behavioral measures of depression but not on behavioral measures of anxiety – despite the fact that their hypothyroid model had significant effects on both. This emphasizes the idea that THs may not necessarily have identical effects on different behavioral processes.

Secondary measure: Total consumption. PTU/thyroxine mice additionally consumed significantly more water – both tap and sweetened – than hypothyroid and control mice. This finding is not surprising given that PTU/thyroxine mice also consumed significantly more food than the other groups throughout the study. They additionally weighed significantly less than the other groups of mice and exhibited rapid weight loss during the last week of treatment; which, when taken together, is indicative of high metabolic activity. This is also consistent with unsystematic observations of increased brown adipose tissue of PTU/thyroxine mice, which is known to drive metabolic activity. While increased food intake and metabolic rates can reasonably explain an increase in water consumption during the saccharin preference test, they importantly do not explain why PTU/thyroxine mice preferred sweetened water to tap water to such a high degree. Saccharin was intentionally used instead of sucrose in the present study because it lacks caloric value, so increased preference for saccharin cannot be attributed to caloric intake.

Methodological concerns. In the present study, there were several concerns worth discussing that arose during saccharin preference testing. Appendix C elaborates

on the issue of water spillage during the procedure, noting how solution tended to drip out of the test bottles as they were being inverted into each cage. The saccharin preference procedure was replicated with empty cages in order to estimate the average amounts of solution lost overnight by reasons other than mouse consumption. Because spillage amounts were distributed normally across the cages – and there is no reason to suspect that experimental groups were differentially affected by this issue – it was found unlikely that water loss affected the general patterns of saccharin preference that were observed between the treatment groups. However, it is possible that the water loss decreased the obtained values of saccharin preference scores. Pilot test data found saccharin preference to be around 77% (Appendix A), while control mice in the present study had an average preference of only 58%. The PTU/thyroxine group, which showed the highest levels of preference, additionally had 10% less preference than the pilot mice. Since mice were tested five to a cage during these preliminary tests, the problem of spillage was not as apparent.

In addition, the test procedure was slightly modified from the methods established a priori due to health concerns in the PTU/thyroxine group. This group exhibited dramatic weight loss during the final week of treatment, and one mouse was found deceased shortly before behavioral tests began. Another was humanely euthanized before the testing procedures after losing over 20% of its maximum body weight, which is ethically required according to IACUC protocol. The original procedure for saccharin preference involved depriving mice of water overnight, and then exposing them to bottles of sweetened and tap water for a period of 2-4 hours. Because drops in body weight are common in mice after overnight water deprivation, it was decided this would pose too big

a risk for the PTU/thyroxine mice. Instead, mice were housed in individual cages overnight with the two water types and each bottle was subsequently weighed the following morning. Both these procedures have been utilized in the literature, so it is unlikely that this change altered the results.

EPM/Anxiety

Primary measure: Open arm time. The primary finding in this experiment is that PTU-induced hypothyroidism had a significant effect on anxiety-like behavior in the EPM. Hypothyroid mice spent a significantly lower percentage of time in the maze's open arms compared to the other groups; which, in this paradigm, is thought to reflect more fear and avoidance for unfamiliar places (Walf & Frye, 2007). Anxiolytic treatments like diazepam (Pellow, Chopin, File, & Briley, 1985) and chronic fluoxetine (Silva et al., 1999) have consistently been found to reverse this effect, supporting the predictive validity of this measure.

Other labs have reported similar findings associating animal models of hypothyroidism with a range of different measures of anxiety (e.g. Pilhatsch et al., 2010; Buras et al., 2013; Bocco et al., 2016; Ge, Xu, Qin, Cheng, & Chen, 2016). On one hand, the fact that different behavioral paradigms have been used to support a relationship between anxiety and hypothyroidism seems to suggest that their association is quite robust. For example, Buras et al. (2013) found consistent patterns of hypothyroidism-induced anxiety on four behavioral tasks: the EPM, open field test, light-dark transition, and fear conditioning. Montero-Pedrazuela et al. (2011) found these associations were also present in a more complex conditioning paradigm wherein hypothyroid mice had increased auditory and contextual fear memory (operationalized by freezing behavior) 3

weeks after conditioning began. Pilhatch et al. (2010) used both a dark-light box and a startle response test, which examine very different behavioral responses, to in order demonstrate hypothyroidism-induced anxiety. Together, these findings indicate an effect of TH deficiency on anxiety that goes beyond the EPM-specific definition of staying in the closed, safe arms of a plus-shaped maze.

Closer inspection of each study's findings, however, raises questions about just how generalizable the thyroid/anxiety relationship is. There are a number of different variables that can "indicate" anxiety within the EPM alone, so operationalization of the construct can therefore vary considerably across studies. One report found hypothyroid mice had increased anxiety through greater amounts of freezing and less "head dipping" into the maze's open arms, but the percentage of time spent in the open areas was not significantly reduced as it was in the current study (Bocco et al., 2016). Another only found differences between hypothyroid and control mice in the amount of entries into closed arms (Mundstock Dias et al., 2012). There is a limited amount of research specifically addressing anxiety in hypothyroid animal models; so despite some of the promising findings that were discussed, it is important to keep in mind that differences in behavioral paradigms, definitions of effects, and actual hypothyroid models could exaggerate any perceived effects on anxiety that thyroid status may have. Furthermore, a study by Yu et al. (2015) notably found significantly increased EPM anxiety in hyperthyroid mice while hypothyroidism had an anxiolytic effect.

The effects of hyperthyroidism on anxiety also raise questions about how THs mediate the construct. In the present study, PTU/thyroxine mice displayed several signs of hyperthyroidism that suggested an overproduction or overactive functioning of THs.

Purposely induced hyperthyroidism in the literature has led to increased anxiety in some studies (e.g. Yu et al., 2015) and decreased anxiety in others (e.g. Stohn et al., 2016), with key differences in the specific hyperthyroidism models used. In this study, PTU/thyroxine mice did not differ with controls in terms of anxiety-like behaviors; however, they did have significantly decreased anxiety compared with mice in the hypothyroid group. Since the present study was not designed to induce hyperthyroidism, it is likely that the thyroxine dose used here was not high enough to produce a significant hyperthyroid-induced effect. Our results are similar to those of Buras et al. (2016) who reported that although not all differences were necessarily significant, there was a consistent pattern of euthyroid mice displaying anxiety at levels between hypothyroid and TH-treated animals. They suggested that TH levels likely have a gradient-like of effect on anxiety, which the patterns found here seem to support as well.

Because the present study did not find similar effects of hypothyroidism on anhedonia or spatial memory, our results seem to suggest that TH deficiency has specific effects on anxiety-related processes. It is possible that the widely reported relationships between hypothyroidism and MDD can primarily be attributed to the anxiety-related symptoms that accompany depression. This idea is supported by findings from Bathla et al. (2016) that 73% of hypothyroid patients reported having some degree of anxiety on the HAM-A compared to 60% reporting some degree of depression on the HDRS. There was additionally an extremely high correlation ($r = 0.93$) between scores on the two measures, which helped to illustrate the strong overlap in responses to items targeting symptoms of anxiety. Furthermore, Montero-Pedrazuela et al.'s (2011) work on fear memory demonstrated higher corticosterone plasma levels in hypothyroid rats after fear

conditioning as well as increased glucocorticoid receptor expression in the amygdala. Because elevated cortisol and glucocorticoid levels have neurotoxic effects that lead to decreased synapse formation and cell survival (Liu, 2017), it is tempting to speculate that the depressive-like reductions in synaptogenesis and cell signaling found in hypothyroid models could be secondary to more direct effects of hypothyroidism on anxious behaviors. This speculation speaks far past any concrete evidence in the literature or the present study, however, and further research would be warranted to examine the possibility.

It is more likely that hypothyroidism's selective effect on anxiety in the current study reflects the PTU dosage used, problems with methodology, or limitations of the specific depression measure. There is one study that reported higher TSH and T4 levels in patients specifically with panic disorder which correlated significantly with anxiety severity (Kikuchi et al., 2005); but the majority of reported relationships between TH function and anxiety – both in human populations and animal models – are accompanied by strong relationships to depression as well.

Secondary measure: Locomotor behavior. In addition, PTU-treated mice exhibited significantly lower total distance traveled compared to PTU/thyroxine mice but not controls. Because traveling less in general and slower travel rates could be confounding explanations for less time spent in the open arms, these data help to support the contention that underlying differences in specific anxiety processes drove the observed behavioral differences. Moreover, the observed differences in travel distance could be related to fatigue, which is commonly associated with hypothyroidism (Nussey & Whitehead, 2001). However, these data could also represent lower body temperatures,

which are also a symptom of hypothyroidism and are commonly associated with reduced locomotor activity in rodents (Venero et al., 2005).

OP/Spatial Memory

Primary measure: Novel object exploration. There were no differences in novel object exploration time between hypothyroid and control mice in the OPT. This study's failure to demonstrate a significant relationship between spatial memory and TH function is surprising, especially given the vast amount of literature connecting thyroid function to hippocampal processes. The hippocampus has a notably high amount of TH receptors, making it a particularly important target region for TH activity (Raymaekers & Darras, 2017). Several studies have accordingly demonstrated spatial memory impairment in hypothyroid animals that correlate with impaired hippocampal LTP – both of which are restored by TH treatment (Alzoubi et al., 2009; Dong et al., 2011). It is possible that the discrepancy between previous studies and the current findings has to do with the particular spatial memory task utilized. For instance, the majority of research demonstrating impaired spatial memory in hypothyroid models used radial water maze tasks (e.g. Wilcoxon, Nadolski, Samarut, Chassande, & Redei, 2007; Alzoubi et al., 2009; Galton, Schneider, Clark, & Germain, 2009) rather than novel object placement.

However, the present OP data were particularly concerning in that even control mice did not show the expected preference for novel object placement. This finding contradicts the natural preference for novel over familiar locations reported in the OPT literature (e.g. Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002), as well as findings from our own extensive pilot tests (Appendix A). It is highly likely that the present results were influenced by temperature and humidity problems that arose in the vivarium

on the days that the OPT was being conducted. Experimental animals must be housed under heavily regulated conditions, and the increased room temperatures observed during OP testing led to several interruptions in the procedure by maintenance and veterinary staff. It also likely led to behavioral and biological alterations that make the validity of the present findings questionable.

There are other methodological aspects of the OPT that could have contributed to our negative findings. For example, the video tracking software used in the EPM was not useful for measuring object exploration in this particular task. Exploration was therefore coded entirely by hand using recorded tapes of experimental sessions; and because all tests were conducted in dim lighting conditions, video quality was not always clear. Erring on the side of caution, behavior was not coded as exploration if head orientation was not completely evident. It is therefore possible that not all object exploration was properly accounted for in the present data. Additionally, mice were not habituated to the open field area before the information session due to time constraints. A habituation session would have further familiarized the mice with the testing environment, and potentially resulted in more exploratory behavior for the objects once they were introduced. However, a true effect of hypothyroidism should have revealed behavioral differences between groups whether or not a habituation session was included.

There is additionally no spatial memory data for PTU/thyroxine mice, as rapid weight loss required them to be euthanized before the test could be conducted. This is particularly unfortunate, as object exploration data from this group could have provided further insight into the meaning of our negative results. If PTU/thyroxine mice exhibited a higher novel object preference than the other groups as they did in the saccharin

preference test, for example, it would strongly suggest that the PTU/thyroxine treatment consistently results in altered behavioral responses. It would also raise the possibility that the PTU dose was too low to detect saccharin preference and spatial memory changes, but that these two tests are nevertheless mediated by TH activity. Furthermore, equal performance to control and hypothyroid groups would provide further evidence that our results were confounded by the temperature increase.

Secondary measure: Total exploration time. Hypothyroid mice additionally did not differ from controls in terms of total exploration time. Unfortunately, this finding does not hold much value given the testing conditions described. Exploration time could have also been raised or altered with the addition of a habituation period, which should be included in future experiments.

Limitations and Future Directions

Drug treatments. The biggest limitation of this study was that the thyroxine concentration used in our model was dramatically miscalculated. A daily dose of 50-70 µg/kg of body weight was estimated from previous literature, but the actual dose consumed in this study was calculated to be 87.57 mg/kg of body weight. This dose of thyroxine, which is markedly higher than expected, resulted in hyperthyroid-like symptoms in the PTU/thyroxine group. There were several signs of treatment-related side effects during the beginning of the treatment period; for example, they tended to consume more food than the other groups, their fur became noticeably ragged, and they were somewhat more difficult to handle. During the fourth week of treatment, body weight began rapidly decreasing and one of the mice was lost through attrition. IACUC ethical guidelines require that mice with a 20% decrease in body weight be humanely sacrificed

to minimize potential suffering, and the research team and veterinarian accordingly euthanized all PTU/thyroxine mice before behavioral tests were finished.

The hyperthyroid symptoms in PTU/thyroxine mice makes their observed behavioral effects particularly difficult to interpret. PTU/thyroxine treatment appeared to normalize the anxiety-like behaviors seen in PTU mice, for example, but one would expect a hyperthyroid model to reverse anxiety-like behavior to a level below that of controls. In the saccharin preference test, PTU/thyroxine treatment contrarily raised saccharin preference significantly above control levels. One major challenge to interpreting these results is that it is unclear how TH concentrations in PTU/thyroxine mice compare to that normal, euthyroid levels.

Questions about the strength of hypo- and hyperthyroid effects could be addressed in future studies by including full dose-response curves to both PTU and thyroxine. Comparing the behaviors of a PTU/low-thyroxine to a PTU/high-thyroxine group could provide more information about how TH treatment affects hypothyroid models. Additionally, including a low-PTU and high-PTU group could examine whether the strength of behavioral effects depend on the strength of PTU administration. This information is especially valuable to have in the absence of serum TH and TSH measurements, which is another important limitation in this study. It should be noted, however, that blood samples were collected to examine TH concentrations for future extension of this research. Serum levels of T3 and T4 can then be examined to see whether there was a relationship between TH concentration and specific behavioral responses.

Finally, the PTU and thyroxine treatments used here do little to differentiate TH in the brain vs. TH in the periphery. The present study was designed to investigate a general hypothyroid model, but future studies could benefit from specifically examining TH function in the CNS. Mechanisms targeted by the thyroid that influence depressive-like behavior are more likely related to brain activity than that in the peripheral tissue, and thyroid dysfunction in human MDD patients is rarely an overt thyroid disorder (e.g. Hage & Azar, 2012). One particular study achieved this using mice with selective D2 inactivation in astrocytes (Bocco et al., 2016) and another used mice with the mutant TR α1 (Pilhatsch et al., 2010), which are two interesting models to consider.

Behavioral tests. Unfortunately, very little information about spatial memory was uncovered in the present study due to several limitations in the OPT. First of all, there is no behavioral data from PTU/thyroxine in this paradigm which limits our understanding of how TH function affected exploratory behavior. Second, increased temperatures in the vivarium during testing could have likely influenced the observed behavior. Although veterinary staff advised us to postpone behavioral testing, over half of the mice had been tested in the high-temperature environment by that point. It was our contention that changing testing conditions halfway through the procedure would ultimately be more detrimental to the experiment. It is also possible that there simply was not an effect of PTU on OPT performance, but the fact that even control mice did not prefer the novel placement strongly suggests that the environmental variables negatively affected behavior.

Additionally, a close review of the literature raised the question of whether anhedonia, as measured by the saccharin preference test, was the best choice in our

behavioral operationalization of depression symptoms. While decreased sucrose/saccharin preference has been found in some hypothyroid models (Yu et al., 2014; Ge et al., 2016), the forced swim and tail suspension paradigms have been found to be more strongly affected by hypothyroid treatment (Ge et al., 2014). Additionally, the strong relationship between thyroid function and metabolism could be confounding in a saccharin preference measure. It is highly likely that the increased saccharin preference and water consumption in PTU/thyroxine mice had more to do with metabolic activity than decreased anhedonia.

Future directions. This work was done as part of a broader goal to elucidate the biological and neurological mechanisms implicated in MDD, and these results are the beginning of a larger investigation. Current research on the MDD is focusing, in part, on intracellular signaling, synaptic plasticity, and the glutamatergic system. The thyroid system – despite being notably crucial for many cellular processes and widely associated with depressive-like behaviors – has yet to be explicitly examined in this particular context. The preliminary behavioral evidence gathered here is supportive of a hypothyroid model inducing particular depressive-like symptoms, especially as they relate to anxiety on the EPM. Furthermore, the finding that thyroxine treatment normalized the amount of time spent in the EPM open arms suggests that addition of TH to the hypothyroid model had some antidepressant-like properties. For example, fluoxetine (Silva et al., 1999) and the AMPAR positive allosteric modulator S47445 (Mendez-David et al., 2017) have been found to reverse this same effect.

There is accumulating evidence that the glutamate receptor AMPA could be implicated in the robust antidepressant effects of ketamine (e.g. Koike et al., 2011, Zhou

et al., 2014). Tissue slices from the prefrontal cortex, amygdala, and hippocampus were collected from the mice in the present study so that AMPA receptor binding can later be examined in this hypothyroid model (procedures further described in Appendices D and E). Treatment with antidepressants like fluoxetine, desipramine, and ketamine have all been associated with increased AMAPR expression in these regions (Du et al., 2006; Tizabi et al., 2012), so it will be exciting to see how binding is influenced by the PTU and thyroxine treatments. Additionally, AMPA expression can be studied in relation to the behavioral responses found in the current research. In sum, there is great potential for future research to examine TH function, depressive symptoms, and glutamate neurotransmission as they relate to one another.

Conclusion

The present study provides preliminary evidence that a model of hypothyroidism by administration of PTU can model symptoms of anxiety in the elevated plus-maze paradigm. Addition of the TH thyroxine prevented these anxiety-like effects induced by the hypothyroid model. This study did not find any effect of hypothyroidism on anhedonia, as measured in the saccharin preference test, or spatial memory, as assessed by the object placement test. However, a PTU/thyroxine combination treatment resulted in increased saccharin preference, which could be interpreted as an increase in the reward value of the sweetened solution. Future studies should examine how behavior is affected by different doses of PTU/thyroxine treatment to better understand the role of TH in these paradigms.

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Table 1

Estimated Daily Treatment Dosages

Group	Treatment	Mean dose/day
PTU	PTU	53.04 mg/kg body weight
PTU/thy	PTU & thyroxine	87.57 mg/kg body weight

Note. Estimated from average weekly food consumption per cage.

Table 2

Overview of Experimental Design

Condition	N	Experimental Treatment
PTU (hypothyroid)	15	Standard rodent chow + PTU (0.5 g/kg of chow)
PTU/thyroxine (hyperthyroid)	15	Standard rodent chow + PTU (0.5 g/kg of chow) + thyroxine (0.5 g/kg of chow)
Control	15	Standard rodent chow

Note. PTU = 6-propyl-2-thiouracil

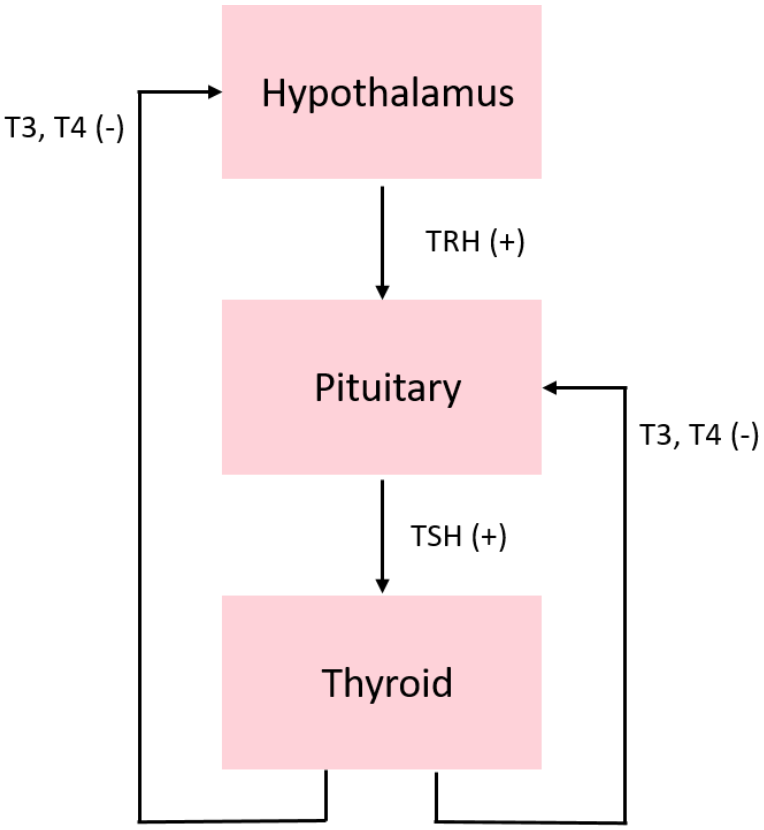

Figure 1. Overview of the hypothalamic-pituitary-thyroid axis.

Thyroid releasing hormone (TRH) produced in the hypothalamus is released to the anterior pituitary, stimulating the release of thyroid stimulating hormone (TSH). This initiates production of T3 and T4 in the thyroid gland. High levels of TH in the bloodstream negatively regulate TRH and TSH release.

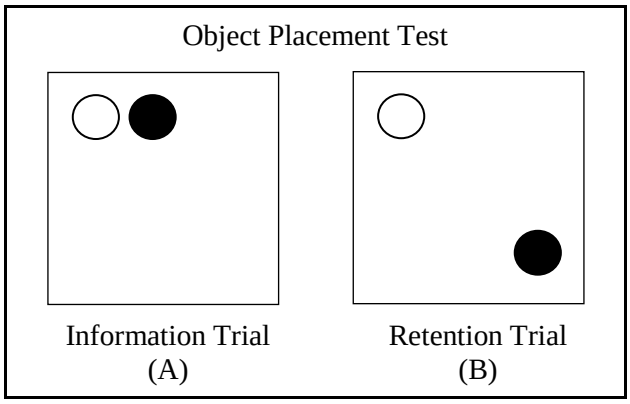

Figure 2. OPT Object Placements.

Approximate positioning of each object during the information (A) and retention trials (B). One object (unshaded) remained in the same position across trials. The other (shaded) was moved to a novel placement during retention.

Nov 19 – Nov 25 *Mouse age: 5 weeks*

Mice arrive at vivarium; 1-week acclimation period with standard chow

Nov 26 – Dec 9 *Mouse age: 6-7 weeks*

Acclimation period ends; mice put on control diet for 2 weeks. Weighed & handled twice a week.

Dec 10 – Jan 6 *Mouse age: 8-11 weeks*

Cages are randomly assigned to treatment groups, and mice receive ear notches for identification. Experimental treatment ensues for 4 weeks.

Note: On 1/5, one PTU/thyroxine mouse was found deceased and another was put down

Jan 7 – Jan 13 *Mouse age: 12 weeks*

Behavioral testing. All mice remain on experimental diets. Mice complete saccharin habituation period, saccharin preference test, and EPM. All PTU/thyroxine mice were put down, and OP continued for PTU and control mice.

Jan 14 – Jan 27 *Mouse age: 13-14 weeks*

PTU and control mice sacrificed; plasma & tissue collected.

Figure 3. Experimental Timeline.

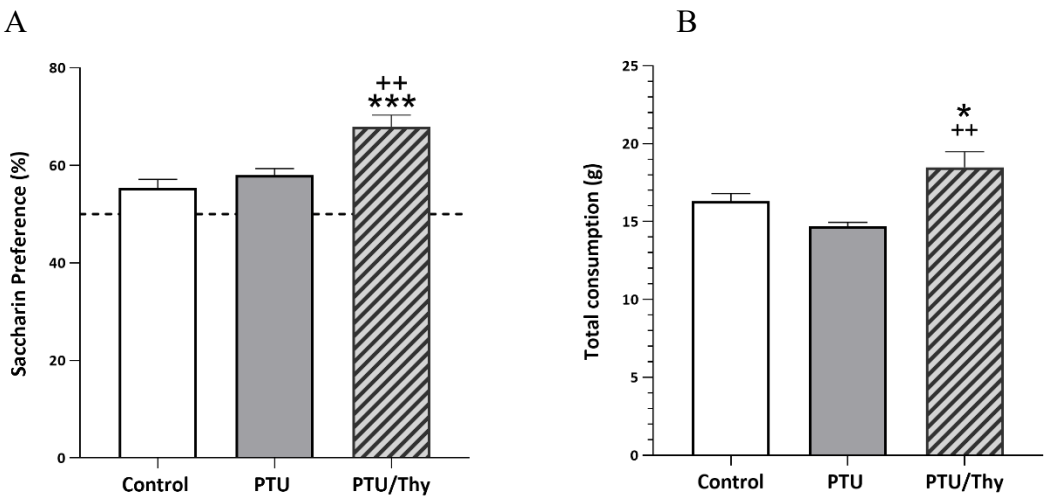

Figure 4. Behavioral Measures on the Saccharin Preference Test.

A) Mean saccharin preference scores (± SEM), expressed as a percentage of total water consumption. ***p<.001 in comparison to controls, and ++p<.01 compared to PTU (Kruskal Wallis Test, Mann-Whitney U post-hoc); n = 12 for PTU/thy, and 15 for PTU and controls. Dotted line represents 50% preference.

B) Total combined consumption of saccharin and tap water (g), expressed mean ± SEM, *p<.05 compared to controls, ++p<.01 compared to PTU (one-way ANOVA, Tukey HSD). N = 12, 14, and 15 for PTU/thy, PTU, and controls (respectively). One outlier was excluded from analysis in the PTU group.

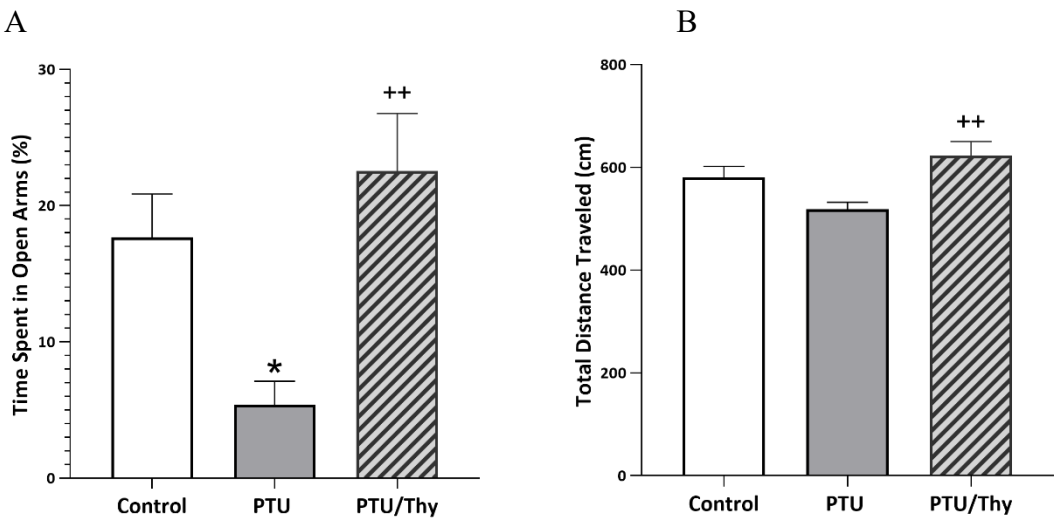

Figure 5. Behavioral Measures on the Elevated Plus-Maze

A) Mean percentage of time spent in the open arms, relative to the closed arms of the maze (± SEM). *p<.05 and ++p<.01 in comparison to controls and PTU, respectively (one-way ANOVA, Tukey HSD); n = 12, 13, and 15 for PTU/thy, PTU, and controls. Two outliers were excluded from the PTU group.

B) Total distance traveled in EPM (cm), expressed mean ± SEM, ++p<.01 compared to PTU (one-way ANOVA, Tukey HSD). N = 12 for PTU/thy and 15 for PTU and controls.

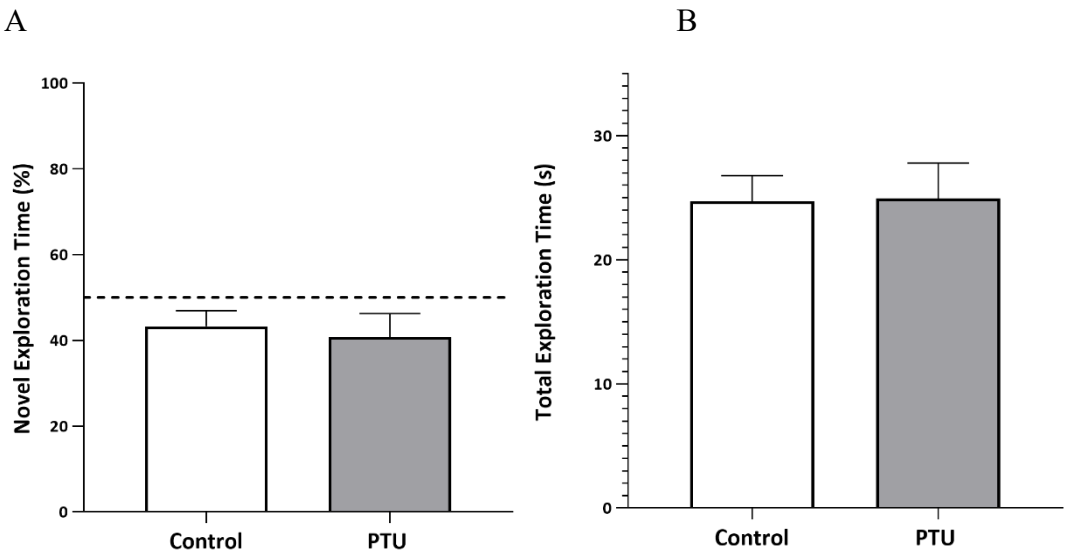

Figure 6. Behavioral Measures on the Object Placement Test

There were no significant differences between PTU and control mice in percent novel exploration time (A; N = 15 for both groups) or total exploration time (B; N = 14 PTU, 15 control). One outlier in the PTU group was excluded from analysis of total exploration.

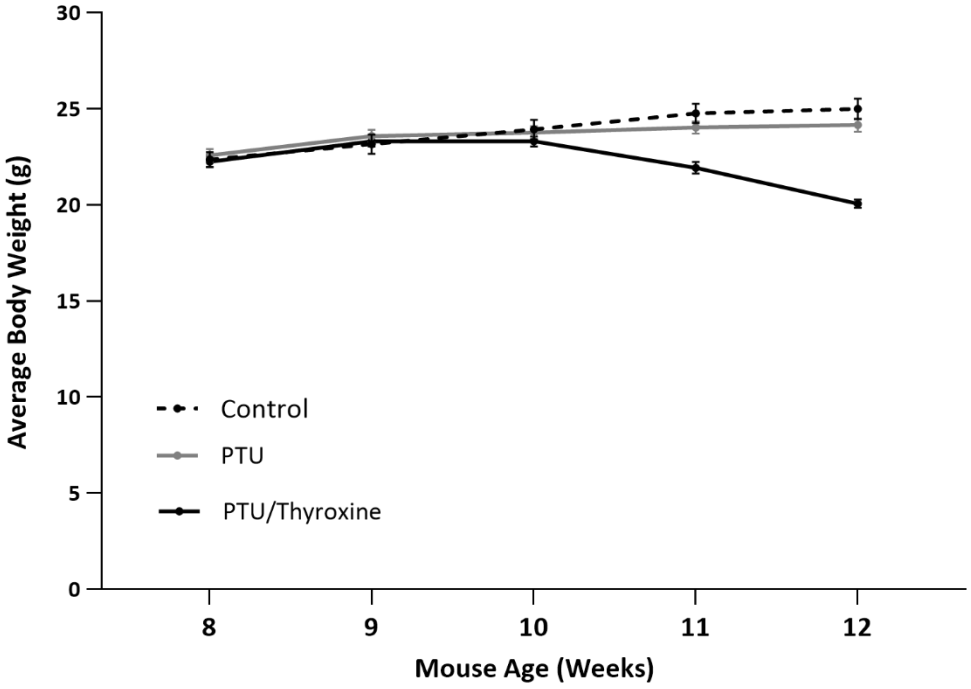

Figure 7. Mean Body Weights.

Mean body weight (g) for each group throughout the duration of the experiment. Error bars represent standard error. There were significant main effects of mouse age ($p < 0.001$) and treatment group ($p = 0.01$), and a significant age x group interaction ($p < 0.001$; mixed ANOVA). PTU/thy mice weighed significantly less than the other two groups ($p < 0.05$, Bonferroni adjustment).

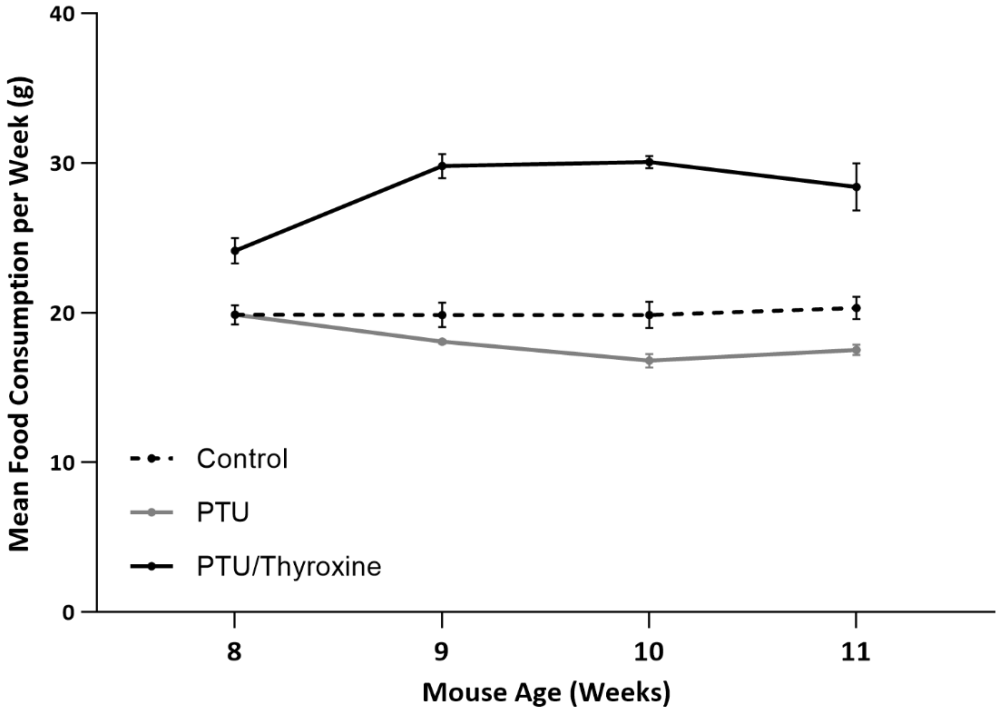

Figure 8. Mean Food Consumption.

Mean food consumption (g) per mouse during each week of treatment, expressed mean ± SEM. PTU/thy mice were fed only through 11 weeks of age. Effects were significant for group and a group/age interaction ($p < 0.01$, mixed ANOVA). PTU/thy mice consumed significantly more compared to the others ($p < 0.001$, Bonferroni adjustment).

Appendix A

Pilot Data for Behavioral Tests

Table A1

Saccharin Preference Pilot Data

Cage	Concentration	Saccharin Preference	Saccharin Consumed(g)	Tap Consumed(g)	Total Consumed(g)
2	0.01%	59.06%	15	10.4	25.4
3	0.01%	70.79%	20.6	8.5	29.1
1	0.03%	77.85%	22.5	6.4	28.9
4	0.03%	77.63%	22.9	6.6	29.5

Note. Cages of mice (n=4, 5 mice per cage, 50% male) were left overnight with one bottle of tap water, and one bottle of either 0.01% or 0.03% saccharin solution. Bottles started with 200mL of solution and were weighed before and after the overnight period. Table A1 shows the amount of tap water consumed, the amount of saccharin solution consumed, and the total amount of solution consumed for each cage. Saccharin preference was calculated by dividing the amount of saccharin consumed by total consumption. The 0.03% solution seemed to yield a higher and more consistent saccharin preference, and was thus the concentration used for the hypothyroid study behavioral tests.

Table A2

EPM Pilot Data

Trial Length (minutes)	N	Mean % of time in open arms	SEM
3	10	30.7%	0.034
4	10	31.7%	0.032
5	10	30.6%	0.033

Note. EPM pilot data was analyzed to determine whether the percentage of time spent in open arms remained stable across different trial lengths. Male mice (n=10) were each given 5-minute sessions in the maze. Video tracking data was examined up to the 3-minute, 4-minute, and 5-minute marks. Because there was no significant difference in percentage of time spent in open arms across time points ($f(2) = 0.035$, $p = 0.97$), a 3-minute trial length was chosen for the hypothyroid study tests.

Table A3

OP Pilot Data: Object Selection

Object	N	Mean exploration time (seconds)	SEM
Flamingo	6	48.6	2.94
Angel	4	35.9	3.19

Note. For the object placement test, it was important to select an object stimulating enough to induce exploration in the mice. Male mice (n=10) were randomly assigned to explore one of two objects: a small ceramic white angel figurine, or a ceramic multicolored flamingo of roughly the same size. Mice were placed in an open field for 5 minutes with two identical figurines of the assigned object. Overall, mice spent more time investigating the flamingo (mean time = 48.6 seconds ± 2.94 SEM) than the angel (mean time = 35.9 seconds ± 3.19 SEM). This difference proved significant in a t-test ($t(16.42) = 2.94$, $p < 0.01$). The flamingo figurines were therefore used for the hypothyroid study tests.

Table A4

OP Pilot Data: Inter-Trial Interval

ITI (minutes)	N	Mean % novel placement exploration time	SEM
30	10	62.6%	2.84
60	10	60.7%	2.45

Note. Male mice (n=10) underwent the object placement task twice, on separate days. In the first session, there was a 60-minute delay between the information and retention trials. In the second session, the delay was reduced to 30 minutes. Percentage of novel placement exploration time in retention trials did not differ based on the inter-trial interval ($t(17.61) = 0.51$, $p = 0.62$). A 60-minute ITI was chosen for hypothyroid study tests because the timing allowed for more efficient testing procedures.

Table A5

OP Pilot Data: Novel Object Placement

Object Placement	N	Mean % novel exploration time	SEM
Modified Placement	6	62.7%	2.06
Original Placement	10	60.7%	2.45

Note. A subset of mice (n = 6) underwent the object placement test with the modified object placements and a 60-minute ITI. There was no difference in percentage of novel object exploration between the old and new placement setups ($t(13.81) = 0.63$, $p = 0.54$).

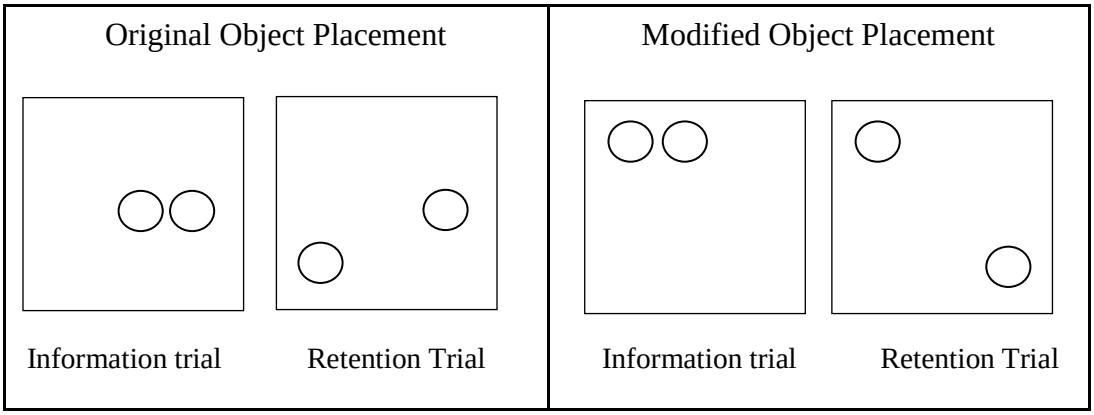

Figure A1. OP Pilot Data: Approximate Object Placement

It became apparent that preference for the novel object placement could alternatively be explained by a preference for the arena corners. Object placements were modified so that during retention trials, novel and familiar objects were approximately the same distance from a corner of the field. Figure A1 shows the old and modified object placements for information and retention trials.

Appendix B

Apparatus Measurements

Figures B1 and B2 are scaled depictions of the apparatus used in the EPM (Figure B1) and OPT (Figure B2). Images were obtained from Maze Engineers (2017).

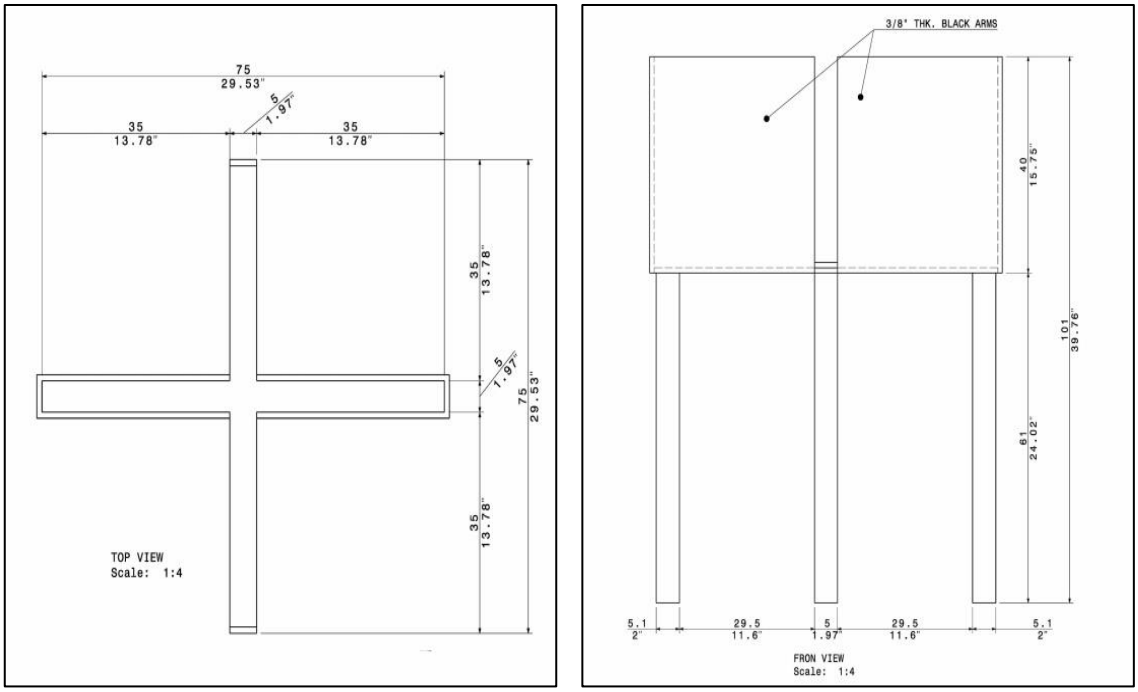

Figure B1. Elevated Plus Maze Dimensions

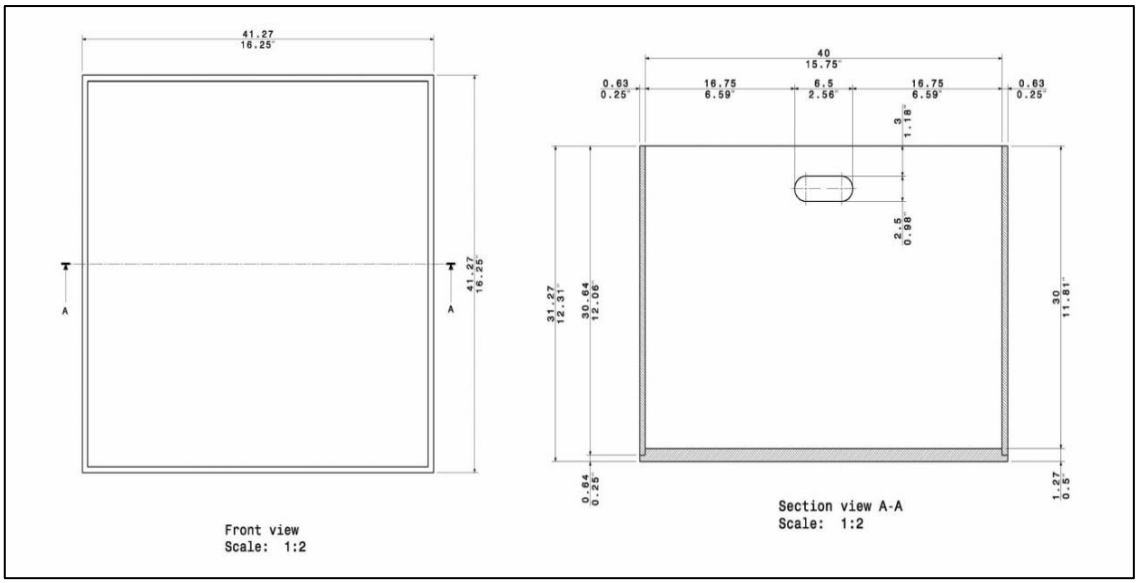

Figure B2. Object Placement Field Dimensions

Appendix C

Investigating Solution Spillage in the Saccharin Preference Test

As noted in the Methods section, there was a concerning amount of solution spillage during the saccharin preference test. Tap water and saccharin solution tended to drip out of the testing bottles as they were placed into the cages. Because the bottles were very sensitive to even the smallest amounts of pressure, we were concerned that the solutions were also dripping throughout the night in response to natural mouse movement.

The obtained preference test results seemed to verify these concerns. Across all treatment groups, mice had an average mean solution consumption of 16.44g ± 0.41 SEM. Average body weight during the week of saccharin testing was 23.21g ± 0.39 SEM. This means that on average, mice would have been drinking 70.83% of their body weight in a 12-hour period — a number which we find extremely unrealistic. An empirical spillage test was conducted to further investigate this issue.

Twenty-four empty cages were fit with two bottles of solution – 200mL of tap water, and 200mL of 0.03% saccharin solution. The bottles of saccharin were randomly assigned to the right or left side of the cage. Cages were prepared and handled in an identical manner to how they were handled in the saccharin preference test. They were left on the same storage racks used in the behavioral tests for the same 12-hour overnight period. Bottles were weighed before and after the testing period to measure the amount of solution lost.

Average spillage was 9.22g ± 0.6 SEM for saccharin-filled bottles, and 10.56g ± 0.29 SEM for tap-filled bottles. Differences between saccharin and tap spillage did not

quite reach significance ($t(32.93) = -2.02$, $p = 0.052$); but the higher variance among saccharin spillage values (2.94 vs. 1.40 SD) and near-significance in the t-test made us suspect that the spillage characteristics could differ between the two solutions, possibly due to different surface properties. We hoped to obtain more accurate consumption values by subtracting the obtained consumption amounts by average spillage. This proved to be problematic, however, as mice consumed less solution than average spillage in several cases.

Concerns about spillage were ultimately dismissed for two reasons. First, a Lilliefors test revealed that spillage values were normally distributed ($D = 0.08$, $p = 0.63$). Second, there was no reason to suspect that spillage amounts differed between mice of different treatment groups. We reasoned that while solution spillage may have affected the total consumption or saccharin preference values, it is unlikely that the degree of difference among treatment groups was compromised. We therefore decided to report our saccharin preference and consumption values as measured, with the assumption that any potential spillage should have been randomly distributed across cages. Still, one should be aware that the measures of saccharin and tap consumption reported here might include a certain degree of error.

Appendix D

Methods for Tissue Sectioning

Slides were created with tissue slices from the hippocampus and prefrontal cortex for binding experiments. Slices were cut 10 micrometers thick along the coronal axis with a cryostat. Paxinos and Franklin's (2012) neuroanatomical atlas was used for reference, which estimates the location of specific brain structures relative to landmarks on the mouse skull. The present study found regions of interest based on their positioning from Bregma, which is the central point on the skull where the sagittal and coronal sutures intersect. Slices approximately 2mm to 1.4mm anterior to Bregma were used for the prefrontal cortex—a range estimated by Paxinos and Franklin (2012). A literature search was conducted to determine an optimal range for the hippocampal tissue, as it was imperative to use locations related to spatial memory function. There is evidence that lidocaine injections blocked spatial memory in mice when injected in the hippocampal CA1 region, -2.0mm from Bregma (Assini, Duzzioni, & Takahashi, 2009). Another study found impaired spatial learning in mice with hippocampal lesions from -1.0 to -3.5mm (Cho, Friedman, & Silva, 1998). This study used a range of approximately -1.3mm, which also included areas of the amygdala implicated in the EPM. Slices were mounted onto glass slides, and approximately 12 tissue slices were collected per region for each mouse. They were then stored at -20℃ for future use.

Appendix E

Examining AMPA Receptor Binding: Overview of Autoradiography and Proposed Methods

AMPA receptor binding in the prefrontal cortex, hippocampus, and amygdala will be measured using autoradiography. This technique involves labeling ligands with radioactive isotopes to study their binding patterns with the receptors of interest. Radioligand experiments and based on the law of mass action, which assumes that the ligand can both bind (associate) and dissociate with a given receptor (binding is thus assumed to be reversible). In this model, equilibrium is said to be achieved when the rate at which ligand/receptor complexes are formed and the rate at which they dissociate are equal. One way to quantify AMPA receptor binding is to determine the amount of specific binding of the radioligand at equilibrium using different radioligand concentrations. Because radioligands bind to nonreceptor sites as well as the receptors of interest—referred to as nonspecific binding—the amount of nonspecific binding must be measured first, and then subtracted from the total binding amount. Nonspecific binding can be determined by using an unlabeled drug at a sufficiently high concentration that will bind to virtually all the receptors of interest. Since the drug will occupy all the receptor sites, binding of the radioligand will be nonspecific and specific receptor binding can be calculated (Motulsky, 1996).

Specific binding at different concentrations of the radioligand can be plotted, and the data can be used to determine the total receptor number. This value, or the B(max), represents total receptor density expressed in fmol of the radioligand per mg of tissue. In this study, the B(max) will quantify AMPA receptor binding as the primary dependent

variable. [^{3}H]AMPA will be used to label AMPA receptors in the sectioned brain tissue. The tissue will be tested for total and nonspecific binding at different concentrations of the radioligand (likely 1, 3, and 10nm). A phosphor screen will be used to collect radioactivity, and the slides will be put in a phosphor imager. Radioactivity can be quantified by the amount of fluorescence detected per unit of area. Average intensity from the nonspecific conditions will be subtracted from the total binding condition to produce a measure of specific binding at each concentration of the radioligand. These measurements can generate the B(max), or receptor density, which can be compared between groups with a one-way ANOVA.

Appendix F

Data with Outliers Included

There were several cases where, while behavioral data was being analyzed, outliers were identified using Pierce's criterion. It was decided *a priori* that these values would be excluded from the final analyses reported in the Results section. The following tables show statistical results including outliers for "percent open" scores in the elevated plus-maze, and total consumption scores in the saccharin preference test. In both cases, the overall differences remain significant. There were also outliers removed for total exploration scores in the object placement test, but those differences were not significant with or without outlier data.

Table E1

EPM Percent Open Scores Including Outliers

	df	**Sum of Squares**	**Mean Square**	**F**	**p**
Group	2	0.136	0.068	4.389	0.02
Residuals	39	0.604	0.015		

Note. A Tukey post-hoc revealed that PTU mice spent a significantly smaller percentage of time in the open arms (m=8.67% ± 0.03 SEM compared to 22.5% ± 0.04 SEM; p = 0.02). However, the difference between PTU and control mice was no longer significant (m=17.7% ± 0.03 SEM for controls).

Table E2

Total Consumption in Saccharin Preference Test Including Outliers

	df	Sum of Squares	Mean Square	F	p
Group	2	84.8	42.4	7.93	0.0013
Residuals	39	208.6	5.35		

Note. A Tukey post-hoc revealed that PTU/thy mice consumed significantly more solution than PTU mice (m=18.5g ± 1 SEM compared to 14.9g ± 0.35, $p < 0.001$). However, the difference between PTU/thy and control mice was no longer significant, with a marginal p-value of 0.051 (m=16.3g ± 0.49 for controls).