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Synaptic plasticity and cognitive ability in experimental adult-onset hypothyroidism

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Running title: Hypothyroidism impairs synaptic plasticity

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Abbreviations:

AC1: adenylate cyclase type I
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP5D: 2-amino-5-phosphonopentanoate
CA1: corno-amonis 1, a hippocampal brain region
CREB: cyclic AMP response element binding
DG: dentate gyrus area of the hippocampus
ERK: extracellular signal-regulated kinase
E-LTP early long-term potentiation
fEPSP: field excitatory postsynaptic potential
GABA: gama-aminobutyric acid
GAD: glutamic acid decarboxylate
GSK3B: Glycogen synthase kinase 3B
I/O: input–output
LTP: long-term potentiation
L-LTP: late long-term potentiation
LTD: long-term depression
MHFS: multiple high frequency stimulation
NMDA: N-methyl-D-aspartate glutamate receptors
P-CaMKII: phosphorylated calcium calmodulin kinase 2
PFC: prefrontal cortex
PKCg: phosphokinase C gama
PPF: Paired-pulse facilitation
PSD: post synaptic density
PTU: 6-n-propyl-2-thiouracil
RAWM: radial arm water maze
RC3: Neurogranin
SGZ: subgranular zone of the dentate gyrus

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Abstract

Adult-onset hypothyroidism impairs normal brain function. Research on animal models of hypothyroidism has revealed critical information on how deficiency of thyroid hormones impacts the electrophysiological and molecular functions of the brain, which lead to the well-known cognitive impairment in untreated hypothyroid patients. Currently, such information can only be obtained from experiments on animal models of hypothyroidism. This review summarizes important research findings that pertain to understanding the clinical cognitive consequences of hypothyroidism, which will provide a better guiding path for therapy of hypothyroidism.

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Keywords: LTP; LTD; neurogenesis; learning and memory; brain structure; synaptic plasticity; neurogranin; NMDA receptors; area CA1, Dentate gyrus

Significance: Cognitive impairment occurs during adult-onset hypothyroidism in both humans and animal models. Findings from animal studies validate clinical findings showing impaired LTP, decreased CaMKII and increased calcineurin. Such findings can only be gleaned from animal experiments to show how hypothyroidism produces clinical symptoms."

Introduction

Impairment of learning and memory is among several dysfunctions of the central nervous system, which occur during adult-onset hypothyroidism in both humans and animals (Burmeister et al. 2001; Gerges et al., 2004; Alzoubi et al., 2006, 2009; Rivas and Naranjo, 2007; Reid et al., 2007; an Koromilas et al., 2010; He et al., 2011; Ge et al., 2012;). Serious cognitive

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impairments have been consistently reported by patients with untreated et al hypothyroidism having symptoms including poor memory for recent events, inability to focus, slow mental activity, and difficulty to understand complex questions (Haggerty et al., 1990; Osterweil et al., 1992; Mennemeier et al., 1993; Leentjens and Kappers, 1999; Burmeister et al. 2001; Samuels et al., 2007; Aghili et al., 2012; Yin et al., 2013). The hypothyroidism-induced disorders are mostly reversible with hormone replacement therapy, suggesting that adult-onset hypothyroidism, when properly treated, may not result in permanent structural and functional deficiencies.

Reports of findings from studies in animal models of adult-onset hypothyroidism validated clinical findings by showing impaired learning and memory coupled with diminished long-term potentiation (LTP), widely considered as a corelate of learning and memory (Gerges et al., 2004; Alzoubi et al., 2005, 2006, 2009; Tong et al., 2007). These reports also described alterations in levels and activities of essential signaling protein molecules in the molecular cascades responsible for synaptic transmission and neuroplasticity (Gerges and Alkadhi, 2004; Alzoubi et al., 2005, 2007).

Thyroid hormone in the adult brain is essential for normal brain functions including neurogenesis. Therefore, cognitive effect of hypothyroidism results when the hormone level in the brain is reduced leading to impaired brain function associated with a range of clinical symptoms, including cognitive deficit, depression and mood swings (Liu and Brent, 2021).

The hippocampus, a brain region essential for development of learning and memory, has a high density of thyroid hormone receptors. It is believed that thyroid hormones promote cell survival, therefore, any change in thyroid hormone levels would be expected to interfere

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with hippocampus-related learning and memory, synaptic plasticity, and neurogenesis. Acquisition of learning tasks, which depend on the hippocampus, can be disrupted when LTP is impaired. A cardinal feature of LTP expression in excitatory synapses in the hippocampus is its dependence on activation of N-methyl-D-aspartate (NMDA) glutamate receptors (Collingridge et al., 1983; but see Alkadhi, 2021). Behavioral tests have shown that blocking LTP by intracerebroventricular infusion of the NMDA receptor blocker D-2-amino-5-phosphono-pentanoate (AP5), impairs spatial learning (Morris et al., 1986; Morris, 1989). This review summarizes reported effects of thyroid hormone deficiency on adult brain structure and function in animal models of hypothyroidism.

Rat models of hypothyroidism

The hypothyroid rat has been the most widely used animal model in the study of thyroid hormone dysregulations in adults. Two common procedures have been commonly utilized to generate hypothyroid rats both of which have advantages and drawbacks. The thyroidectomy procedure, which involves surgical removal of the thyroid gland, is time-consuming and may involve complications including inflammation and/or infection at the site of surgery, which can be avoided by good hygiene and application of proper antibiotics and anti-inflammatory ointments. Other effects of thyroidectomy are incomplete removal of the thyroid gland and unwanted removal of the parathyroid glands. Incomplete excision of the thyroid gland can, sometimes, be considered an advantage as it may mimic some level of clinical severity of the disease.

The second model entails the use of parenteral or oral administration of an antithyroid drug such as 6-n-propyl-2-thiouracil (PTU), carbimazole or methimazole, which inhibits the

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production of new hormone in the thyroid gland (Yoshihara et al., 2019). The convenience of using anti-thyroid drugs is an obvious advantage in perinatal and maternal hypothyroidism models. However, possible toxic effects of these drugs including liver damage and other direct toxic actions are serious disadvantage that could confound the experimental results. The effects of PTU have been thoroughly studied inasmuch as the drug is clinically used in the treatment of hyperthyroidism. Transgenic mouse and rat have also been used to study various aspects of thyroid hormone dysregulation (see Dillmann, 1999).

Excitability and synaptic plasticity in hypothyroidism

Synaptic plasticity in the brain is typically assessed by short-term and long-term activity-dependent modifications of synaptic efficacy. It is widely accepted that intracellular molecular events that underlie synaptic plasticity are the basis of learning and memory (Bliss and Collingridge, 1993; McNaughton, 1993). Various forms of synaptic plasticity can be measured experimentally in animal models of hypothyroidism. A brief discussion of these forms of plasticity is narrated below.

Effect of hypothyroidism on neuronal excitability: The Input/output curve

The input–output (I/O) curves represent the association between stimulus strength and the response it generates. I/O curve is affected by a variety of factors including brain disorders. The slope of the I/O curve can be used as a general measure of cortical excitability (Alavi et al., 2021) and can also be used as a clinical biomarker for a variety of neurological conditions (Ridding and Rothwell, 1997; Pitcher et al., 2003; Potter-Baker et al., 2016; Kojovic et al., 2017; Stefanou et al., 2017; Latorre et al., 2019; Kemlin et al., 2019; Kojima et al., 2019; Derosiere et al., 2020; Sirkka et al., 2020; Sundman et al., 2020; Khedr et al., 2020a, 2020b).

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No significant difference was observed in the I/O relationship between control and thyroidectomized animals in the responses generated by various stimulus intensities in area CA1 and dentate gyrus (DG) of the hippocampal formation (Alzoubi et al., 2005, 2006b; Fernandez-Lamo et al., 2009; Yousef et al., 2019; Babur et al., 2020). However, at variance with these findings is a reported increase in the I/O curve of CA1 and DG areas that has been shown in brain slices from rats treated with PTU (Glombik et al., 2021). Moreover, another group reported that PTU-treated rats showed a significantly attenuated I/O relationship in the DG area of the hippocampus (Artis et al., 2012). This disagreement may be a result of the use of PTU to induce hypothyroidism, recording from brain slices (Gilbert, 2011; Gilbert and Lasley, 2013) and/or the effect of hypothermia, which is exacerbated by anesthesia (Sanchez-Huerta et al, 2015). In my lab we also determined the stimulus intensities required to generate the minimal and maximal responses and our results indicated that they were not significantly different in euthyroid control and thyroidectomized anesthetized animals (Alzoubi et al., 2005). Therefore, basal synaptic transmission seems to be generally unaltered in area CA1 and DG of thyroidectomized rats. Similar conclusion was reached in the medial prefrontal cortex, where PTU-induced hypothyroidism in anesthetized adult rats did not significantly affect basal synaptic transmission (Sui et al., 2006).

Paired-pulse facilitation (PPF): Paired-pulse facilitation (PPF) is a widely used method to test for short-term alteration of synaptic transmission. It is a form of short-term synaptic plasticity in which the response to a second stimulus, delivered promptly after the first one, is increased (Zucker and Regehr, 2002). It measures variations in the probability of neurotransmitter release at the presynaptic nerve terminal (Thomson, 2000; Zucker and

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Regehr, 2002; Lauri et al., 2007). The PPF phenomenon may be an important mechanism for learning, since learning impairment has been linked to deficits in PPF (Chapman et al., 1995; Silva et al., 1996).

It has been demonstrated that PPF is normal in the DG area of thyroidectomized rat (Fernandez-Lamo et al., 2009). However, impaired PPF was reported in area CA1, but not the DG area in brain slices from PTU-hypothyroid rat (Glombik et al., 2021). Impaired PPF in PTU-treated rats was also reported in the dorsomedial prefrontal cortex (mPFC) pathway, which is important for learning and memory (Sui et al., 2006). These diverse findings may be a function of different areas of the brain examined, methods of recording of PPF and/or different methods of induction of hypothyroidism in experimental animals.

Long-Term Potentiation (LTP): Modifications of synaptic strength is widely accepted as the associate of learning and memory. Short- and long-term increases in synaptic transmission strength can result in specific patterns of afferent stimulation of the synapse. Long-term potentiation (LTP), a synaptic transmission enhancement resulting from repetitive activation of the synapse (a model of learning experience), is widely regarded as a cellular correlate of learning and memory (Bliss and Collingridge, 1993; Barnes,1995).

The two extensively studied forms of LTP; the early (E-LTP) and late (L-LTP), differ from each other in several features although both forms are mostly N-methyl-D-aspartate (NMDA) receptor-dependent. The E-LTP, which mainly requires CaMKII phosphorylation, and L-LTP, which requires de novo protein synthesis through activation of transcription factors such as CREB (Kandel 2001; Kandel and Schwartz 2001). The two forms, E-LTP and L-LTP have been suggested to correspond to short and long term memory (Kandel 2001)**.** E-LTP is distinguished by a significant potentiation of

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the synaptic responses that lasts up to 3 hours. To experimentally induce E-LTP, one train of stimuli at 100 Hz applied for a period of 1s, is needed. In comparison, L-LTP requires four trains of stimuli, applied with 2.5 min intervals between trains, for induction and is more durable as it lasts much longer than 3 h (Frey et al., 1993b; Huang et al., 1994). LTP is measured electrophysiologically in two independent ways: (1) changes in population spike amplitude, which is a measure of number of neurons reaching firing threshold and (2) changes in field excitatory postsynaptic potential (fEPSP) slope, which measures synaptic strength.

We and other investigators have reported that hypothyroidism markedly impairs both E-LTP and L-LTP in area CA1 (Figs 1A and 1B) without significantly affecting those of the DG area of thyroidectomized rats (Gerges et al., 2005, 2001; Alzoubi et al., 2005, 2006a, b, 2007, 2009; Alzoubi and Alkadhi, 2007) or PTU-treated rats (Babur et al. 2020). From molecular studies, we have shown that while the activity and protein levels of the phosphatase calcineurin were significantly increased in area CA1, they were markedly decreased in the DG area of thyroidectomized rats. This may explain the conservation of LTP of the DG area in these hypothyroid rats (Gerges et al., 2005, 2001) as reduced dephosphorylation results in sufficient phosphorylated calcium calmodulin kinase II (P-CaMKII) to support LTP. In contrast, other groups reported a marked decrease of LTP in the DG of freely moving thyroidectomized rats (Fernandez-Lamo et al., 2009) and anesthetized thyroidectomized rats (Yousef et al., 2019). In PTU-treated rats, Glombik et al (2021) reported impairment of LTP of the DG area but not that of area CA1. Using the PTU-hypothyroid model, Bitiktas and coworkers (2016) reported that in the DG area, LTP measured with the population spike was decreased, but was potentiated when measured with fEPSP in these rats. These conflicting findings may be the consequence of

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using different hypothyroid animal models, anesthesia and hypothermia and/or the levels of thyroid hormones deficiency.

Long-Term Depression (LTD): The enduring decline in synaptic strength form of synaptic plasticity, LTD, has been described in various parts of the brain including the hippocampus (e.g., Anwyl, 2006). The function of this form of synaptic plasticity is not clear but it is thought to be involved in a possible fine-tuning mechanism for learning and memory processes (Bear and Abraham, 1996). Support for this proposition is suggested by the observation that behavioral learning in a novel environment resulted in clear LTD expression in hooded Lister rat (Manahan-Vaughan and Braunewell, 1999). The same researchers also showed that LTD evoked by low frequency stimulation during exploration of a novel environment was accentuated in freely moving Wistar rats (Manahan-Vaughan and Braunewell, 1999).

Early on, induction of LTD by low frequency stimulation was achievable only in slices from hippocampi of young animals (Dudek and Bear, 1992). However, presently LTD can be induced in anesthetized or freely moving adult animals using the two-pulse protocol (Thiels et al., 1994, 1996; Doyere et al., 1996; Manahan-Vaughan and Braunewell, 1999; Aleisa et al., 2006). The expression of LTD requires a moderate increase in the concentration of free intracellular calcium ion (Ca^{2+}), which is achieved by postsynaptic depolarization and activation of glutamate NMDA receptors (Fujii et al., 1991).

In another set of experiments, stimulation of area CA1 with a brief train of 5 pulses at 100 Hz did not change the slope of fEPSP in euthyroid rat but caused a significant reduction of the same response in thyroidectomized rats. This indicated that expression of LTD in area CA1 synapses was facilitated in hypothyroid rats (Alzoubi et al., 2007b). In euthyroid rats, the

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normal two-pulse stimulation protocol evoked a robust LTD, but the same stimulation protocol in thyroidectomized rats markedly increased the magnitude of LTD (Alzoubi et al., 2007b, 2008b).

Effect of hypothyroidism on brain structure

It is well-known that thyroid hormones are essential for the development and function of the central nervous system where they regulate differentiation of neurons and neuroglial cells (Gomes et al., 1999; Billon et al., 2001; Lima et al., 2001; Jones et al., 2003; Baxi et al., 2014; Dezonne et al., 2015; Noda, 2015). Hypothyroidism may cause pathological damage in the ultrastructure of various regions of the brain, in addition to alterations of levels and activities of a variety of signaling molecules and neurotransmitters. These changes in brain structure and function may affect short- and long-term memory, gene expression, cell signaling and cell migration (Madeira et al., 1990, 1991, Gerges et al, 2004; Gilbert, 2004; Alzoubi et al., 2009; Koromilas 2010).

The structural integrity of the neurons and the efficacy of synaptic transmission in the brain are vital for various forms of cognitive activities. The integrity of structural elements including synapses and myelin sheath is essential for information transfer among neurons. Morphological alterations during hypothyroidism have been described in many areas of the brain including hippocampus (Madeira and Paula-Barbosa, 1993; Cortes et al., 2012).

It has been shown that the number of nascent brain cells is reduced in adult hypothyroid animals (Montero-Pedrazuela, 2006; Babur et al., 2020*).* At the cellular and molecular levels, cognitive symptoms of hypothyroidism are probably caused by alterations in the levels and activities of neurotransmitters and signaling molecules, which may result in

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disruption of synaptic plasticity. This may lead to impaired learning as well as short-term and long-term memory (Sui et al., 2005; Alzoubi et al., 2009; Glombik et al., 2021).

Transmission electron microscopic study of the prefrontal cortex (PFC) of thyroidectomized adult rats revealed ultrastructural changes in neurons, synapses and even the myelin sheath. The morphological changes in the myelin sheath included disrupted and much less

compacted laminae with reduced thickness and irregular outline (Wang et al., 2017). Moreover, the neurons displayed thin cytoplasm with less organelles and enlarged endoplasmic reticulum with mitochondria showing deteriorated cristae (Wang et al., 2017).

In the pyramidal neurons of visual area of the cerebral cortex of thyroidectomized rats, there was reduced number of synaptic spines. In addition, there was fusion of pre- and postsynaptic regions, with reduction in the number of synaptic vesicles in the presynaptic regions (Ruiz-Marcos et al., 1980, 1988). Similar findings were reported in the PFC of thyroidectomized rats (Wang et al., 2017).

Apoptosis and gliosis are suggested as signs of brain inflammation. Neuronal damage with increased expression of apoptotic markers Bax/Bcl2 in area CA3 and other brain regions have been reported during drug-induced hypothyroidism *(*Ambrogini et al., 2005; Desouza et al., 2005; Alva-Sanchez et al., 2009). It is not clear, however, whether these outcomes are due to toxic effects of the anti-thyroid drugs or a consequence of hypothyroidism.

The hippocampus of PTU hypothyroid rats showed a reduction in the number of granule cells of the dentate gyrus and pyramidal cells of area CA1 with a corresponding decrease in the weight, and volume of the hippocampus due to neuronal death (Madeira et al., 1991, 1992).

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Additionally, vacuolar deterioration and alteration of the pyramidal cells have been reported in hippocampi of hypothyroid rats (AbdAllah et al 2014). This may reflect some of the cognitive deficiencies reported in hypothyroidism as these areas are involved with learning and memory. In agreement with results from animal experiments, studies of untreated hypothyroid patients revealed serious structural alterations in the hippocampus and other areas of the brain. These include significant reduction in the volume of the hippocampus (Cooke et al., 2014) and volume of gray matter in various cortical regions with significant increases in the volume of gray matter in bilateral cerebellar crus and left precentral gyrus (Su et al., 2023).

In PTU-hypothyroid rats, magnetic resonance imaging (MRI) study revealed a progressive decrease in brain volume accompanied by impaired memory and decreased expression of signaling molecules important for synaptic plasticity and memory, including CaMKII, CREB, neurogranin, ERK and GSK3B as well as decreased expression of the transcription factor EGR1 (Zif268*)* among others (Chaalal et al, 2014). The same group reported increased hyperphosphorylation of tau and enhanced levels of several proinflammatory cytokines in the hippocampus of PTU-hypothyroid rats (Chaalal et al, 2014). It is possible that the very large doses of PTU used by these authors to induce hypothyroidism may have played a role in causing these deficits. However, although these authors also reported reversal of the hypothyroid symptoms by hormone replacement therapy, some toxic effects of the antithyroid drug may have been overlooked in their report.

Synaptic plasticity-related signaling molecules in hypothyroidism:

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High-frequency stimulation causes release of abundant glutamate, which activates glutamate NMDA receptors on the postsynaptic membrane leading to elevation of intracellular Ca^{2+} levels (Malenka et al., 1988; Nicoll et al., 1989; Fukunaga, 1993). This transient increase of free intracellular Ca^{2+} causes dissociation of the neurogranin/calmodulin complex (Gerendasy et al., 1994, 1995; Gerendasy and Sutcliffe, 1997; Krucker et al., 2002). Elevated free intracellular Ca^{2+} also activates PKCg, which phosphorylates neurogranin causing release of calmodulin (Gerendasy and Sutcliffe, 1997). The unbound calmodulin forms a calcium/calmodulin complex, which forms a critical signaling molecule called calcium calmodulin kinase II (CaMKII), which is capable of autophosphorylation (Giese et al., 1998; Wang and Kelly, 1995). The phosphorylated CaMKII (P-CaMKII) activates several molecules important for LTP expression (Fukunaga et al., 1996; Nayak et al., 1996; Barria et al., 1997a, b). The activation of these substrates by P-CaMKII continues, even after Ca^{2+} reverts to basal levels, and only stops after dephosphorylation by calcineurin (Fukunaga et al., 1996; Wang and Kelly, 1996; Fukunaga and Miyamoto, 2000).

Neurogranin (RC3), is like a holding pool for calmodulin; it is expressed particularly in the dendritic spines of the brain, where it is an important part of the PKC signaling cascade (Gerendasy et al., 1994,1995; Gerendasy and Sutcliffe 1997; Krucker, et al., 2002). The RC3 protein controls availability of calcium-calmodulin (CaM)-complex through an assortment of different kinases and phosphatases (Koromilas et al., 2010). This molecule is regulated by thyroid hormones (Bernal et al., 1992; Iniguez et al., 1992, 1993.1996; Piosik, et al., 1996) and is essential for the expression of E-LTP (Chen et al., 1997; Pak et al., 2000; Wu et al., 2003; Huang et al., 2004). Phosphorylation of RC3 by PKC in the presence of Ca^{2+} decreases its binding ability to calmodulin. The brain-specific RC3 gene is implicated in synaptic plasticity and memory

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formation through modulation of calcium calmodulin (CaM)-dependent signaling (Marambaud et al., 2009). We have measured the PKC protein levels in thyroidectomized rats and found it to be markedly decreased compared to euthyroid control rats (Fig 2A, Alzoubi et al., 2005a; 2006a). The RC3 mRNA level (Iñiguez et al., 1992; Alzoubi et al., 2005a) as well as its protein level were markedly reduced in hypothyroid rodents, (Fig 2 B; Alzoubi et al., 2005a, 2006a; Iñiguez et al., 1993; Enderlin et al., 2004; Vallortigara et al., 2008).

Protein kinases are required for the induction and/or maintenance of LTP and memory formation. For example, E-LTP expression is dependent on phosphorylation of existing CaMKII molecules (Malenka et al., 1989; Pettit et al., 1994; Lledo et al., 1995; Giese et al., 1998; Roberts et al., 1998). In contrast, L-LTP depends on de novo protein synthesis that involves kinases-induced activation of transcription factors such as cyclic AMP response element binding (CREB) protein. Other kinases implicated in LTP expression include PKA (Abel et al., 1997), MAPK (English and Sweatt, 1997; Impey et al., 1998; Huang et al., 2000) and CAMKIV (Bito et al., 1996; Ho et al., 2000).

The highly localized marked influx of Ca^{2+} induced by multiple high frequency stimulation (MHFS), activates adenylate cyclase type I (AC1) (Wong et al., 1999; Poser and Storm, 2001; Ferguson and Storm, 2004), which, in turn, activates PKA, by itself or through MAPKp44/42 (ERK1/2), which then phosphorylates and activates CREB (Impey et al., 1998; Huang et al., 2000; Rosenblum et al., 2002). The resulting P-CREB activates genes essential for L-LTP expression (Kandel, 2001; Barco et al., 2002, 2003; Alarcon et al., 2004). CREB can also be directly activated by CaMKIV (Bito et al., 1996; Tokuda et al., 1997; Kasahara et al.,2001). Active P-CREB can be

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inactivated through dephosphorylation by protein phosphatases including calcineurin (Bito et al., 1996).

In the hippocampus of thyroidectomized rats, specific cellular messengers including P-CaMKII (Fig 2C), and its precursor calmodulin (Fig 2D) were decreased (Gerges et al., 2005; Alzoubi et al., 2005a, 2006a, 2006b). Other messenger molecules critically required for expression of synaptic plasticity and learning including P-CREB (Fig 2E), MAPKp44/p42 (Figs 3A, 3B), CaMKIV (Fig 3C), AC1 (Fig 3E) and were also reduced in the hippocampal area CA1 in adult thyroidectomized rats (Alzoubi et al., 2005a, 2006a, 2007a; Alzoubi and Alkadhi, 2007). Hypothyroidism markedly diminished the protein levels of phosphorylated MAPKp44/p42 in area CA1 with no significant effects on levels of these molecules in the DG of the same thyroidectomized rats (Fig 3A, 3B: Alzoubi et al., 2007a). Interestingly, our molecular findings have shown that whereas hypothyroidism significantly enhanced the levels and activity of calcineurin in area CA1 (fig 3D; Alzoubi, 2005a, 2006a), it decreased its level and activity in the DG area. We suggested that these findings may account for preservation of PKC and phosphorylated CaMKII protein levels in the DG area of thyroidectomized rats (Gerges et al., 2005). However, Fernandez-Lamo et al. (2009) reported diminished synaptic transmission and LTP magnitude in the DG of thyroidectomized animals. The long time-lag between removal of the thyroid gland and recording of LTP and the multiple use of anesthetics may account for the different results in these experiments.

It is well established that pre-synaptic proteins including SNAP-25, synaptotagmins and syntaxin-1 are necessary for neurotransmitter release. Western blot analysis showed that the vesicle release mechanism proteins, synaptotagmin-1, munc-18 and SNAP-25, were significantly

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increased in the PFC of PTU-hypothyroid adult rats (Wang et al., 2017). Immunoblot analysis and immunostaining revealed that levels of synaptotagmin-1 were significantly decreased in hippocampal CA1 and CA3 areas but not in the DG of hypothyroid rats (Liu et al., 2011). In contrast, levels of SNAP-25 were significantly upregulated in all three areas; CA1, CA3, and DG compared to euthyroid controls (Liu et al., 2011). In thyroidectomized rats, the SNAP-25 protein expression was increased while that of syntaxin-1 was decreased in the anterior pituitary (Quintanar and Salinas, 2002). However, in the hippocampus, syntaxin-1 levels were increased in PTU-hypothyroid adult rats (Wang et al., 2014, 2015). The mechanism for these changes in protein molecules is not well understood, however, these changes may be a repair mechanism to preserve the integrity of synaptic transmission during hypothyroidism. Supporting this suggestion is the finding that decreasing the concentration of neurotransmitter acetylcholine (ACh) may lead to upregulation of the synaptic proteins synaptotagmin-1 and SNAP-25 expression; possibly a mechanism to enhance vesicle exocytosis to preserve normal synaptic transmission (Wang et al., 2017; but see Yang et al., 2012).

The postsynaptic density (PSD) in glutamatergic synapses in area CA3 of the hippocampus of PTU-hypothyroid rats were analyzed by ELISA, in situ hybridization and electron microscopy; it was found that in addition to gliosis and deterioration of PSD, there was a significant loss of neurons and astrocytes due to apoptosis (Cortes et al., 2012). PSDs of area CA3 neurons were significantly thinner in PTU-hypothyroid rats compared to those of control euthyroid rats. This was a consequence of reduced density of receptors in the postsynaptic membrane (Cortes et al., 2012). The same authors reported hypothyroidism induced a decrease in the number of NMDA receptor subunits NR1 and NR2A/B fractions as well as in the number

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of NR1 subunits associated with NR2A subunits, suggesting that PSDs in the hypothyroid state contained reduced amounts of NMDA receptors (Cortes et al., 2012). In agreement, immunoreactivity of NMDA receptor subunits of thyroidectomized rats revealed a significant decrease of NR1 and NR2B subunits in hippocampal area CA1 (fig 4A, 4C), but a slight increase (not significant) in the levels of NR2A (fig.4B) (Alzoubi et al., 2007b). Reduction in NMDA receptors at the postsynaptic neuron would be expected to interfere with synaptic plasticity.

The mRNA levels of the NMDA receptor subunits NR1, NR2A, NR2B, the AMPA receptor subunit GluR1, and the kainate receptor subunit KA2 were determined in the hippocampi of thyroidectomized rats by in situ hybridization histochemistry (Lee et al., 2003). This work showed decreased expression of NR1 subunit mRNA in the hippocampus without affecting the expression of KA2 or GluR1 subunit mRNA. However, no significant change was found in the mRNA expression of NR2A subunit in the same brain region (Lee et al., 2003). In support of the findings of Lee et al (2003), we reported a significant decrease in protein level of NR1 subunit and an increased level of NR2B subunit with no significant change in NR2A subunit in thyroidectomized rats (Alzoubi et al., 2007b), Similarly, no change was reported in KA2 and GluR1 subunits mRNA levels (Lee et al., 2003) or kainate receptor density following thyroidectomy (Calza et al., 1997). These variable findings are unlikely to be caused by reduction in the number of dendritic spine or a decrease in overall hippocampal cell number (Rami et al., 1986; Madeira et al., 1991,1992), which would have caused widespread, nonselective, decreases in the mRNAs of all these receptors.

Hypothyroidism significantly impacts other receptors. For instance, PTU-induced hypothyroidism was reported to cause a significant decrease in the expression levels of 5-HT2A

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receptors in the hippocampus and PFC (Jin et al., 2021). Similar decrease in 5HT2 receptors was reported in the striatum of thyroidectomized rats (Mason et al., 1987). In the cerebellum of thyroidectomized rats, Mason and coworkers (1987) also reported a significant decrease in beta-adrenergic receptors.

To look at another major receptor, we used immunoblot analysis to measure the subunits levels of nicotinic acetylcholine receptor (n-AChR) and found no significant changes in the levels of alpha 4-AChR, beta 2-AChR (fig 4D, 4E) or alpha 7-AChR (fig 4F) in area CA1 of thyroidectomized rats (Alzoubi et al., 2007b).

Ample research work has been done on the role of thyroid hormones in brain GABAergic system. In fact, clinical evidence suggests that certain human nervous system disorders such as anxiety and seizure susceptibility may involve hypothyroidism-induced alterations in brain GABAergic circuits (See review: Wiens and Trudeau, 2006). Carbimazole-induced hypothyroidism in adult rats caused increased levels of the major inhibitory neurotransmitter, gama-aminobutyric acid (GABA), in the cortex and hypothalamus but lower glutamate levels in the cortex and thalamus (Upadhyaya and Agrawal, 1993). In the visual cortex of thyroidectomized adult rats, there was an increased GABA level as well as glutamic acid decarboxylase (GAD) activity, which is an enzyme that converts glutamate to GABA (Kalaria and Prince, 1986; Chapa et al., 1995).

In thyroidectomized male and female rats there was an increase in the number of benzodiazepine (BZ) sites on $GABA_A$ receptors of the cerebral cortex (Medina and De Robertis, 1985). In contrast, Ortiz-Butron et al. (2003) reported that in methimazole-induced hypothyroid female rats, BZ binding sites of the medial amygdala were decreased by 24%. This is another

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example of variable findings resulting from using two different models of experimental hypothyroidism.

Neurogenesis during hypothyroidism

Neurogenesis continues throughout life but is reduced with age. The two major sites in the adult mammalian brain that contain stem cells capable of producing new neurons are the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles. It is estimated that in the adult rodent SGZ, about 9000 progenitor cells are generated daily but only nearly half reach maturity (Fanibuda et al., 2018) and it takes 4-6 weeks to become fully functional neuron (Ming and Song, 2011; Concalves et al., 2016). In the human hippocampus, approximately 700 new neurons are generated per day (Spalding et al., 2013). Adding new neurons can modify the connectivity of the standing networks, which may repair damaged networks, cause behavioral modifications, or form new connectivity.

Thyroid hormones, T4 and T3 can enter the CNS carried by specific transporters in the adult brain where more T3 is produced from T4 by deiodination (Rodrigues et al., 2013; Morte and Bernal, 2014; Liu and Brent, 2021). In the adult brain, thyroid hormones are involved in the regulation of hippocampal neurogenesis where they influence the survival of adult hippocampal progenitor cells (Desouza et al., 2005). The fact that the DG of hippocampus contains a high density of thyroid hormone receptors (de Jong, 2006), makes it an important target for thyroid hormones. Thus, thyroid hormones play a critical role in regulating new neuron production in the DG area of rat hippocampus (Ambrogini et al., 2005).

The DG area of the hippocampal formation is one of few brain regions where neurogenesis occurs throughout adulthood. The SGZ of the DG of the hippocampal formation

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can generate new neurons all through adult life (Hodge et al., 2008; Sibbe and Kulik, 2017). This is a highly ordered process initiated from the slow dividing type-1 primary neural stem cells, which produce the intermediate neuronal type-2 progenitor cells which, then lead to type 3 cells, which give rise to mature neurons (Hodge et al., 2008; Toni and Sultan, 2011; Sibbe and Kulik, 2017; Tanaka et al., 2019).

Adult hypothyroidism impairs normal neurogenesis by causing a significant decrease in production of new cells from the SGZ of the DG area (Babur et al., 2020). Moreover, hypothyroidism interferes with proliferation and maturation of the nascent neurons (Lemkine et al., 2005; Desouza et al., 2005; Montero-Pedrazuela et al., 2006).

In the PTU hypothyroidism model, the DG showed abnormal neuronal growth, migration, and maturation, which resulted in reduced number of new cells (Tanaka et al., 2019; see Koromilas et al., 2010 for review). Madiera et al., (1991) estimated the total number of granule cells in PTU-hypothyroid rats by analyzing the volume of the dentate gyrus granular layer and the density of its neurons, which were found to be reduced and concluded that the total number of granule cells was decreased probably due to increased cell death.

Assessment of neurogenesis with the thymidine analogue 5-bromo-2'-deoxyuridine in PTU-hypothyroid rats showed that while mitotic activity of the neural precursors was not affected by thyroid hormone deficiency, nascent cell survival was curtailed, and new neurons exhibit a delay in neuronal differentiation under these conditions (Ambrogini et al., 2005; Sánchez-Huerta et al., 2016).

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Hypothyroidism impairs learning and memory

It is clinically established that untreated thyroid hormone deficiency leads to impaired learning and memory. In experimental hypothyroidism several laboratories, including mine, reported effects of thyroid hormones deficiency on cognitive ability in various hypothyroid animal models using different testing methods. We showed LTP was impaired with significant changes in the levels of plasticity-related proteins, including CaMKII, calmodulin, and calcineurin (Alzoubi et al., 2005, 2006). Additionally, it has been reported that in hypothyroidism, brain glucose uptake was impaired, thus diminishing the energy resource necessary for neural activity (Contsant et al., 2001; see reviews: Begin et al. 2008, Jahagirdar and McNay, 2012). Therefore, hypothyroidism-induced cognitive impairment may be partly due to reduced glycolysis in the brain.

In my laboratory, we have tested thyroidectomized rats for performance on the radial arm water maze (RAWM*),* a sensitive and dependable model for analyzing hippocampus-dependent spatial learning and memory (Buresova et al., 1985; Hodges, 1996; Diamond et al., 1999; Alamed et al., 2006). The RAWM is a hybrid of the radial arm maze (RAM) and Morris water maze (MWM). The RAWM maintains the advantages while decreasing the disadvantages of both the RAM and MWM (Buresova et al., 1985; Hodges, 1996; Diamond et al., 1999; Alamed et al., 2006). Each rat was tested for memory in the RAWM. For each rat, the daily training in the RAWM consisted of 4 consecutive learning trials and three memory tests: a 20-min short-term memory test was followed by a 5-hr and 24-hr long-term memory tests (Fig 5A). The testing was conducted for a minimum of 8 consecutive days or until the rats attained the “days

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to criterion" (DTC; Fig 5B). The DTC was defined as the number of days in which a rat made no more than one error in three successive days in the fourth learning trial and the memory tests. Thus, two sets of data were obtained from these trials: the number of errors in each trial/day and the DTC. From these two parameters we concluded that hypothyroidism slowed the learning process and impaired short-term and long-term memory (Fig 5A; Alzoubi et al., 2006b).

Another test is the classical Pavlovian fear conditioning paradigm, which was used on adult thyroidectomized Wistar rats. In these experiments, results showed that fear memory was enhanced but learning acquisition was normal, whereas spontaneous recovery of fear memory was maintained, and memory extinction was delayed (Montero-Pedrazuela et al., 2011). In another testing model methimazole-hypothyroid rat were used in the Morris water maze, it was reported that the distance and latency to reach the survival platform were increased while the swim speed was significantly reduced (Hosseini et al, 2010; Diaz et al, 2012). In addition, the radial arm maze testing of hypothyroid rats revealed significant impairment of the working memory as well as reference memory (AbdAllah et al., 2014). The cognitive impairment finding was supported by results from passive avoidance test, which revealed learning deficits as well as from novel object recognition test, which disclosed impaired intermediate and long-term memory (AbdAllah et al., 2014).

Therefore, there is a general agreement that hypothyroidism in different experimental animal models and different testing systems invariably causes cognitive impairment as reported by several independent laboratories. These findings agree with results in patients with untreated hypothyroidism (e.g. Su et al., 2023).

Concluding remarks

Thyroid hormones deficiency in adulthood negatively affects brain structure and function. While the clinical effects of adult-onset hypothyroidism in humans are well documented, experiments on animal models of hypothyroidism provided detailed insights into the physiological, morphological and molecular events that may be involved in triggering clinical symptoms. The sometime contradictory reports on the effects of hypothyroidism on the brain revealed in animal experiments, may be a manifestation of regional differences in the brain. Additionally, significant challenge is posed by differences in experimental designs. For instance, findings from in vivo studies vary in whether hypothyroidism was induced by thyroidectomy or administration of an anti-thyroid drug with different doses and varied time courses. Other variables may include the use of different ages and sexes of rodents and the use of different technical methods of measurement in various brain regions. These variations must be taken into consideration when studying these data. Generally, investigators did not measure serum hormone levels after each experiment, and this may affect the findings from these experiments. In addition, the discrepancies in the effects of hypothyroidism reported by some investigators may also result from other factors including animal models used, method of testing and the effect of anesthesia and the resulting hypothermia, which is accentuated by thyroid hormones deficiencies.

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Figure Legends

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Figure 1: Effect of hypothyroidism on synaptic plasticity. (A) Adult-onset hypothyroidism impairs E-LTP in area CA1 of rat hippocampus. E-LTP was induced by **high frequency stimulation (HFS**) at time zero. E-LTP was measured as an increase in fEPSP slope and expressed as percentage of the baseline. The f-EPSP slope of the hypothyroid group (closed circles, *) in all points after HFS were significantly (P<0.01) lower than those of the euthyroid control groups (open circles). Each point in each group is the mean ± SEM from 7-8 rats. (B) Adult-onset hypothyroidism caused impairment of L-LTP in area CA1 of rat hippocampus. L-LTP, measured as an increase in fEPSP slope was induced, at time zero, by multiple HFS (MHFS). The fEPSP slope at all points after MHFS were significantly (P<0.05) lower in the hypothyroid group (closed circles *) than in the control euthyroid group (open circles). Each point in each group is the mean ± SEM from 6-8 rats. (C) Enhanced LTD in the hypothyroid rats (closed circles). Hippocampal LTD of the CA1 area evoked by stimulation of the Schaffer collateral pathway by twin-pulse stimulation. In the hypothyroid group (closed circles *), all points after twin-pulse stimulation were significantly (P<0.05) lower than those of the control euthyroid group (open circles). Each point in each group is the mean±SEM from 5–7 rats. In all 3 panels, experiments were done on urethane-anesthetized Wister rats, (*) indicated significant difference (P<0.05) from the euthyroid groups and hypothyroidism was induced by thyroidectomy.

Figure 2: Immunoblot analysis of the effect of adult-onset hypothyroidism on protein levels of plasticity-related signaling molecules in area CA1 of the hippocampus. Protein levels of (A) PKCg, (B) neurogranin, (C) P-CaMKII, (D) calmodulin and (E) P-CREB were markedly decreased in thyroidectomized rats. Each bar is the mean±SEM from 5-7/rat group. *Significant (P<0.05) difference from the euthyroid (Control) rats.

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Figure 3: Immunoblot analysis of the effect of adult-onset hypothyroidism revealed marked decreases in protein levels of (A) MAPKp42, (B) MAPKp44, (C) CaMKIV and (E) adenylate cyclase, while the protein levels of the phosphatase calcineurin (D) were significantly increased in hippocampal area CA1 of thyroidectomized rats. (*) Indicates significant difference ($P < 0.05$) from levels of the same area of euthyroid rats. Values are mean±SEM of 6–8 rats/group.

Figure 4: Immunoblot analysis of hypothyroid and euthyroid (Control) protein levels of receptor subunits of glutamate NMDA receptor (A) NR1, (B) NR2A, (C) NR2B, and subunits of nicotinic acetylcholine receptor (D) alpha 4-nACh, (E) beta 2-nACh and (F) alpha 7-nACh in cell membrane of the hippocampal area CA1 of thyroidectomized and euthyroid rats. Each point in each group is the mean±SEM from 5–8. (*) Indicate significant difference ($P<0.05$) from that of control euthyroid rats.

Figure 5: (A) Learning and memory performance in the radial arm water maze (RAWM) were impaired in thyroidectomized rats. The first four trials (Trials 1-4) represent the acquisition (learning) phase. Then, memory tests were done at 15m (Trial 5; short-term memory), 5h and 2h (Trials 6 and 7; long-term memory) after the last learning-phase trial. Trials and tests were performed every day for a period of 8 days. On all days, the hypothyroid group exhibited slowed learning ability, shown as significantly higher numbers of errors in trials 2 and 3. There was a consistently significant impairment of the short-term (Trial 5) and long-term memory (Trials 6 and 7). Each point is averaged from days 7 and 8 of testing. (*) Indicates significant difference from

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those of euthyroid control. Each point is the mean +SEM from 10–14 rats/group. Inset is a sketch of the RAWM. (B) Days to criterion (DTC) measurements for 24h long-term memory confirmed hypothyroidism-induced spatial memory impairment. The criterion is reached for a particular trial if the animal makes no more than one error in 3 consecutive days in a trial.

Even though learning was clearly slow in days 2–3 (panel A), no difference was observed in DTC for trial 4 (learning phase), indicating that, eventually, all animals have learned to the same extent by the end of the acquisition phase (panel A). The values are mean +SEM from 10–14 rats. (*) Indicates significant difference from those of euthyroid control. ($P<0.05$; ANOVA followed by Tukey test).

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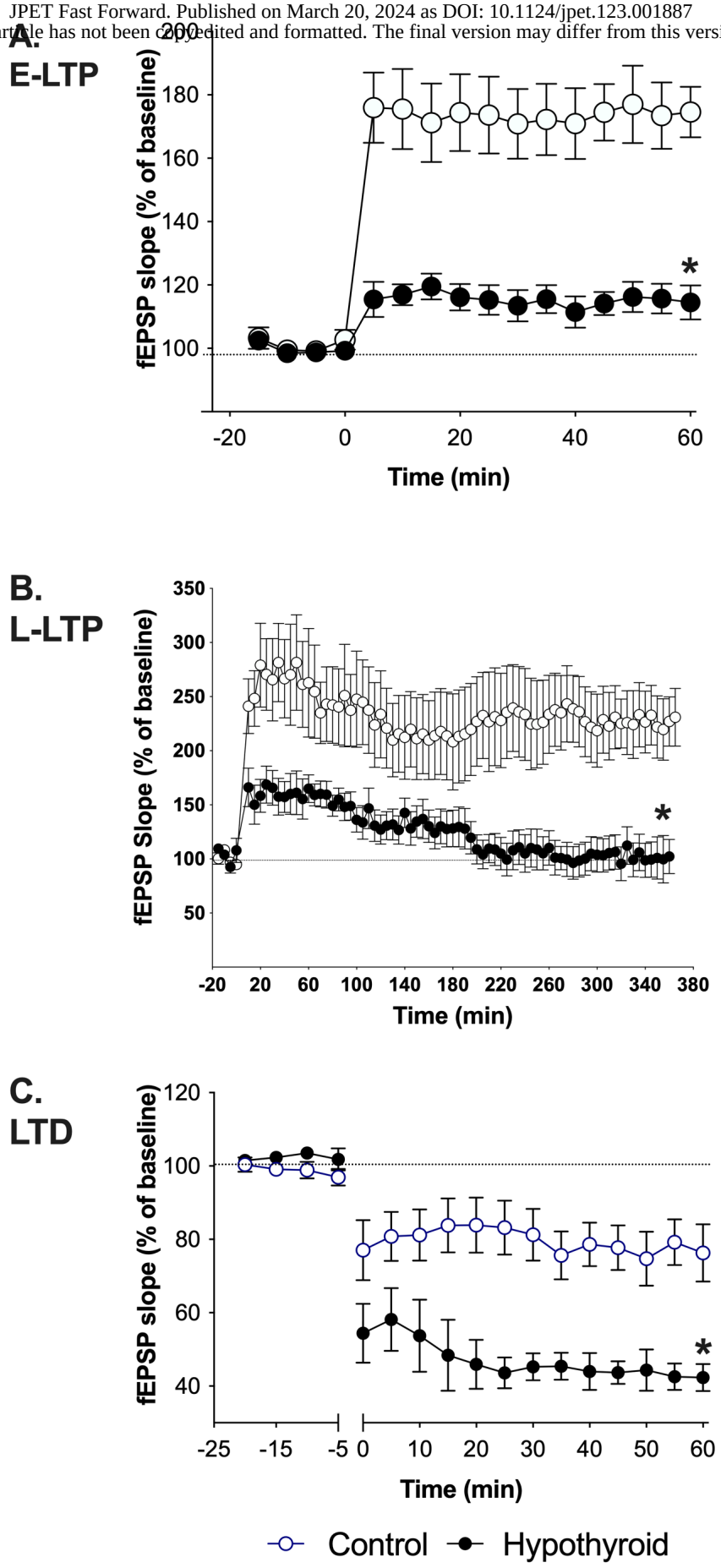

Figure 1

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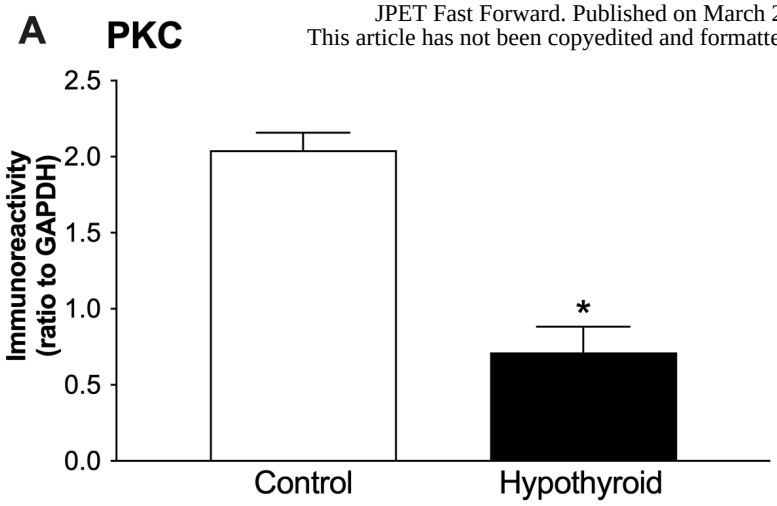

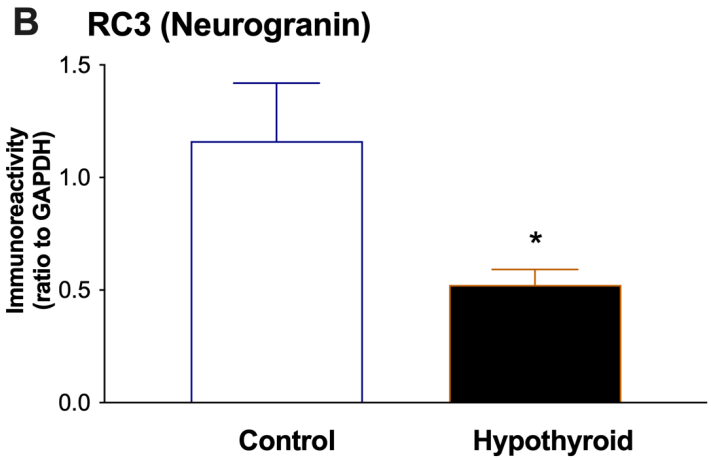

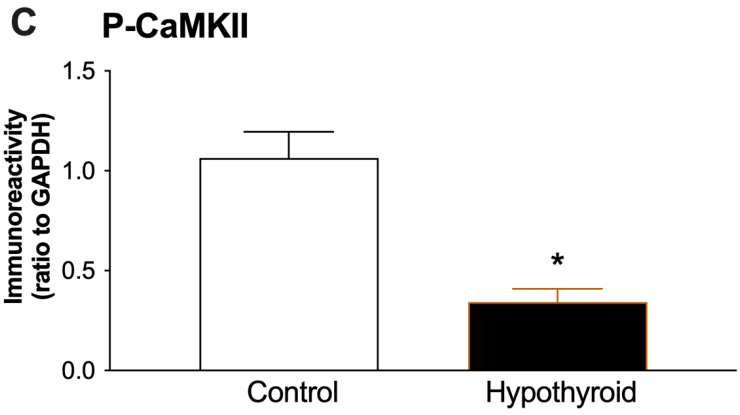

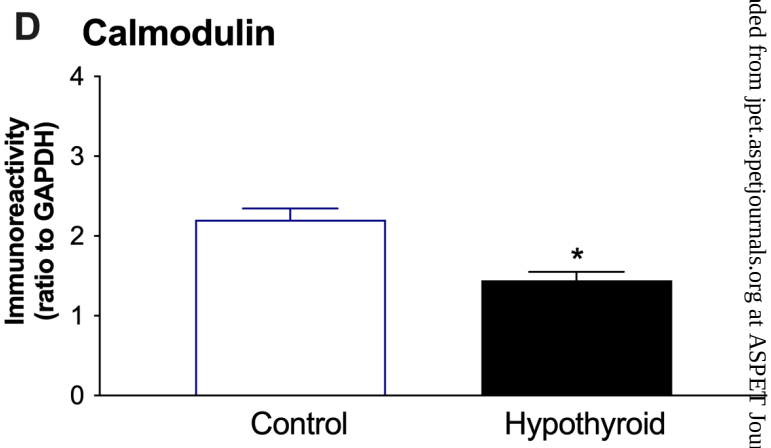

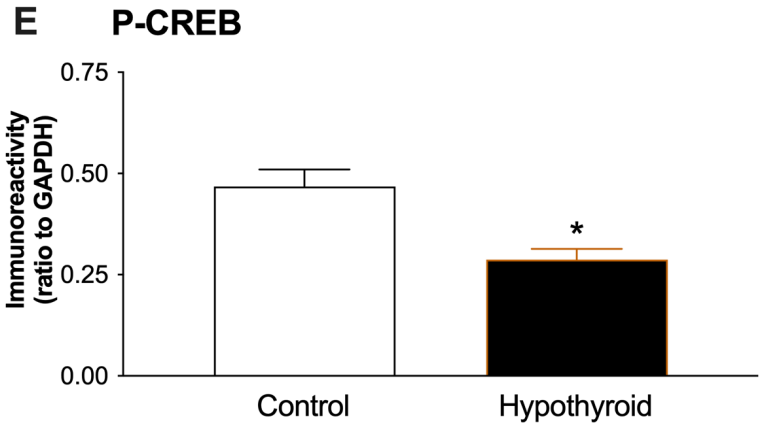

FIGURE 2

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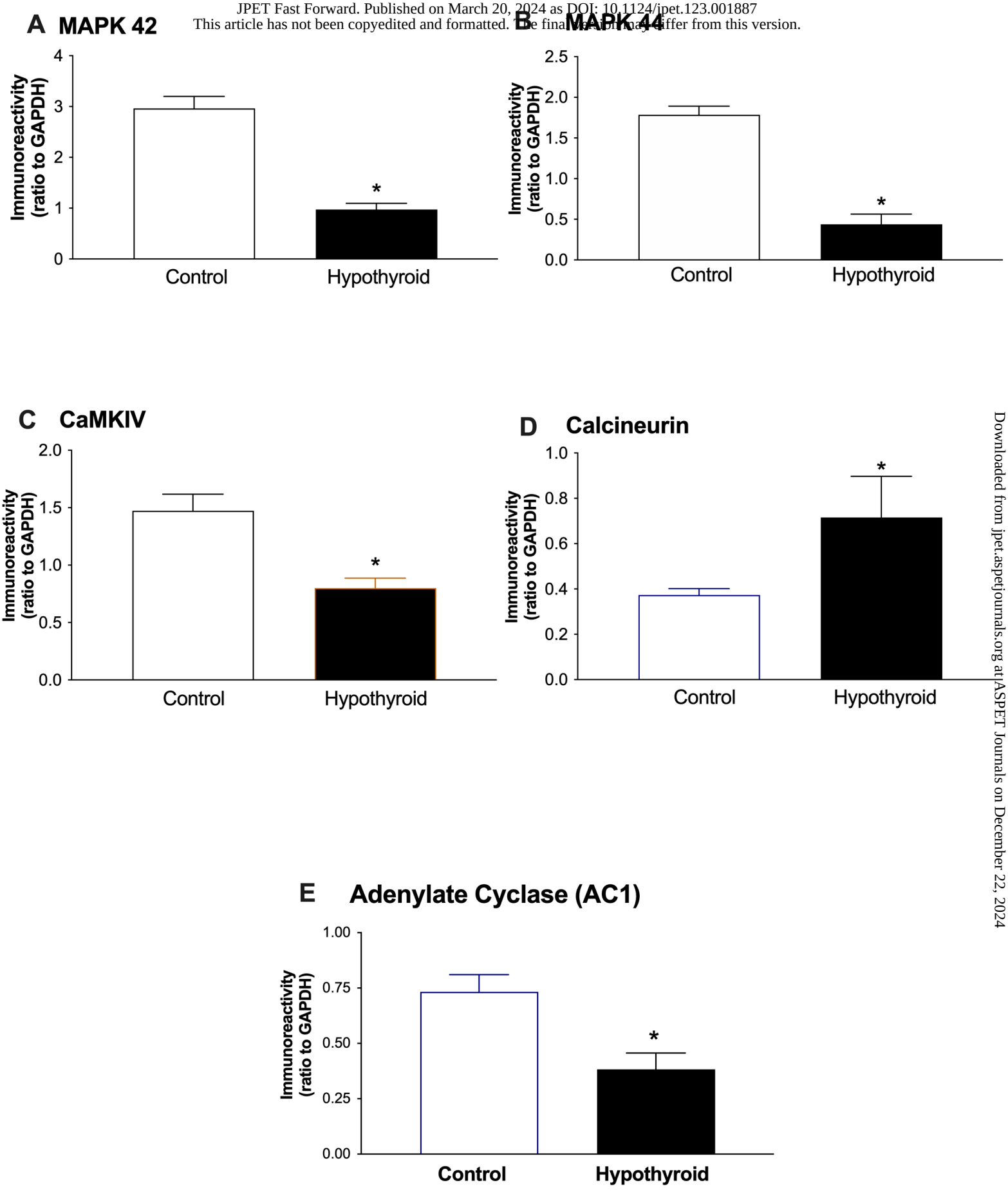

FIGURE 3

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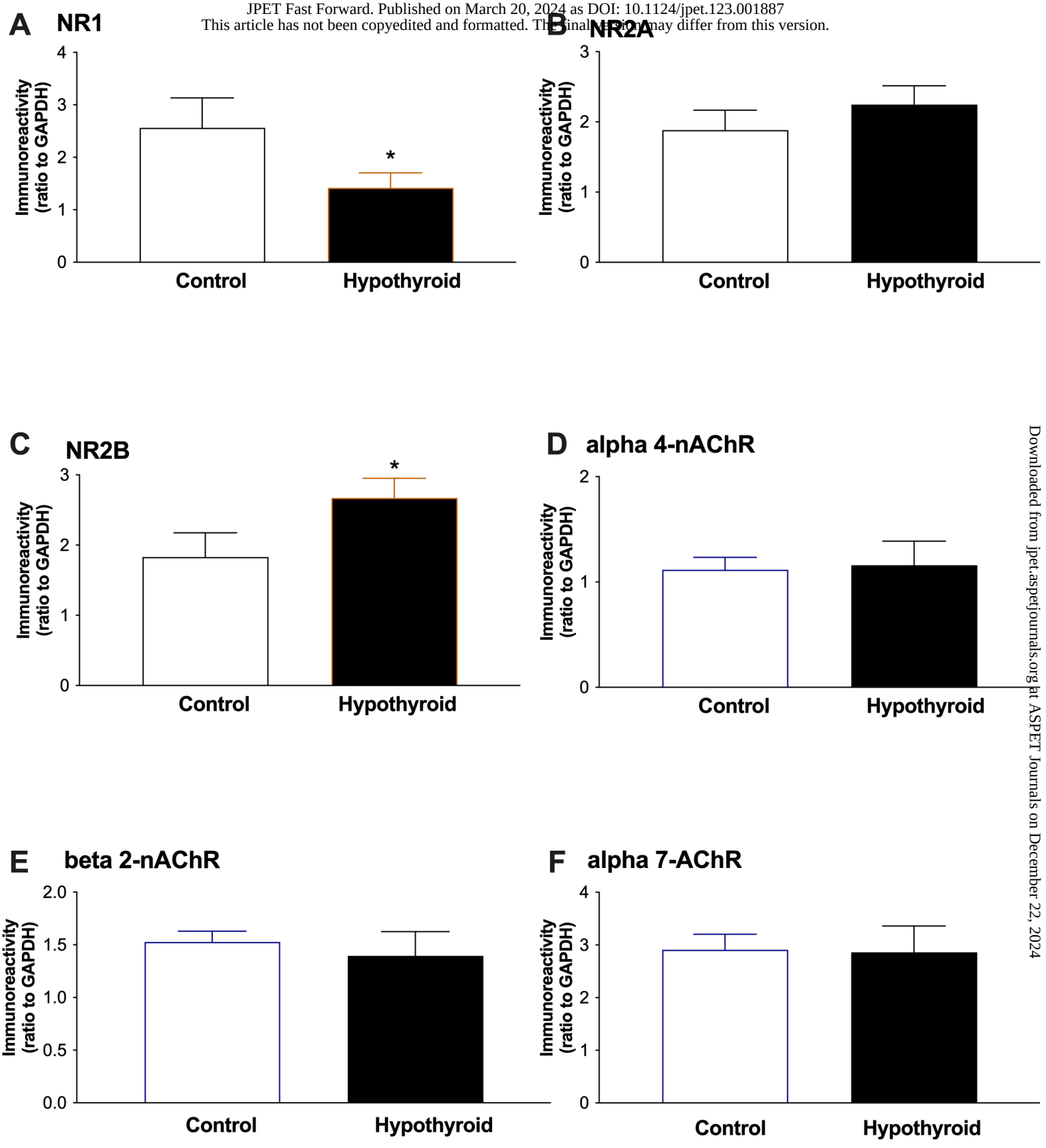

FIGURE 4

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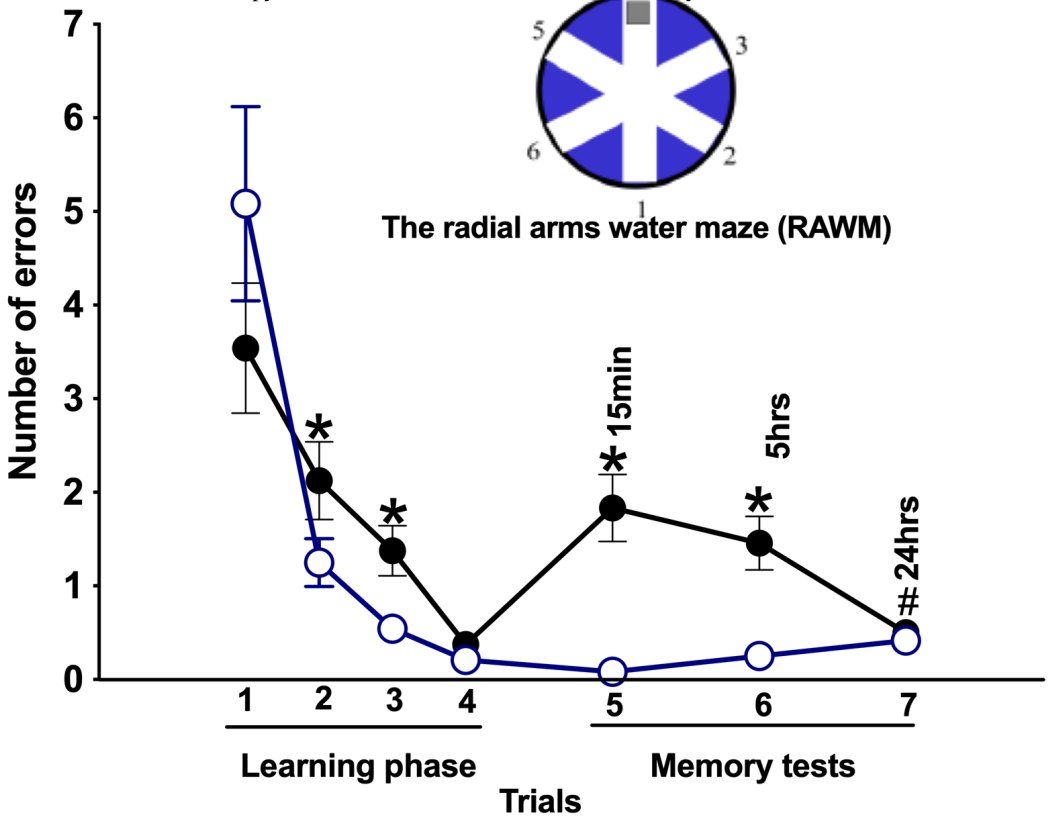

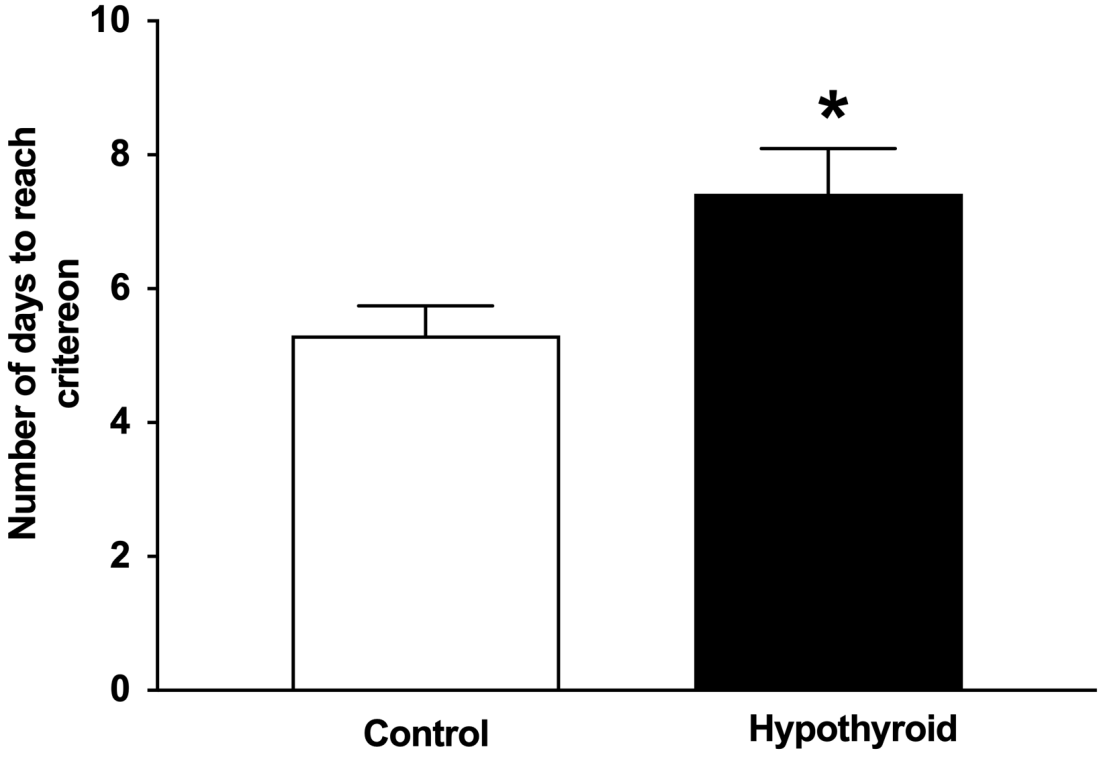

FIGURE 5