



Review

Thyroid hormones, brain function and cognition: a brief review

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Abstract

In addition to their role in cellular metabolic activity, thyroid hormones (THs), also regulate neural development; the central nervous system is particularly dependent on TH for normal maturation and function. Specifically, there appears to be extensive inter-reliance between TH and acetylcholine (ACh), nerve growth factor and hippocampal function. These associations led us to investigate the possible effects of thyroxine (L-T4) on performance of a spatial learning task, where cholinergic activity and hippocampal function are known to be important. Groups of rats ($n = 20$) received saline (controls) or L-T4 at 2.5 or 5 mg/kg daily for 4 days as a sub-chronic treatment, or 0, 5 or 10 mg/kg doses administered every third day for 28 days prior to testing as a chronic regimen. Rats were assessed in a watermaze for their ability to find a submerged or visible platform. Forty minutes prior to watermaze testing, half the animals in each group received 1 mg/kg scopolamine to elicit a cognitive deficit. Following testing, rats were decapitated, blood samples taken, and the frontal cortex and hippocampus were dissected out for acetylcholinesterase (AChE) assay. The results showed that L-T4 treatment, administered both sub-chronically and chronically, significantly enhanced the ability of rats to learn a spatial memory task, compared with controls. Moreover, both short-term and long-term L-T4 treatment reduced the cognitive-impairing effects of scopolamine. Improvements in performance were shown to occur alongside significantly increased cholinergic activity in frontal cortex and in the hippocampus of treated animals. These findings demonstrate an augmentative effect of L-T4 upon cognitive function, possibly mediated by an enhancement of cholinergic activity. The results support previous findings of a relationship between L-T4 and acetylcholine, and underscore possible mechanisms by which disorders of thyroid function may be associated with cognitive decline. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Hippocampus; Rat; Acetylcholine; Spatial learning; Thyroxine; Development; NGF; Ageing

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1. Introduction

1.1. Overview of thyroid hormones and regulation

Thyroid hormones (THs), including tri-iodothyronine (T3) and tetra-iodothyronine (T4), are recognised as key metabolic hormones of the body, with T3 being the most functionally active form. THs have many physiological actions, and essentially modulate all metabolic pathways through alterations in oxygen consumption and changes in protein, lipid, carbohydrate and vitamin metabolism. Through the direct manipulation of protein expression associated with such pathways, THs also affect the synthesis and degradation of many other hormones and growth factors and, thus, indirectly, influence additional endocrine signalling. THs are formed within the follicular cells of the thyroid gland, and are released into the systemic circulation in response to thyroid stimulating hormone (TSH). Measurements of thyroid gland secretion have shown the secretory molar ratio of T4 to T3 to be 14:1 in humans [1,71], whilst being 5:1 in the adult male rat [121].

In the circulation, THs are bound to a variety of plasma proteins, although a small percentage of both T3 and T4 exist in their free form. Whilst T4 is produced entirely by the thyroid gland, the majority of circulating T3 is derived from the deiodination of T4 in non-thyroidal tissues such as the liver and kidney. Deiodinase enzymes act to catalytically remove iodine and may do so at different sites to produce either T3 via 5'-deiodination or the inactive metabolites rT3 or T2 through 5-deiodination. Three deiodinase isoforms have been identified, each varying in substrate selectivity, reaction product, locality, and enzyme kinetics [81]. Many tissues contain more than one deiodinase enzyme, and their expression also alters with age, especially during development [71].

Although an extracellular site of action has been suggested, THs act on target cells at the genomic level through associative binding with specific nuclear receptor proteins [107]. Transport of THs into target cells appears to be through passive diffusion or via a transporter mechanism, and further attachment to proteins for intracellular transport occurs once inside the cell [27,81]. Thyroid hormone receptors (THRs) are members of a large superfamily of nuclear receptors including those for oestrogen, glucocorticoids and retinoic acid. Two genes, c-ERB-A a and b encode THRs

[120,135]. These genes have been alternatively spliced to give several isoforms [81,97].

Within its structure, the THR contains two highly conserved regions, a ligand-binding domain (LBD) and a DNA-binding domain (DBD). The LBD enables homodimeric or heterodimeric binding of T3 [52], and interactions of this domain with co-activators or co-repressors gives further modification of transcription [69]. The DBD enables the THR to bind to sites known as thyroid-responsive elements (TRE) located within the promoter regions of target genes. THRs may bind to a TRE as a monomer, homodimer, or as a heterodimer in association with an accessory protein such as retinoic acid [52]. This provides a further method by which adaptive modulation of protein transcription regulation may occur [13,51]. The receptor subtype defines the activity of both the LBD and DBD. For example, THR a2 does not bind THs, but does bind DNA. The variety of the proteins modulated by THs partly explains the wide range of effects mediated by THs upon cellular function.

THs are important for cell differentiation, growth and maturation. The integration of effects mediated by THs enables necessary metabolic alterations to accompany developmental changes of the tissues. In accordance with differences in functional and maturational timeframes, the effects of THs are cell, tissue and organ specific. THs are active in most tissues and through the relative integratory nature of their effects play a major role in matching cellular activity with necessary biochemical and energy requirements. Thus, THs enable metabolic homeostasis to be maintained during changes in cell development and function.

1.2. Thyroid hormones and the central nervous system

In addition to the systemic effects of THs and the localisation of receptor subtypes and deiodinase enzymes to selective peripheral tissues, many recent investigations have focused on activity within the central nervous system (CNS). Since the demonstration of high affinity T3 binding sites in rat brain [122], spatial and regional distribution patterns of THRs and their mRNAs have been described in the brain at both developmental and adult stages [12,22,56,95]. In the human, the presence of THRs can be seen as early as the tenth week of gestation [8].

A strong, dependent relationship is now recognised

to exist between the CNS and THs, and is not restricted to neuronal cells. Metabolic effects, such as those affecting mitochondrial respiratory enzyme activity [28], and acetate metabolism [17], have been shown to occur in the brain in response to THs. Astrocytes have been found to: (1) display THRs [87,88]; (2) possess a dependency upon THs for glucose transport [116]; (3) depend on THs for the expression of specific structural proteins [18,47,125]; and (4) show a positive correlation between TH levels and B-adrenoceptor expression [25,75]. Furthermore, in the foetal rat hippocampus, thyroid deficiency impairs the maturation of glial cells, and consequently affects the migration of other cells in this region [93].

Whilst relationships between THs and neurotransmitters, structural proteins, growth factors and other proteins continue to be investigated, THs have proven to dramatically affect neuronal development, notably the maturation of specific neuronal populations [37,46,62,64,68]. For example, in postnatally hyperthyroid rats, the distribution of mossy fibres to CA3 and CA2 areas in the hippocampus is altered, with expansion of these target areas occurring in conjunction with the development of an apparent accessory infrapyramidal bundle [80]. Some of the morphological alterations occurring as a result of thyroid status have been more fully illustrated through studies at the cellular level. Recent elucidation of a neuronal dendritic protein, RC3, which displays TH-dependency in the adult cortex and striatum [72,73] may represent a possible mediator of these morphological alterations. This relationship supports the likelihood of a trophic role for THs upon inter-neuronal associations. Effects on such neuronal sites may prove important with respect to synaptic connectivity of neurones. Such changes may be in combination with TH-mediated influences on cytoskeletal elements. At an ultrastructural level, cytoskeletal elements such as actin and tubulin have specific relationships with thyroid status, since these proteins share close relationships with microtubule associated proteins (MAPs), and their expression is influenced by THs [43,58].

A very close association exists between THs and acetylcholine (ACh) and cholinergic function [64,113] which persists throughout life. These effects appear to be isolated to specific cholinergic nuclei and their pathways, notably the basal forebrain (BFB) and hippocampus [110]. Furthermore, THs interact with various neurotrophic factors via an influence on their expression, and regulation of their receptor populations [2]. The most notable of these is nerve growth factor (NGF) [41,42], which has a well-documented association with cholinergic activity [61].

Alterations in structure, function and behaviour as a consequence of thyroid dysfunction, have highlighted the importance of these hormones, especially for CNS development and the maintenance of optimum function of particular neuronal systems throughout life. These influences of THs are both cell and region specific, and also dependent on developmental stage.

1.3. CNS dependency on thyroid hormones during development

The importance of THs for mammalian brain development has long been recognised particularly during the perinatal period. Perinatal hypothyroidism results in various abnormalities including mental retardation and delayed myelination [33]. Since the recognition of THs effects on myelination [10,134], the expression of genes encoding proteins specific to myelination, such as myelin basic protein and myelin-associated glycoprotein have been identified as being dependent on normal thyroid function [39,115]. These findings lend support to possible reasons behind the altered myelination with changes in thyroid status during development.

TH deficiency during foetal and neonatal periods in the rat produce deleterious effects such as reduced synaptic connectivity, decreased myelination and alternations in levels of neurotransmitters [41,42,48,110]. Similarly in humans, deficiencies in thyroid function in and around these times predisposes the infant to a condition known as endemic cretinism. Although variable in severity, endemic cretinism primarily involves neurological deficits through impairments of cerebral cortex, basal ganglia and cochlear development. The major motor disorder presents as rigidity with added spasticity due to extrapyramidal dysfunction thought possibly to involve a putameno-pallidal lesion [1,126,130]. These irreversible effects on the brain appear to be incurred during the second trimester as a direct result of foetal iodine deficiency producing thyroid dysfunction. Investigations of cretinism have provided valuable information as to the neuronal systems that are dependent on THs during early stages of development. Auditory deficits associated with this condition and the dependency of cochlear development on THs are partly explained by findings that TH deficiency reduces the number and distribution of dendritic spines in the auditory cortex [119]. Similar effects are also noted in the pyramidal cells of the visual cortex [117,118].

The long-term effects of early thyroid dysfunction on CNS function have been well demonstrated in adult rats following neonatal thyroidectomy [30]. Profound reductions of glucose use are observed, not only in the cerebral cortex and auditory regions, but throughout the brain. The reliance of neuronal tissue on T3 is also well illustrated by the strict regulation of T3 seen to occur in the CNS [75]. Furthermore, rapid alteration (within 4 h) of cerebrocortical, type II 5' diiodinase levels are seen in response to changes in circulating T3 concentration [83].

1.4. Thyroid dysfunction in the adult, and associations with neurological disease

As THs mediate important effects within the CNS throughout life, it is not surprising that dysfunction of the

thyroid axis during later life appears to contribute to a variety of CNS-related pathologies. The prevalent nature of thyroid dysfunction concomitant with CNS symptomatology has prompted further investigation into possible connections between the two. Adult-onset thyroid dysfunction is associated with both neurological and behavioural abnormalities [27], emphasising the importance of THs for normal brain function. For instance, ‘Myxoedema Madness’, relating hypothyroidism to depressive psychosis was characterised many decades ago, while more recently, the symptoms of hypothyroidism have also been characterised as similar to those of depression [33,65,70,106].

Neurologically, hypothyroidism has been associated with cerebellar ataxia, memory impairment, hallucinations, confusion, delusions, psychotic behaviour and loss of the alpha-rhythm during EEG examination. In contrast, hyperthyroid symptoms include an increased frequency of the alpha-rhythm, irritability, nervousness and tremulousness, whilst extreme cases may suffer delirium, stupor and coma [1]. Symptoms of hyperthyroidism have been further correlated with the severity of the condition, with sweating, tachycardia, trembling, anxiety and restlessness progressing to nervousness, irritability and sleep disturbances, whilst paranoia, and symptoms of mania and depression affect the most severe patients [57].

In the elderly hypothyroid patient, dementia appears to be a dominant secondary characteristic [86]. Hypothyroidism has also been considered a reversible cause of secondary dementia in the elderly [24,70], and thyroid function tests have been adopted as a valuable tool in the evaluation of dementia. Interestingly, TH abnormalities have been shown to occur in 38% of frontotemporal dementia and in 36% of non-specified dementia cases, in a group of psychogeriatric patients [38]. A past history of thyroid dysfunction has also been previously postulated as a risk factor for Alzheimer’s disease (AD) [14,66], and a positive, but not significant correlation has been reported between myxoedema and AD [139]. Cortical atrophy, a characteristic of AD and ageing has also been identified in some [130], but not other [48] studies of hypothyroid patients. Interestingly, pyramidal cells of the hippocampal CA1 region show altered dendritic spine density in response to THs in adult subjects [55]. Furthermore, the hippocampus, which has an important role in memory and learning and is a major site of AD pathology, exhibits reductions in THR mRNAs in AD patients [77]. This finding may, however, relate to the general cell loss and, thus, reduction in cells containing THRs, which is prevalent in this region in AD.

Cognitive decline often appears concomitant with ageing, and is particularly associated with many age-related neurodegenerative diseases, notably AD, along with various psychiatric and demyelinating disorders. Whilst alterations in specific components of the thyroid axis have been associated with these conditions [86], treatment strategies involving THs have provided benefit to some of the symp-

tomatology in such cases, notably for psychiatric symptoms [65,137]. Cognitive disturbances related to thyroid axis dysfunction have also been augmented through such treatment. For example, subclinical hypothyroidism is characterised by an increased serum TSH alongside normal TH concentrations. Normalisation of the altered hormonal concentrations via L-T4 treatment has been shown to significantly improve some aspects of memory performance in affected patients. Despite being effective treatment for mood disorders, both lithium and electro-convulsive therapy also induce unwanted cognitive side effects, yet the provision of THs as an adjunct to such treatment has proven to effectively reduce these problems.

1.5. Ageing and the hypothalamo-pituitary-thyroid axis

Ingbar [71] postulated that a relationship between altered thyroid function and ageing existed by characterising associations between rising serum cholesterol and lipoprotein concentrations, and a lowering of the basal metabolic rate in aged subjects. Like many endocrine systems, the hypothalamo-pituitary-thyroid (HPT) axis is subject to alteration with age. However, contrasting evidence exists with regard to such changes concerning many of the elements constituting the HPT axis. This presents difficulty in determining possible implications upon CNS function, that may stem from changes in the HPT with age. The hormonal elements of the HPT are THs, thyroid-stimulating hormone (TSH) derived from the anterior pituitary, and TRH produced in the paraventricular nucleus of the hypothalamus. THs are secreted in response to stimulation by TSH, and similarly, TSH release is controlled by TRH levels. A negative feedback relationship exists between THs and both TSH and TRH.

In aged subjects, serum basal and free T3 concentrations have been shown to be inversely correlated with age [20], while free T4 concentrations have been reported as higher [100], unchanged [59,131], or reduced [20]. Aged subjects also display increases in rT3 levels [91,92]. TSH is released following synthesis by the thyrotrophs of the anterior pituitary. Despite its negative regulation by THs, and stimulated release by TRH, as with many hormones TSH exhibits a distinct circadian rhythm. In ageing rats, this circadian rhythm of TSH has shown to progressively deteriorate [76], yet in humans it is reported to be unaffected [131]. More recently, however, reductions in TSH levels during the morning period have been shown to be reduced both in humans [92,100,131] and rats [11], whilst increased TSH levels have been reported in those suffering from dementia [45]. Notably, there are conflicting reports for both animal and human studies [36,59,76]. A reduced response of TSH to TRH stimulation is recognised in ageing humans [100,131], whilst conversely, in rats, a significantly increased sensitivity of TSH to TRH has been reported [76]. Interestingly, patients with depression show blunted TRH–TSH responses, while AD patients display this characteristic

less consistently [99]. With reference to the same study, those AD patients with a blunted TRH–TSH response also had a significantly higher free T4 level and were also more severely demented than the AD patients with a non-blunted TRH–TSH response.

The diversity of actions mediated by the THs occurs through the various receptor populations and location of the different deiodinase enzymes which directly affect the availability of both active and inactivated metabolites of THs. In rats, ageing presents changes in deiodinase activity which appear to be sex-dependent. In female rats, significant reductions of types I and II deiodinase enzymes are seen in the pituitary accompanied by no change in the thyroid gland [23], contrasting with increases in activity of the two enzymes in the pituitary and reductions in thyroidal 5'DI(I) in males [29]. Alterations in deiodinase levels may be explained by changes in the availability of selenium. Inhibition of deiodinase enzyme activity during selenium deficiency [7] led to these enzymes being identified as selenoproteins [9]. A highly significant correlation between T3/T4 ratio and selenium status has since highlighted the importance of this element to TH functioning [105], and selenium deficiency has also been associated with Down's syndrome and the presenile changes occurring in children with Kashinbeck disease [85].

In addition to the alterations in receptor populations and activity of related enzymes, changes in circadian rhythms, sensitivity to stimulus and plasma concentrations of the THs, TSH and TRH, indicates this system to be globally affected with age. The conflicting data surrounding many of the variables forming the HPT only make it more difficult to determine the possible mechanisms behind any such alterations. It may be a singular affected component which triggers modification to other steps of the pathway or simultaneous changes in a number of individual elements which leads to a cascade of uncoordinated activity. The consequences of changes in activity of the different elements of the HPT will not only affect thyroid status, but indirectly affect the ageing process through many other mechanisms upon which THs have influence.

Finally, an increased prevalence of autoimmune antibodies against elements of the thyroid axis is correlated with ageing. Early forms of autoimmune thyroid disease have been found significantly associated with rapid cycling of bipolar disorder [106]. Moreover, a positive correlation has also been made between free T3 and dihydroepiandrosterone (DHEA), a steroid which is known to progressively decrease with age [114]. This supports previous findings in females where a raised and reduced serum DHEAS was observed in hyperthyroidism and hypothyroidism, respectively [94]. In aged rats, positive correlations have been made between serum TSH levels and superoxide dismutase activity in the liver [11], one of many antioxidant enzymes which appear to decrease with age predisposing the body to greater damage from free radicals.

1.6. Cognition and the hippocampal formation

The term 'cognition' refers to mental activities involved in the acquisition, storage, retrieval and use, of knowledge. Such activity requires the integration of a wide variety of mental processes including perception, memory, imagery, language, reasoning, problem solving and decision making. It has been established that mnemonic processes are associated with areas within the medial temporal lobe, following reports of amnesic symptoms in patients subsequent to surgical removal of these regions, notably the hippocampal formation [124]. These findings and ongoing studies with such patients, have contributed significantly to the understanding of memory processes. Structures for the formation of recent memory have been grossly localised and memory established as a neural substrate separate to other cognitive functions. The prominent role of the hippocampal formation (HF) in learning and memory, is constantly under review, and more recent indications are that the HF is a region on which only certain properties of memory processing depend. Furthermore, the nature of such properties remain ill-defined and arguments continue to surround such philosophies [35]. In rodents, the HF has been more specifically implicated in the formation of spatial memory. This form of memory is defined as the awareness of one's position in relation to the surrounding environment. Lesion studies in conjunction with spatial tasks have provided evidence for the memory-related HF function to be as a form of representational processing of spatial information. A close relationship is shared by the HF and basal forebrain (BFB), the nuclei of which bear prominent cholinergic cell groups. Evidence strongly supports the existence of an important ACh influence upon hippocampal function. In support of this is the characteristic neuropathology of AD involving dramatic reductions of cholinergic cell bodies and ACh levels within the BFB and HF, respectively. Patients with this disease present with a debilitating loss of cognitive function, and loss of spatial awareness [40].

1.7. The relationship between the hippocampal formation and the basal forebrain

Cholinergic neurones of the pedunculopontine and dorso-lateral tegmental nuclei of the brainstem project to forebrain structures including the medial and lateral septal nuclei and the diagonal band of Broca [138]. Evidence suggests that these cholinergic projections make contact with cholinergic cells within these forebrain areas [78]. Cholinergic cell groups from BFB areas include the substantia innominata, magnocellular preoptic area, ventral globus pallidus, nucleus basalis of Meynert, diagonal band and medial septum. These project topographically to front, parietal, occipital and temporal neocortices and also to cingulate cortex and hippocampal formation [40,67,82,128]. More specifically, the medial septum (MS), horizontal and vertical limbs of the diagonal band of Broca, and nucleus basalis

of Meynert (nBM) send projections to the hippocampus proper, dentate gyrus, cingulate cortex, and neocortex, respectively [40]. The cholinergic cells of the medial septum (MS) and the vertical limb of the diagonal band of Broca (vDB) project to the HF, most prominently to the stratum oriens and stratum radiatum of the cornu ammonis, and the supra and infragranular regions of the dentate gyrus [3,4,84]. The reticulo-septo-hippocampal pathway was first demonstrated following the stimulation of the midbrain reticular formation or the MS, leading to ACh release in the hippocampus [32]. Septal neurones projecting to the hippocampal formation originate in the MS and vDB, innervating the HF via the fimbria, dorsal fornix, and supracallosal striae. A fourth route through the amygdala complex has also been demonstrated, although this appears to mainly innervate the subiculum [44,96]. Whilst a small contralateral pathway also exists, the main ipsilateral projection is highly topographically organised providing a laminar pattern of termination within the HF. For example, in the dorsal hippocampus, CA1 pyramidal cells along with dentate granule cell layers receive vDB afferents, whilst more ventrally, afferents are from both the vDB and MS. Different types of septal fibres have been further differentiated in terms of the transmitter released. MS/vDB neurones receive reciprocal inputs from hippocampus, amygdala and brainstem including locus coeruleus, raphe nuclei, and laterodorsal tegmental nuclei.

The medial septal area comprising the MS and vDB, contribute fibres to form a projection known as the septo-hippocampal pathway (SHP). This predominantly cholinergic projection has become well characterised in structure and function [34]. Similar to the BFB projections to the cortex, the SHP is not purely cholinergic, and contains a large contribution from GABA neurones and various additional neuropeptidergic contributions. Functionally, the SHP relays brainstem, thalamic, and hypothalamic information to the hippocampus, and plays a significant role in the genesis and maintenance of hippocampal electrical activity, notably theta rhythm [127]. Lesion studies have also demonstrated the SHP to have an involvement in memory and learning, especially spatial tasks [104]. These findings have been supported further by a wealth of information derived from lesion studies and pharmacological manipulations examining hippocampal function. Notably, in aged rats displaying spatial learning impairments, intrahippocampal grafts of foetal septal tissue rich in cholinergic neurones have led to improvements in such learning abilities [44]. Interestingly, age-dependent degeneration of the SHP has been observed in rats [49], with such degeneration being accelerated by stress.

In patients with AD, loss of neurones in the nBM [112,136] alongside reductions in cholinergic markers in the cerebral cortex [6] have indicated a possible involvement of this cholinergic forebrain projection system in the learning and memory deficits associated with the disease. In rats, lesions of the nucleus basalis magnocellu-

laris, the equivalent nucleus to the nBM of humans, have also produced disturbances of learning [21,31]. Cholinesterase inhibitors facilitate central cholinergic function and provide positive effects on various measures of learning and memory [19,26], although this is not always the case [15,16]. Similar facilitatory effects have been demonstrated in rats with lesions of the nBM [60,89,90].

1.8. Thyroid hormones, cholinergic function and nerve growth factor

During both developmental and adult stages, THs exert great influences upon and within selected brain regions, notably hippocampal, cortical, basal forebrain, and cerebellar areas. Perinatal thyroid deficiency produces a variety of alterations in CNS development including effects on the development of several neurotransmitter systems. Marked retardation of cholinergic system maturation has been well documented [108–111]. Interestingly, despite long-term TH replacement therapy, the effects of thyroid deficiency on cholinergic function have shown to be irreversible in all regions of the brain except the basal forebrain [110]. Following (nerve growth factor) NGF or L-T3 treatment, neuronal cultures derived from embryonic rat cerebral cortex display dose-dependent increases in CAT and AChE in conjunction with neuritic sprouting [46]. Separately, each treatment led to enhanced CAT expression, whilst combined treatment produced a synergistic response. Similar findings have been reported *in vivo* [111]. The development of forebrain cholinergic terminal fields in telencephalic areas is also regulated by TH status and, again, these effects are sensitive to NGF [103]. Moreover, the effects of T3 upon neuronal differentiation resemble those induced by NGF [74]. Whilst a summative interaction between NGF and THs on cholinergic activity exists [62,111], THs appear to mediate direct effects on NGF-induced expression in neonate and adult mice [132,133]. TH status also modulates NGF expression in the cerebellum of perinatal rats [41].

1.9. Impetus for the present studies

This investigation evaluated the effects of sub-chronically and chronically administered thyroxine on cognitive ability in rats. A modified version of the Morris watermaze was used to assess spatial learning. Performance in this task is closely associated with the functional integrity of the septo-hippocampal pathway and in particular, cholinergic function. However, other cholinergic pathways from the basal forebrain to the cortex may also be implicated. Scopolamine, a muscarinic antagonist, is an effective pharmacological tool for inducing selective cognitive deficits. Use of a scopolamine-induced cognitive deficit enabled further evaluation of changes in cognition associated with TH treatment and possible interactions with cholinergic systems. Following drug treatments and testing in the watermaze, selected brain regions were removed and

analysed for cholinesterase activity as a marker of cholinergic function.

2. Methods

2.1. Animal housing

Adult male, (350–480 g) Hooded-Lister rats from the University of Bradford breeding unit, were used for all experiments. The animals were housed in standard cages (30 × 20 × 60 cm) in groups of five. Lighting was kept to a normal, 12 h on/12 h off cycle. Humidity was maintained at 45–50% and temperature at 22–23°C. Water and standard rat chow were available ad libitum. All animals were closely monitored in accordance with Home Office guidelines. The animals were moved to a holding room 1 h prior to drug administrations and experimental testing, to allow the rats to become acclimatised to the new environment.

2.2. The watermaze

The watermaze was a slightly modified version compared to that used by Morris et al. [102]. The circular pool was formed of white plastic and measured 1.2 m in diameter and 0.6 m deep. Two heating elements on the bottom maintained the temperature of the water at 25–28°C. The island was cylindrical with a diameter of 12 cm and was 25 cm in height; the water level of the pool was maintained at approximately 2 cm above the top of the island when in position. The walls of the maze rose a further 30 cm above the surface of the water. The water was rendered opaque using 500 ml of E308 (liquid latex), and this ensured that the top of the island was invisible through the surface of the water. A square black metal frame supported the pool tank and extended above the pool to give attachment to spotlights in each corner at 1.6 m above the water. These were positioned to provide uniform light across the whole pool area, whilst black curtains surrounded the whole frame to minimise interference from external stimuli. On two adjoining sides of the pool enclosure, 28 cm above the water surface, two posterboards with large black and white stripes (one vertical and the other horizontal) were positioned to provide visual cues. A video camera was positioned 2.5 m above the centre of the pool. This monitored all maze performances and provided input to a VHS video recorder and television unit. A digitising image analysing unit (VP112) connected in parallel to the video camera and a computer enabled the precise tracking and measurement of the rats' movements. For testing purposes the maze was divided into four equal quadrants, with the platform located in the same quadrant each test day, but rotated to other quadrants on subsequent test days.

2.3. Drug preparation and administration

Application of a Latin square paradigm was used to select

animals for the drug treatment to be administered. All drugs were given by intraperitoneal injection, in a volume of 1 ml/kg body weight. L-thyroxine (L-T4; Sigma) was dissolved in a minimal amount of sodium hydroxide, with hydrochloric acid added to bring the pH of the solution to pH 7.4. A drug-free solution was used as the vehicle control. Scopolamine HCl (Sigma) was made up in a saline solution to a concentration of 1 mg/kg. All control injections were done with normal saline.

2.4. Animals and treatment protocol

Sixty animals were used for the sub-chronic experiment and 52 for the chronic treatment procedure. For experiment 1, the group of 60 animals was divided into three groups (A, B or C) of 20 subjects. On each of the test days, group A received saline as a control, whilst groups B and C received 2.5 and 5 mg/kg L-thyroxine (L-T4), respectively, 80 min prior to testing. Forty minutes prior to testing and 40 min following L-T4 treatment, half of groups A, B and C were administered saline and the other half injected with 1 mg/kg scopolamine (only on day 1). Behavioural testing was then conducted as outlined below. For experiment 2, the cohort of 52 subjects was again divided into three groups of 16–18. Based on preliminary data from experiment 1 the doses of L-T4 were adjusted upwards, with doses of 0, 5 and 10 mg/kg given to groups A, B and C, respectively. Administration of L-T4 or saline was given in the morning of every third day for 28 days inclusively, whilst the scopolamine was again administered on the behavioural testing day (two days following this 28-day injection period). Scopolamine or saline were administered 40 min prior to behavioural testing as per experiment 1. Data for three chronologically-treated subjects were inadvertently lost due to a computer failure.

2.5. Watermaze procedures

The rats were tested in the watermaze [102] for four successive days following sub-chronic L-T4 treatment, but tested on one day only following chronic treatment. The task took the form of six trials, repeated consecutively, with 30 s between each trial. On each of the first five trials the swim starting point was alternated between the three locations other than the site of the immersed island, and the rat given a maximum of 60 s to find the island. Trial six involved replacing the submerged platform with one which was visible (cued test).

2.6. Brain removal and preparation for assay

One to two days following completion of behavioural testing, randomly selected rats were decapitated by guillotine and the brains rapidly extracted. Brains were dissected on ice-cooled glass plates, and frontal cortex and hippocampus were removed and placed into Eppendorf tubes containing 1 ml of saline. Samples were then sonicated using a

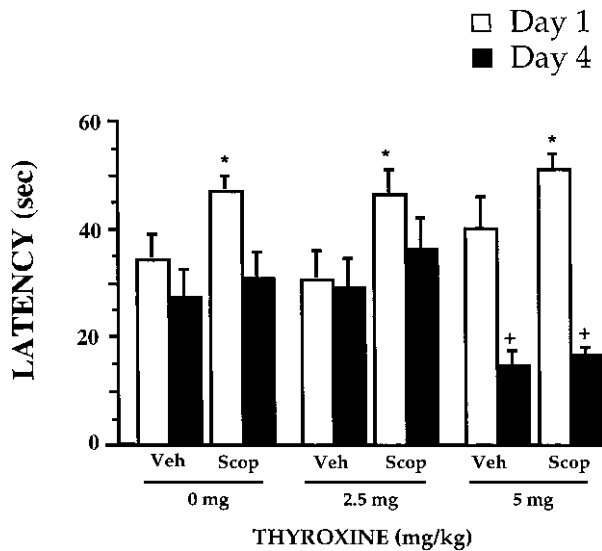


Fig. 1. The effects of sub-chronic thyroxine and acute scopolamine on latencies to locate a submerged platform in a watermaze task. Overall, thyroxine treatment improved spatial performance as is evident from the performance of the 5 mg/kg dose of L-T4 on day 4. Moreover, scopolamine impaired performance, leading to higher latencies to locate the platform, but in combination with L-T4 this effect was lost. This is again notable on day 4, where animals receiving 5 mg/kg L-T4 and scopolamine were not different from vehicle-treated rats given L-T4. Data shown are means \pm sem. $N = 10$ /group; * significantly different from vehicle-treated rats on day 1 ($p < 0.05$); + significantly different from corresponding 0 mg/kg L-T4 groups on day 4 ($p < 0.05$).

Sonoplus ultrasonic homogeniser and MS72 probe for 10 s prior to assay.

2.7. Cholinesterase assay

Brain cholinesterase activity was measured using commercially available kits purchased from Sigma Chemical Co. (Poole, UK). Individual samples of tissue homogenate from hippocampus and cortex were analysed. Briefly, 0.1 ml sodium chloride was added to 0.1 ml of homogenate in a test tube, and the solution vortexed. Reference tubes were then placed in a waterbath at 60°C for 10 min in order to inactivate the cholinesterase, before being cooled back to room temperature. All tubes then received 1.5 ml water, 1.0 ml nitrophenol solution, and 0.1 ml acetylcholine chloride solution. Exactly 30 min from the addition of the acetylcholine chloride, 0.2 ml of each sample was transferred to cuvettes for reading in a spectrophotometer at 440 nm.

2.8. Protein assay

Protein standards were made up using deionised water and bovine serum albumin (Biorad) to dilutions ranging from 0.1 to 3.2 mg/ml. Blank tubes contained 0.1 ml of normal saline, while 0.1 ml of protein standard solutions were added to a set of test tubes alongside those containing 0.1 ml samples. Samples had previously been diluted, adding 0.08 ml to the 0.02 ml samples tissue homogenates.

5 ml of diluted (as 1:4 solution) and filtered Biorad dye reagent was added to all the tubes prior to vortexing. After 15 min, 200 μ l samples were taken from all tubes and transferred to a microplate. Optical density was then measured at 595 nm in an automated platereader. Optical density values were plotted against protein standard concentrations and regression analysis performed to determine protein concentrations for the samples. Using the protein values, cholinesterase concentrations were then calculated according to the levels of protein in the tissue samples.

2.9. Statistical analysis

Latencies to locate the submerged platform (s) were averaged over trials 1–5. All data were subjected to a 3-way, split plot factorial ANOVA with pre-treatment (vehicle or L-T4), and scopolamine level (0 or 1 mg/kg) as orthogonal factors, and test day as a repeated measures variable. Where appropriate, the degrees of freedom associated with the repeated measures factor have been corrected for by applying a Greenhouse–Geisser procedure with ($N - 1$ df for all within-subjects error terms). Post hoc testing was performed using Bonferonni-corrected t -tests with the experiment-wise alpha level maintained at 0.05.

3. Results

3.1. Effects of sub-chronic L-T4 administration on cognitive performance

Fig. 1 illustrates the effects of thyroxine on latencies to locate the submerged platform in the MWM task. For ease of comparison, we present only data obtained for days 1 and 4 of testing. Overall, L-T4 treatment resulted in improved performances, i.e. lower latencies, to locate the platform $F(2,54) = 7.83$, $p < 0.05$. There was no immediate improvement in cognitive performance, but improvements emerged by day 4. This improvement was only notable in the group receiving the highest dose (5 mg) of L-T4, and for this reason we eliminated the use of the 2.5 mg dose in the chronic study. Superimposed on the L-T4 treatment, rats were challenged with the cognitive-impairing drug scopolamine. On day 1, SCOP-treated animals were significantly impaired to VEH-treated counterparts $F(1,54) = 11.1$, $p < 0.05$; however, sub-chronic administration of L-T4 completely eliminated the impairing effects of SCOP. This was most notable on day 4 at the 5 mg/kg dose of L-T4, where there are no differences between the VEH and SCOP-treated rats.

In order to ensure that neither L-T4 nor SCOP produced significant effects on locomotor abilities, measures of swim speeds were calculated. As shown in Fig. 2, neither drug produced any consistent effects on swim speeds on either test day. Thus, changes in performance are not the result of either a paucity of motor skills, or hyperactivity.

Another useful control measure arises from the data

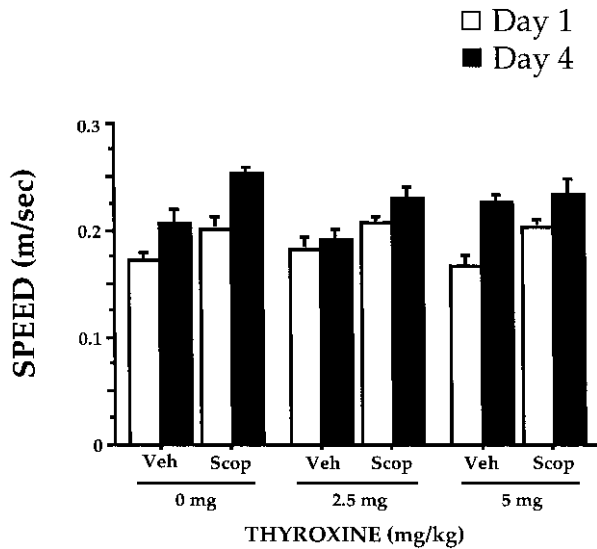


Fig. 2. The effects of sub-chronic thyroxine and acute scopolamine on swim speeds during a watermaze task. There were no statistically significant effects, although there was a slight tendency for scopolamine to increase swim speeds irrespective of thyroxine treatment level. Data shown are means \pm sem. $N = 10$ /group.

obtained for the visible platform test. We monitored the animals' abilities to swim to a platform that was positioned above the water surface, in order to ensure that they were able to use visual cues to orient themselves. There were two main effects noted here: (1) all rats, regardless of drug treatment swam to the visible platform more quickly on test day 4 $F(1,54) = 47.2, p < 0.05$. Thus, by day 4 the animals knew they could escape the water and seek refuge on the platform, and were able to immediately view the platform; and (2) SCOP-treated rats were slower to swim to the visible platform, but only on day 1 of testing $F(1,54) = 14.9, p < 0.05$. If this were merely an effect on light accommoda-

tion and papillary size, presumably it would persist and occur on day 4 as well, but this is not the case. These data are shown in Fig. 3.

3.2. Effects of chronic L-T4 administration on cognitive performance

The effects of thyroxine and scopolamine treatment on cognitive performance in rats following the chronic L-T4 administration regimen are shown in Fig. 4. Overall, L-T4 administration significantly lowered latencies to locate the submerged platform in the MWM $F(2,46) = 4.23, p < 0.05$. Part of this improvement lies in the fact that prior SCOP administration, significantly impaired performance, as evidenced by the higher latencies to locate the platform $F(1,46) = 13.49, p < 0.05$. The cognitive-improving effects of L-T4 were particularly evident in the SCOP-treated rats although overall, the L-T4 \times SCOP interaction was not significant $F(2,46) = 0.82, n.s$.

Similar results were found for the swim speed data and are presented graphically in Fig. 5. As is evident, L-T4 treatment significantly reduced swim speeds $F(2,46) = 5.75, p < 0.05$; an effect that was largely absent in VEH-treated rats, but emerged only in the SCOP-treated rats. Overall, SCOP-treatment produced significant increases in swim speeds $F(1,46) = 18.44, p < 0.05$, an effect that was much less apparent in the L-T4 chronically-treated rats. That this is the case is shown by the significant L-T4 \times SCOP interaction with $F(2,46) = 9.88, p < 0.05$. It is not clear whether or not the cognitive-impairing effects of SCOP can be attributed to this alteration in swim speeds. It is interesting to note that the enhanced swim speeds in response to SCOP occurred only in the chronically treated L-T4 rats and not those treated sub-chronically.

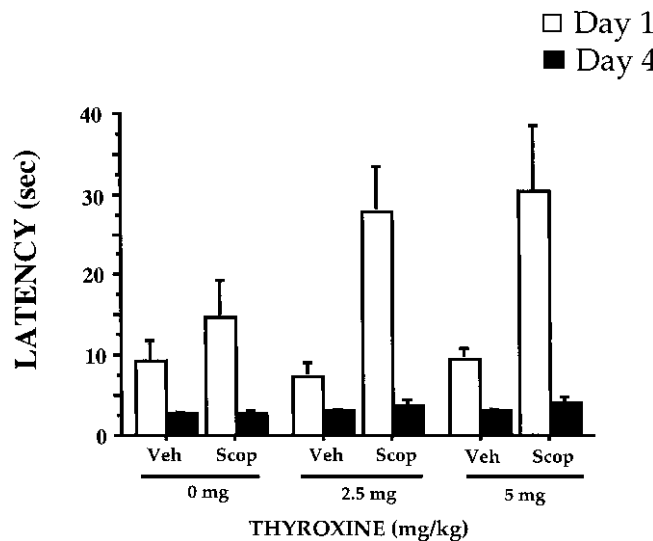


Fig. 3. The effects of sub-chronic thyroxine and acute scopolamine on latencies to locate a visible platform in a watermaze task. Overall, there was no obvious effect of thyroxine on this measure. However, scopolamine impaired performance, leading to higher latencies to locate the platform, but only on day 1 of testing. By day 4, all rats were equally proficient at escaping the water. Data shown are means \pm sem. $N = 10$ /group.

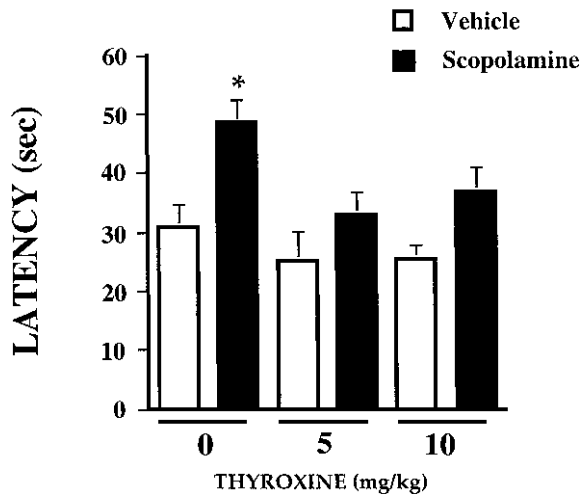


Fig. 4. The effects of chronic thyroxine and acute scopolamine on latencies to locate a submerged platform in a watermaze task. Overall, thyroxine treatment improved spatial performance, while scopolamine impaired performance, leading to higher latencies to locate the platform. Moreover, L-T4 treatment reduced the cognitive-impairing effects of scopolamine, although it did not completely reverse scopolamine's effects. Data shown are means \pm sem. $N = 7-8$ /group; * significantly different from vehicle-treated rats given 0 mg/kg L-T4 ($p < 0.05$).

Thus, these animals are strikingly different than those with less exposure to L-T4.

Following sub-chronic L-T4 administration, data for latency to swim to a visible platform (depicted in Fig. 3),

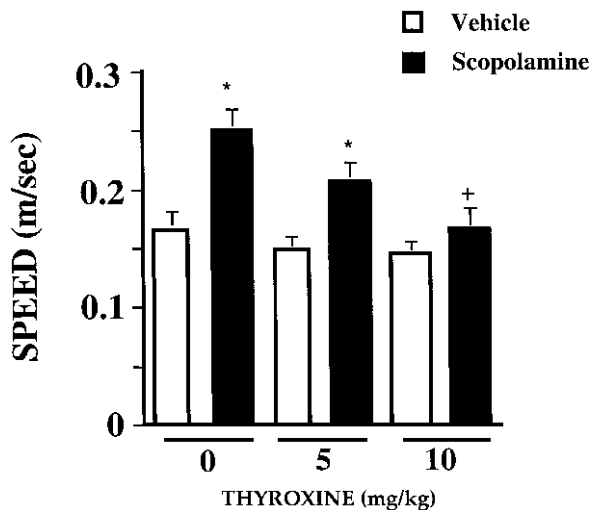


Fig. 5. The effects of chronic thyroxine and acute scopolamine on swim speeds during a watermaze task. Thyroxine on its own produced no obvious effects; however, scopolamine-treated rats were significantly more active than vehicle-treated counterparts. Interestingly, this hyperactive response to scopolamine was dose-dependently reduced by chronic L-T4 administration. This is especially evident in the rats which received the 10 mg/kg dose of L-T4. Data shown are means \pm sem. $N = 7-8$ /group; * significantly different from vehicle-treated rats receiving the same dose of L-T4 ($p < 0.05$); + significantly different from scopolamine group given 0 mg/kg L-T4 ($p < 0.05$).

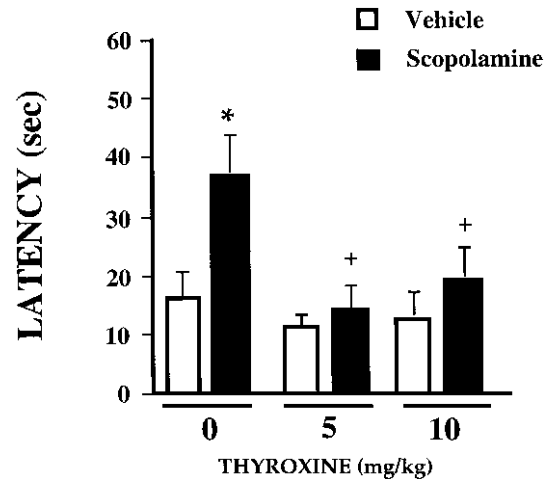


Fig. 6. The effects of chronic thyroxine and acute scopolamine on latencies to locate a visible platform in a watermaze task. Thyroxine on its own produced no obvious effects; however, scopolamine-treated rats were significantly slower to locate the visible platform and escape the water. Interestingly, this impairing response to scopolamine was abolished by chronic L-T4 administration. Data shown are means \pm sem. $N = 7-8$ /group; * significantly different from vehicle-treated rats given 0 mg/kg L-T4 ($p < 0.05$); + significantly different from corresponding 0 mg/kg L-T4 group given scopolamine ($p < 0.05$).

showed a predominant effect of SCOP-treatment only, which delayed swimming to the visible platform. Fig. 6 shows the corresponding data obtained following chronic L-T4 administration. ANOVA revealed only a significant effect of SCOP-treatment which again delayed swimming to the visible platform $F(2,46) = 4.37$, $p < 0.05$. Interestingly, this effect was markedly reduced in the L-T4 treated rats, which located the platform and escaped from the water as quickly as vehicle-treated cohorts. Post hoc tests did demonstrate that both the 5 and 10 mg/kg doses of L-T4, given chronically, abolished the impairing effects of SCOP-treatment.

3.3. Effects of sub-chronic and chronic thyroxine on brain AChE activity

Fig. 7 shows the results obtained for AChE activity, assayed following L-T4 treatment. ANOVA revealed significant effects of both sub-chronic and chronic L-T4 treatment for hippocampal areas with $F(2,54) = 3.4$, $p < 0.05$, and for sub-chronic treatment in the frontal cortex $F(2,54) = 3.5$, $p < 0.05$. L-T4 treatment led to increased AChE activity in both regions suggesting that cholinergic activity is up-regulated and enzymatic degradation is enhanced in response to this.

4. Discussion

In this investigation, thyroxine treatment administered both sub-chronically and chronically significantly enhanced the ability of rats to learn a spatial memory task. Rats given

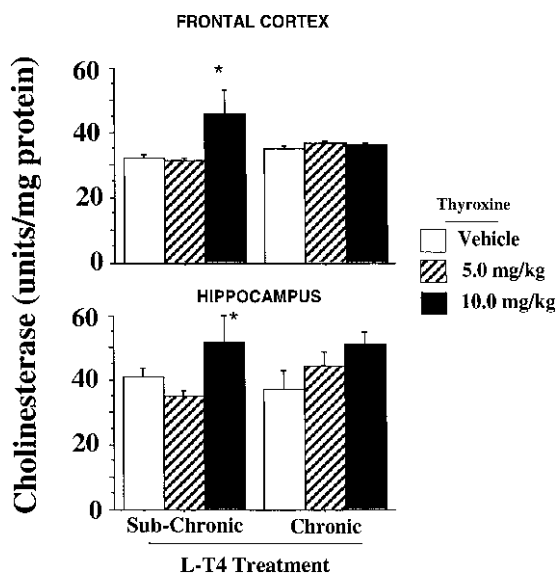


Fig. 7. The effects of sub-chronic or chronic thyroxine treatment on AChE activity in frontal cortex and hippocampus of subjects tested in the water-maze. Sub-chronic L-T4 administration led to elevated AChE activity in both frontal cortex and hippocampus, while chronic L-T4 administration increased AChE activity only in the hippocampus. Data shown are means \pm sem. $N = 10$ /group; * significantly different from corresponding 0 mg/kg L-T4 group ($p < 0.05$).

thyroxine learn and complete a spatial task quicker than control animals. In the chronically treated animals, both 5 and 10 mg/kg thyroxine doses significantly enhanced performance, whilst the 5 mg/kg dose improved performance of the rats receiving the drug sub-chronically. A wealth of information derived from lesion and pharmacological studies relate hippocampal function, the septo-hippocampal pathway and spatial performance tasks. Notably, in aged rats displaying spatial learning impairments, intra-hippocampal grafts of foetal septal tissue rich in cholinergic neurones leads to improvements in learning abilities [44]. Stimulation and lesion studies of the septo-hippocampal pathway of the rat have also shown ACh turnover in the hippocampus to be involved [101]. Our results suggest that TH effects may possibly be mediated through enhanced hippocampal cholinergic function.

A complex relationship exists between THs and cholinergic function [63,64,113] during development stages and in adulthood. Marked retardation in the development of the cholinergic system following perinatal thyroid deficiency is well documented [110,111], with such effects isolated to specific cholinergic nuclei notably those of the BFB. In the present study, we observed increased AChE activity in both hippocampal and frontal cortical regions; we suggest that this is indicative of enhanced cholinergic output in nerve terminals innervating these areas. The rise in degradative enzymes suggests that ACh release and/or terminal densities are augmented and the cholinergic system is upregulated overall. It might be argued that the corresponding rise in AChE activities might act to dampen

cholinergic function, but such an interpretation would necessitate arguing that the enhanced cognitive performance seen in L-T4 treated rats was accompanied by decreased ACh function, and this is counter-intuitive to many research reports. Thus, our data support the view that thyroxine enhanced cholinergic function, and that the augmentative effects on cognitive performance may be mediated through this increased cholinergic activity. That such an effect is possible is also demonstrated by other studies, for instance, the dependency of septal cholinergic neurones upon THs for development has been demonstrated in culture [62]. Moreover, postnatal thyroxine treatment has been shown to increase cholinergic cell body density in the MS and cholinergic fibre concentrations in CA3 [123].

In rats, lesions of the nucleus basalis magnocellularis (nBM), also produce disturbances of learning [21,31]. Cholinesterase inhibitors acting on central cholinergic function have been shown to positively affect various measures of learning and memory [19,26] although this is not always the case [15,16]. Similar benefits have been demonstrated in rats with nBM lesions [60,89], although such treatment was thought to enhance the activity of other central cholinergic pathways. In rats with lesions of the nBM which receive continual physostigmine infusions, cortical muscarinic receptor binding is downregulated [90], supporting the possibility that such treatment is effective via other pathways. Furthermore, no correlation was found between impaired spatial learning and cortical cholinergic deficits in chemical or mechanical lesion studies of rats [5] indicative of greater involvement of the septo-hippocampal pathway in such learning [104]. Alterations in spatial learning ability may, therefore, occur through improved cholinergic function at hippocampal sites, with enhancement in cortical regions providing a less well understood role. In this study, if the increases in AChE are indicative of increased cholinergic function, then this implicates involvement of both sites.

The effects brought about by THs upon cognition may be more clearly demonstrated through analysis of the effects and interaction with scopolamine. Scopolamine is an effective pharmacological tool for invoking a cognitive deficit, through its action as a muscarinic antagonist and, thus, reflects the important role acetylcholine has with respect to cognitive processes. Scopolamine proved effective in invoking a cognitive deficit in the animals, with significant impairments seen in animals receiving L-T4 sub-chronically and chronically. In the sub-chronic treatment group, the rats administered scopolamine were observed to repeatedly swim around the circumference of the water-maze, displaying a characteristic scopolamine-induced deficit in cognitive ability. Interestingly, the effect of scopolamine was only seen at the start of the trial period when performances on days one and four were compared. However, the chronically treated animals showed a less marked display of pool circling, probably indicative of THs reducing the scopolamine effect. In the sub-chronically treated group,

neither thyroxine nor scopolamine had effects upon motoric performance. However, in animals treated chronically, thyroxine reduced swim speed, independent of the dose of thyroxine administered. The combined effect of these drugs was an obvious reduction of the scopolamine-induced hyperactivity that occurred in the 10 mg/kg group. Systemically administered scopolamine has been shown to increase extracellular concentrations of ACh approximately 10- and 20-fold in frontal cortex and hippocampus, respectively, with an increased spontaneous motor activity seen over the same time course in freely moving rats [129]. Thus, our data suggest that THs diminish SCOP-induced hyperactivity by augmenting cholinergic function.

THs are broadly defined as metabolic hormones, yet it is becoming evident that such a term appears to encompass a wide variety of actions. THs particularly affect transcription of proteins associated with neuronal structure, growth factors, and neurotransmitters. THs appear to support both the development and maintenance of cholinergic function. These effects may be mediated via a complex interaction between NGF, ACh and THs. During development, NGF augments cholinergic activity as reflected by alterations in levels of cholinergic enzymes [53,79,98]. For example, intra-cerebroventricular administration of NGF markedly increases choline acetyl-transferase (CAT) in neonatal rats in septal, hippocampal and cortical regions [53]. In adult rats with unilateral septo-hippocampal lesions, intra-cerebroventricular NGF treatment increases levels of CAT in ipsilateral septum and hippocampus [63,64] demonstrating that the relationship continues throughout life. Furthermore, THs enhance NGF expression in both neonate and adult mice [132,133]. More specifically, in adult rats, THs increase NGF concentrations in hippocampus without a corresponding rise in CAT, whilst cortical levels of CAT increase without alteration in NGF [50].

A synergistic relationship between NGF and THs on cholinergic activity has been identified [62,111]. While cholinergic neurones in culture exposed to either NGF or T3 show enhanced CAT expression, treatment with both agents produces a response greater than the sum of the maximal effects brought about by the individual treatments. Similar findings have been seen in vivo [111]. The development of forebrain cholinergic terminal fields in telencephalic areas are TH-dependent, with such effects also being sensitive to NGF [103]. Furthermore, the effects of T3 upon neuronal differentiation resemble those induced by NGF, also pointing to a T3–NGF interaction [74]. This is supported further by evidence that in neuronal cultures of embryonic rat cortex, dose-dependent increases in CAT and AChE occur alongside neuritic sprouting following NGF or L-T3 treatment [46].

This study has highlighted the important interaction between THs and the CNS. The findings suggest that THs augment cognitive function as measured by a spatial learning task, when given either sub-chronically or chronically. The mechanisms by which either form of

administration evokes such effects may be different, but both appear to involve modulation of cholinergic activity. Interactions between THs, ACh and NGF may offer another possible mechanism by which such changes might have been elicited. The involvement of altered thyroid function in ageing and associated changes in cognition have been previously well described. Yet, whilst THs have proven effective in enhancing spatial learning, this may only represent one particular aspect of cognition that may be targeted therapeutically.

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