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Effects of Hypothyroidism on Programmed Cell Death in Forebrain Structures of the Rat during Development and in the Adult

Ph.D. Thesis

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Introduction

Thyroid hormones (THs) play a pivotal role in differentiation, growth, and metabolism of nearly all tissues. In the brain they have multiple actions on neurogenesis, neuronal cell migration and differentiation, synaptogenesis and myelination (Bernal et al., 2003; Zoeller and Rovet, 2004; Bernal, 2007). THs exert their physiological role mainly through binding to specific nuclear receptors, which are encoded by two different protooncogenes, c-erbAα and c-erbAβ (Lazar, 1993). Each gene has several alternative mRNA splicing products including the predominant isoforms of thyroid hormone receptors, TRα (1,2) and TRβ (1,2,3) (Thompson et al., 1987; Koenig et al., 1988; Mitsuhashi et al., 1988; Hodin et al., 1989; Koenig et al., 1989). Neurons in the cerebral hemispheres are characterized by a large number of nuclear triiodothyronine receptors, which increase under hypothyroidism (Valcana and Timiras, 1978).

Hypothyroidism during development results in profound mental retardation, deaf-mutism and spastic diplegia known in humans as cretinism (Konig and Moura Neto, 2002). In rats, hypothyroidism during the critical period, corresponding to the first two postnatal weeks, causes growth and neurological abnormalities similar to human cretinism (Eayrs, 1960, 1971; Schwartz, 1983; DeLong, 1996). Adult thyroid dysfunction is also associated with both neurological and behavioral abnormalities (Bauer et al., 2008). Nevertheless, the mechanisms of action of thyroid hormones on neurogenesis and neuronal apoptosis, which regulate the optimization of neuronal number during the course of development, are poorly understood. Several studies relate hypothyroidism with programmed cell death.
(apoptosis) during brain development (Patel and Rabie, 1980; Rabie et al., 1980; Xiao and Nikodem, 1998; Ambrogini et al., 2005; Huang et al., 2005a; Huang et al., 2005b). Hypothyroidism has been shown to increase not only the extent of apoptosis in the developing cerebellum, but also its duration by down regulating the anti-apoptotic genes \textit{bcl-2} and \textit{bcl-xL} and maintaining a high expression of the pro-apoptotic gene \textit{Bax} in mitochondrial fraction, compared to a limited expression in euthyroid state. \textit{Bax}, translocated from cytosol to mitochondria plays an important role in the initiation of apoptosis possibly by triggering off the translocation of cytochrome c, apoptosis-inducing factor (AIF) and second mitochondrial-derived activator of caspases (SMAC) from mitochondria to cytosol (Muller et al., 1995; Singh et al., 2003a; Singh et al., 2003b).

Programmed cell death is generally defined as a series of stereotypical, biochemical and morphological steps leading to cell demise. As opposed to cell death by necrosis, programmed cell death is an active process by which dying cells are removed in a safe non-inflammatory manner. The term “programmed” stresses that this cell death modality has an intrinsic, genetically defined character regulated by various signalling pathways and that it is clearly distinct from the accidental induction of necrosis. Programmed cell death comprises the processes of classical apoptosis, apoptosis-like and necrosis-like. Classical apoptosis may be the predominant type of cell death occurring during synaptogenesis. The importance of classical apoptosis as a critical form of programmed cell death in the adult nervous system is still debated. The precise types of neuronal death associated with “normal” physiological brain aging are currently unknown. Accumulated evidence suggests that under pathological conditions, during both acute and chronic neurodegeneration all types of programmed cell death phenotypes can occur.
This study aimed to evaluate the effect of hypothyroidism on cell survival in developing and adult rat forebrain structures in two experimental models of pharmacologically induced hypothyroidism; the first with congenital and the second with acquired hypothyroidism. Two forebrain structures were selected for qualitative and quantitative investigation during development and in the adult: the striatum and the lateral septum. Other forebrain structures were qualitatively analysed. The striatum was selected because (1) the thyroid hormone-regulated gene \textit{Nrgn} (also known as \textit{RC3}) is responsive to triiodothyronine (T3) in the striatum but not in other regions of the adult brain (Iniguez et al., 1992); (2) the thyroid hormones-regulated gene \textit{Srg1}, involved in synapse formation and/or function, which is highly expressed in striatal neurons, is dramatically reduced in hypothyroid brain (Thompson, 1996); (3) the striatum shows altered metabolic activity after administration of thyroxine (T4) in bipolar patients (Bauer et al., 2005); (4) hypothyroidism decreases capacity of the mitochondria for oxidative phosphorylation in the striatum, and therefore the striatum should be considered as a “sensitive” area to thyroidal state (Martinez et al., 2001) and (5) thyroid hormones interact reciprocally with GABAergic systems in mammals (Wiens and Trudeau, 2006). GABAergic medium-spiny projection neurons represent more than 90% of the total cell population in the striatum.

On the other hand, the lateral septal nucleus is viewed as a medial component of the striatum, as it receives abundant glutamatergic afferents from the cortex (hippocampal formation) that innervate GABAergic spiny neurons in this structure (Risold et al., 1997; Swanson and Petrovich, 1998; Swanson, 2000; Swanson and Risold, 2000). The lateral septum is rich in GABAergic neurons that express glutamic acid decarboxylase (Jakab and Leranth, 1995). No strictly local interneurons have been described (Phelan et al., 1989). Known that there is
reciprocal regulation of thyroid and GABAergic systems in vertebrates (Wiens and Trudeau, 2006), lateral septum would be another good forebrain structure for studying the effects of hypothyroidism on cell survival in developing and adult rat brain.

The present thesis consisted of four major chapters the Literature Review, the Materials and Methods, the Results and the Discussion. The first chapter contains an extended review of the current knowledge and views on the role of thyroid hormones and hypothyroidism in the developing and adult brain, the current knowledge on the programmed cell death and the anatomy and connections of the forebrain structures selected in this study. The second chapter contains the materials, the histological techniques used, the experimental protocol used for inducing the hypothyroidism in neonatal and adult animals, and the statistical methods used for the evaluation of the experimental results. The third chapter contains the qualitative and quantitative analysis of the results and the fourth chapter the discussion of the present results in relation to the literature.

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Literature Review

**Thyroid gland**

The thyroid gland of the rat lies ventrolaterally and on either side of the trachea just behind the larynx. It spreads over the fourth and the fifth tracheal ring and it consists of two lobes connected by an isthmus (Krinke, 2000). Much of our knowledge regarding the maturation of the thyroid gland and its function in the foetus and neonate is derived from studies in the rat (Legrand, 1986; Schwartz et al., 1997). The rat belongs to altricial species, born with a relatively underdeveloped brain and with the thyroid–pituitary–hypothalamic axis not fully matured (Oppenheimer and Schwartz, 1997). Brain maturation in rodents occurs early in postnatal development. There is evidence for a role of thyroid hormones in brain maturation during the intrauterine period in human (Pharoah et al., 1971; Grant et al., 1992; Boyages and Halpern, 1993) and sheep (McIntosh et al., 1979; McIntosh et al., 1982). The placenta plays a key role in the transfer of hormones and factors other than T4 that have an impact on thyroid function. In the first half of pregnancy, maternal T4 provides an important source of hormone for the developing fetus (Brown et al., 2005; Ahmed et al., 2008).

There are several reports on the harmful effect of thyroid hormone deficiency during development (Nicholson and Altman, 1972; Lauder, 1977b, 1977a; Stein et al., 1991; Porterfield and Hendrich, 1993; Bernal and Nunez, 1995; Oppenheimer and Schwartz, 1997; Anderson, 2001; Wong and Leung, 2001; Lavado-Autric et al., 2003; Lee et al., 2003; Farwell et al., 2005; Koibuchi, 2006; Ahmed et al., 2008). Generally hypothyroidism, is the most common pathological hormone deficiency (Roberts and Ladenson, 2004). Taken together, hypothyroidism can be classified on the basis of the time of onset (congenital or acquired), the severity (overt “clinical” or mild “subclinical”), and the degree of endocrine aberration (primary or secondary) (Roberts and
Ladenson, 2004). Primary hypothyroidism follows a dysfunction of the thyroid gland itself, whereas secondary hypothyroidism results from the dysfunction of metabolic or messenger pathways associated with thyroid hormone production and metabolism (Kirsten, 2000; Shagam, 2001; Guha et al., 2002; Ahmed et al., 2008). Primary hypothyroidism is characterised by reduced free T4 levels and elevated thyroid-stimulating hormone levels (Brown et al., 2005). Also, the most prevalent cause of hypothyroidism is a defective development of the hypothalamus or pituitary leading to multiple pituitary hormone deficiencies, while defects of hypothalamic and pituitary peptides and their receptors have rarely been identified as the cause of congenital hypothyroidism (Grueters et al., 2002).

Prenatal development of the thyroid gland
The thyroid follicular cells or thyrocytes derive from the endoderm (Noden, 1991; Walker and Liem, 1994). In rat, the thyroid gland is derived from the fusion of a medial outpouching from the floor of the primitive pharynx, the precursor of T4-producing follicular cells, and bilateral invaginations of the fourth pharyngeal pouch, which give rise to the parafollicular, or calcitonin (C)-secreting cells. The development of the thyroid gland is classically subdivided into discrete steps (Wendl et al., 2007); first, a group of cells buds off the floor of the primitive pharynx; second, these cells reposition dorsocaudally to reach the ventral wall of the trachea; third, the precursor cells proliferate and, fourth, differentiate into thyroid follicular cells (Macchia, 2000; Ahmed et al., 2008). In addition, other cells, including neural crest derived C cells, merge with the group of endoderm derived precursor cells (Manley and Capecchi, 1998). Any disorder of the thyroid that leads to reduced T4 production at birth is called congenital hypothyroidism (Macchia, 2000). Although, abnormal embryonic development of the thyroid gland leads to congenital hypothyroidism in humans
and other mammals, the principles of thyroid organogenesis are largely unknown (Wendl et al., 2007). At foetal day 15 in the rat, despite early evidence of thyroglobulin, thyroid peroxidase, and thyroid stimulating hormone (TSH) receptor gene expression, the thyroid gland is not evidently distinguished from the surrounding structures. At this time, neither iodine organification nor thyroid hormonogenesis is present and only a primitive follicular structure can be discerned. On foetal day 17, TSH receptor gene expression is significantly up regulated and this is accompanied by significant growth and rapid development in both structural and functional characteristics of the rat thyroid gland (Brown et al., 2000). Expression of both thyroglobulin and thyroid peroxidase mRNA is also increased at this time, thyroid follicles first appear on morphological examination, thyroid peroxidase function can be demonstrated and there is evidence of thyroid hormonogenesis (Remy et al., 1980; Kawaoi and Tsuneda, 1985; Brown et al., 2000). The aforementioned findings suggest that the TSH receptor plays an important role at later stages of prenatal development.

**Structure and function of the thyroid gland**

The epithelial cells of the thyroid gland, the thyrocytes, are organized in follicles. The function of the thyrocytes is to produce the THs T3 and T4. T3 and T4 are important regulators of growth, development, and differentiation. These hormones are also very important regulators of the metabolic rate. Between the follicles, there are individual or small groups of parafollicular cells or C cells. These cells secrete calcitonin, when there is an increase in serum calcium level. TSH is a major regulator of the thyroid gland morphology and physiology, as it affects a wide variety of aspects of thyroid function. TSH is responsible for the morphological appearance of thyroid follicles and the synthesis and secretion of thyroid hormones (Rajkovic et al., 2006). It is synthesized and released by the pituitary gland. TSH binds to the TSH receptor
(TSHR) on the basolateral side of the thyrocytes and regulates hormone production. The synthesis of THs requires uptake of iodide across the basolateral membrane into the thyrocytes, mediated by sodium iodide symporter (NIS), transport across the cell and efflux through the apical membrane into the follicular lumen. The efflux of iodide across the apical membrane is mediated, at least in part, by pendrin. Once iodide reaches the cell-colloid interface it is oxidised and rapidly organified by incorporation into selected tyrosyl residues of thyroglobulin. This reaction, referred to as organification, is catalyzed by thyroperoxidase (TPO) and results in the formation of mono- and diiodotyrosines (Taurog, 1996; Royaux et al., 2000; Yen, 2001). TPO also catalyzes the coupling of the two iodotyrosines to form either T3 or T4. To release thyroid hormones, thyroglobulin is endocytosed by pinocytosis digested by lysosomes, and then secreted into the bloodstream at the basolateral membrane (Wendl et al., 2002).

**Thyroid hormones**

The synthesis and storage of THs predominately occurs in the thyroid gland and the principal hormone is T4 (DeVito et al., 1999). Although a small proportion of the thyroid-localized hormones is T3, most T3 comes from the deiodination of T4 by tissue specific deiodinases. The processes involved in the synthesis, storage, release, transport, and metabolism of THs are complex and consist of several steps as follows (DeVito et al., 1999): (1) uptake of iodide ion by the thyroid gland; (2) oxidation of iodide and the iodination of tyrosine residues within thyroglobulin; (3) coupling of iodotyrosine residues to produce iodothyronines; (4) proteolysis of thyroglobulin and release of T4 and T3 into the blood; (5) binding to serum transport proteins; (6) target tissue synthesis of T3 from T4; (7) catabolism of T4 and T3 in peripheral tissues; and (8) catabolism and biliary elimination of THs in the liver. Actually, there are many
examples of pharmaceutical, environmental, and naturally occurring chemicals that alter one or more of these processes in mammals or interfere with the production, transport, and metabolism of these hormones as they have been reviewed by several authors (Gaitan, 1989a; Hill et al., 1989; Brucker-Davis, 1998).

Thyroid hormones enter the brain through two routes (Porterfield, 2000): (1) the predominant route is via the blood–brain barrier involving direct transport through the capillary endothelium and into the brain cells. Hormone transport via this route depends on the serum hormone levels (influenced by serum protein-binding relationships), the transport systems through the endothelium, and the transport systems into the brain cells; (2) the second and less significant route is via the choroid plexus–cerebrospinal fluid (CSF). The thyroid hormone-binding protein transthyretin (TTR) binds T4 but not T3. It is produced by the choroid plexus and secreted into the CSF. The TTR then binds to T4 that enters CSF through the choroid plexus and may play an important role in T4 transport to the brain cells.

**Thyroid hormone receptors**

Thyroid hormones exert their major effect through T3 that bind to nuclear thyroid hormone receptors (TRs). TRs as ligand-dependent transcription factors regulate expression of T3-responsive target genes (Bernal, 2002; Forrest, 2002). TRα1 is the major isoform expressed during foetal life (Forrest et al., 1991; Mellstrom et al., 1991). Prior to birth, there is an increased expression of TRβ1 (Forrest et al., 1990; Forrest et al., 1991), which is distributed widely as development proceeds (Strait et al., 1990; Mellstrom et al., 1991; Bradley et al., 1992). Nevertheless, TRα has been estimated to account for 70-80% of total TRs expression in the brain (Schwartz et al., 1992). TRα1 and TRβ1 also exhibit differential spatiotemporal expression in neurons throughout the postnatal and
adult brain (Mellstrom et al., 1991), suggesting discrete roles for the two isoforms during development and in the mature CNS. THs influence gene expression, either positively or negatively, through binding to nuclear TRs (Brent, 1994; Glass, 1994). Certain genes expressed in the brain have been shown to be under thyroid hormones control. These include myelin genes (Farsetti et al., 1992), the Purkinje cell specific gene, PCP2, (Zou et al., 1994) the transcription factor NGFI-A (Mellstrom et al., 1994), and neuron specific enolase gene (NSE) (Xiao et al., 1993).

The presence of TRs in neural tissue at early stages of development suggests that foetal brain is a target for T3, even before the onset of foetal thyroid gland function. Indeed, maternal THs reaches the foetus in significant amounts (Vulsma et al., 1989) and has specific effects on the transcriptional activity of hormone-responsive genes in the foetal cerebral cortex before the foetal thyroid gland is fully active (Dowling et al., 2000). Although small amounts of T3 are produced directly by the thyroid gland, more than 80% of this form of THs in the brain derives from the local conversion of T4, the major circulating product of the thyroid gland (Crantz et al., 1982), to T3. T3 is measurable in human brain from at least the 10th week of gestation, in contrast to other organs where only T4 is present at this stage. Thus, a selective accumulation of T3 in foetal brain occurs in the presence of low foetal thyroid activity and low or undetectable T3 concentrations in serum and other tissues (Burrow et al., 1994). Brain receptor occupancy increases in parallel with T3 levels and reaches 60% at a time when the occupancy in the liver is only 10% (Ferreiro et al., 1990). This early high occupancy of brain receptors may be due to early local expression of Type II 50-deiodinase, an enzyme responsible for the conversion of T4 to T3 (Croteau et al., 1996; Bianco et al., 2002).
Role of Maternal Thyroid Hormones

Thyroid hormones are essential for normal neonatal development in both humans (Zoeller et al., 2002) and rodents (Çalıkoglu, 1999). Experimental works have indicated that THs are transported from the mother to the foetus, albeit in limited amounts and that the foetal brain is exposed to THs before initiation of foetal thyroid hormone synthesis (Porterfield and Hendrich, 1991; Morreale de Escobar et al., 1997; Sinha et al., 1997; Pickard et al., 1998). However, until recently, it has generally been accepted that the effects of THs on brain development occur only after birth (Fisher, 1999). THs of maternal origin are important in foetal brain development and neurological outcome of the offspring survival (Gaitan, 1989b; Zoeller et al., 2002). First, THs of maternal origin cross the placenta and reaches the foetus (Vulsma et al., 1989; Contempre et al., 1993). In addition, the TRs are expressed in the foetal brain before the onset of foetal thyroid function, and receptor occupancy is within the range known to elicit physiological effects (Bernal and Pekonen, 1984; Ferreiro et al., 1988). Second, iodine therapy prevents neurological cretinism in regions of endemic goiter only if initiated before the beginning of the third trimester (Cao et al., 1994). In rat, the THs of maternal origin can reach the foetus (Morreale de Escobar et al., 2004b) and the TRs are expressed in the foetal rat brain before the onset of foetal thyroid function (Falcone et al., 1994; Morreale de Escobar et al., 2004a). These findings show that, the THs are essential for brain maturation from early embryonic stages onward (Bernal and Nunez, 1995; Morreale De Escobar et al., 2000). However, thyroid hormones-dependent stages of foetal brain development remain to be characterized. Notably, the maternal thyroid is the only source of T4 and T3 for the brain of the foetus because its thyroid gland does not start contributing to foetal requirements until midgestation in human, and embryonic days (E) 17.5–18 in rats (Ausó et al.,
2004). Therefore, the amount of maternal T4 that the foetus receives early in pregnancy will determine thyroid hormone action in its brain.

**Hypothyroidism and brain development**

Reduction or absence of thyroid hormones during brain maturation yield molecular, morphological, and functional alterations in the cerebral cortex, hippocampus, and cerebellum (Schwartz, 1983; DeLong, 1996; Lee et al., 2003). Hypothyroidism during foetal and neonatal development results in delayed neuronal differentiation and decreased neuronal connectivity (Nunez et al., 1991). Disorders of neuronal migration are considered to be the major causes of both gross and subtle brain abnormalities (Rakic, 1990). In general, in human and rat, the maternal hypothyroidism during pregnancy disturbs brain development of the foetus, resulting in neurological deficits in offspring (Man et al., 1991; Porterfield and Hendrich, 1993; Sinha et al., 1997; Pickard et al., 1998; Evans et al., 1999; Haddow et al., 1999; Pop et al., 1999; Forrest, 2004; Mirabella et al., 2005). Many studies have characterized the neuroanatomical consequences of developmental hypothyroidism. The perinatal hypothyroidism alters the density and size of neuronal perikarya within cerebral cortex, as well as fibre density and orientation within adult cortical layers (Eayrs and Taylor, 1951; Eayrs, 1955; Eayrs and Horne, 1955). Additionally, Berbel and colleagues (Berbel et al., 2001) have published a study that characterizes the effect of developmental hypothyroidism on a variety of anatomical features, including spine density of pyramidal neurones in the cerebral cortex and the organization of callosal connections. Hypothyroidism produces changes in callosally projecting neurons, which may be due to the maintenance of a juvenile pattern of projections. Thus, THs deficiency during a brief perinatal period produce severe neurological defects in humans and experimental animals (Thompson, 1996). During the critical period of development, hypothyroidism causes
abnormalities of the CNS such as incomplete maturation of neuronal and glial 
cells, reduction in synaptic densities and myelin deficits (Wong and Leung, 
2001). In rat, thyroid hormone deficiency during foetal and neonatal periods 
produces deleterious effects, such as reduced synaptic connectivity, delayed 
myelination, disturbed neuronal migration, deranged axonal projections, 
decreased synaptogenesis and alterations in levels of neurotransmitters (Geel et 
al., 1967; Dussault and Ruel, 1987; Oppenheimer and Schwartz, 1997). 
Deficiencies of myelination have been observed in the cerebral cortex, 
hippocampus and cerebellum (Balázs et al., 1969; Rosman et al., 1972). All 
these effects in rats can be reversed by thyroid supplementation but only if 
supplementation is started before the end of the second week of extra uterine life 
(Thompson and Potter, 2000). The greater the delay in thyroid replacement the 
less the chance of recovery (Eayrs, 1971; Legrand, 1986). However, 
hypothyroid animals can maintain a close to normal level of T3 in the brain for 
extended periods. This phenomenon is due to at least three regulating 
mechanisms: (1) uptake of thyroid hormones is enhanced. It was shown that the 
uptake by the telencephalon of labelled T3 was much higher in 
thyroidectomized animals than in controls or in thyroidectomized and T3 
supplemented ones; (2) conversion of T4 into T3 is increased. One of the most 
important elements of this process is the adjustment of the expression and 
avtivity of the type II deiodinase of the brain to a higher level. Enzyme kinetic 
studies, expression of TRα- and β-nuclear thyroid hormone receptors and after 
cloning the chicken type II deiodinase-in situ hybridization studies clearly 
supported the central role of the conversion process; (3) the rate of loss of T3 
from the brain is slowed down under hypothyroid conditions as evidenced by 
hormone kinetic studies (Rudas et al., 2005).

Generally, hypothyroidism during brain development results in permanent 
functional deficits (Schoonover et al., 2004). Hence, it is apparent from the
previous results that thyroid hormone deficiency causes a series of abnormalities in the CNS that may lead, in turn, to an irreversible impairment, disorganization, and abnormal development. This drastic effect may be responsible for the loss of neurons’ vital functions. Adult thyroid dysfunction is also associated with both neurological and behavioural abnormalities (Calzà et al., 1997); however, the mechanisms of actions of thyroid hormones in the adult CNS are poorly understood.

**Experimental induction of hypothyroidism**

Inhibition of thyroid hormone synthesis occurred experimentally by a group of thioureylene drugs [methimazole (MMI), propylthiouracil (PTU), thiourea or carbimazole], which can inhibit the synthesis of thyroid hormones. These drugs interfere with the conversion of iodide to iodine and consequently the iodination of tyrosyl groups. The tyrosyl groups are also inhibited from joining to form T3 and T4. The congenital hypothyroidism in rats was induced because foetuses had access to the drug by placental transfer and neonates through milk secretion (Hasebe et al., 2008). Neonatal rats receiving the antithyroid drug MMI in their mothers’ milk are rendered hypothyroid (Chiovato et al., 1997; Kumar et al., 2006). Hypothyroidism during the postnatal period, induced by the administration of either MMI or PTU, is known to markedly retard both maturation and development of the nervous system (Darbra et al., 2003).

**Cell death**

The term programmed cell death (PCD) was introduced in 1964, proposing that cell death during development is not of accidental nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin and Williams, 1964; Gewies, 2003). Multicellular organisms have a requirement to adjust their cell number in different tissues to establish and
maintain proper function and morphology. This is accomplished by mitosis to increase the cell number and cell death to decrease the number. Many diseases are caused by either excess of cells, such as cancer and autoimmune diseases or by inappropriate cell loss in, for example, neurodegenerative diseases. PCD is generally defined as a series of stereotypical biochemical and morphological steps leading to cell demise. As opposed to cell death by necrosis, it is an active process by which dying cells are removed in a safe, non-inflammatory manner (Leist and Jäättelä, 2001). The term “programmed” implies that this cell death modality has an intrinsic, genetically defined character, regulated by various signalling pathways (Danial and Korsmeyer, 2004) and that it is clearly distinct from the accidental induction of necrosis by cell damage or “bioenergetic catastrophe” (Edinger and Thompson, 2004). Furthermore, PCD is a fundamental physiological phenomenon, as it is involved in controlling the proliferation and differentiation during development (e.g., mammalian organogenesis) and in the optimization of cell/tissue functions throughout adulthood. Recently, PCD has also been associated with pathological processes such as cancer (Jäättelä, 2004) and neurodegeneration (Mattson, 2000). The controlled type of cell death that occurs during development but also in some pathological events is in many cases performed by distinct molecular mechanisms and characterised by a particular morphology first described by Kerr and colleagues who established the term apoptosis (Kerr et al., 1972). Apoptosis “Απόπτωσις” in Greek means “falling off” in terms of leaves from trees, and this natural phenomenon can be seen as a symbol for the controlled clearance of cells during this process.

Light and electron microscopy have identified the various morphological changes that occur during apoptosis (Häcker, 2000; Elmore, 2007a). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Kerr et al., 1972). With cell shrinkage, the cells are smaller in size,
the cytoplasm is dense and the organelles progressively degenerate. Pyknosis is the result of chromatin condensation and this is one of the most characteristic features of apoptosis. The apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. Electron microscopy can better define the subcellular changes. Early during the chromatin condensation phase, the electron-dense nuclear material characteristically aggregates peripherally under the nuclear membrane although there can also be uniformly dense nuclei. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called “budding.” Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. Macrophages that engulf and digest apoptotic cells are called “tingible body macrophages”. The tingible bodies are the bits of nuclear debris from the apoptotic cells. There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003; Elmore, 2007).

The hallmark for apoptosis is the chromatin condensation and DNA fragmentation into internucleosomal fragments of multiples of approximately 180 basepairs (Wyllie, 1980; Wyllie et al., 1984) and/or high molecular weight fragments (HMW) of 50 or 300 kilobasepairs (Oberhammer et al., 1993). Yet another characteristic is the externalisation of the membrane lipid
phosphatidylserine, a process that appears to be crucial for the phagocytosis of the apoptotic cell (Fadok et al., 1992; Martin et al., 1995). Apoptosis is usually an active process that requires ATP and is in many cases dependent on mRNA and protein synthesis (Wyllie et al., 1984), whereas necrosis is passive. The differences between apoptosis and necrosis are summarised in Table 1.

In addition to these morphological and biochemical characteristics, apoptosis utilises a special molecular program, which is described below. However, some of the proteins involved in this program are also involved in necrosis. It should be noted that the border between apoptosis and necrosis is not always clear and in some cases the dying cells do not exhibit all features characteristic of one or the other.

Table 1: Characteristic features for apoptosis and necrosis

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological or pathological</td>
<td>Pathological</td>
</tr>
<tr>
<td>Cells shrinkage and formation of apoptotic</td>
<td>Cell swelling and bursting</td>
</tr>
<tr>
<td>bodies</td>
<td></td>
</tr>
<tr>
<td>No change in plasma membrane permeability</td>
<td>Increased plasma membrane permeability</td>
</tr>
<tr>
<td>Organelles intact</td>
<td>Organelle destruction</td>
</tr>
<tr>
<td>Nuclear condensation and fragmentation</td>
<td></td>
</tr>
<tr>
<td>DNA fragmentation in intranucleosomal fragments</td>
<td>Random DNA fragmentation</td>
</tr>
<tr>
<td>Externalisation of phosphatidylserine</td>
<td></td>
</tr>
<tr>
<td>Phagocytosis, no inflammation</td>
<td>Release of intracellular content, inflammation response</td>
</tr>
<tr>
<td>Active process, often requires RNA and protein synthesis</td>
<td>Passive process</td>
</tr>
</tbody>
</table>
**Classification of programmed cell death**

The existence of multiple types of PCD is well documented, but difficulties in reaching a consensus on the criteria used to distinguish them have hampered efforts to establish a universal classification (Sloviter, 2002; Golstein et al., 2003; Kroemer et al., 2005; Krantic et al., 2007). As mentioned above, the “programmed” aspect is clearly a PCD hallmark, but the term is often employed to refer to particular characteristics of the phenomenon. For instance, it is used to indicate the developmentally programmed occurrence of PCD, or to reflect the programmed stereotypical succession of morphological and biochemical events in non-developmental PCDs. However, the term programmed is generally employed as a synonym of regulated, leading to a broad classification of PCDs into apoptosis, necrosis, and autophagy (Golstein et al., 2003; Kroemer et al., 2005). One of the more restraining classifications, based on the criterion of nuclear morphology, divides PCDs into classical apoptosis, apoptosis-like PCD and necrosis-like PCD (Fig. 1). These are, respectively, characterized by “crescent-like” (type 2), partial/peripheral (type 1) or absent nuclear chromatin condensation (Leist and Jäättelä, 2001; Jäättelä and Tschopp, 2003; Lockshin and Zakeri, 2004; Krantic et al., 2005). The other common criteria used for classification are biochemical (based on the activation of a specific class of proteases) and pharmacological (based on the capacity of specific protease inhibitors to block a given type of PCD). Experimentally, however, these parameters have only been well documented for classical apoptosis, currently proposing nuclear morphology as the most accurate criterion. This issue, however, is still debated, as the understanding of apoptosis-like PCD is rapidly evolving despite the absence of selective pharmacological inhibitors of the proteases involved. In contrast, the knowledge about necrosis-like PCDs remains very limited, and will thus be considered together with apoptosis like PCD.
Fig. 1. Current PCD classifications: apoptosis, autophagy and paraptosis, the major types of regulated death, are subdivided according to morphological, pharmacological and biochemical criteria (Krantic et al., 2007).

**Classical apoptosis**

Classical apoptosis, the best known phenotypic expression of PCD, consists of at least two phases, initiation and execution, resulting from the activation of cystein-dependent, aspartate-directed proteases termed caspases. The death receptors (extrinsic) and mitochondrial (intrinsic) pathways represent the common routes of caspase activation during the initiation phase. The death receptors-mediated recruitment of procaspases-8 or procaspases-2/-10, as well as the mitochondrial pathway-induced activation of caspase-9 via cytochrome c release, both converge to the activation of procaspase-3 and of caspases-6 and -7, further downstream. These pathways are all associated with caspase activated DNase (CAD) activation, leading to “typical” inter-nucleosomal DNA
fragmentation (Hengartner, 2000). Considerable evidence suggests that the initiation of the intrinsic apoptotic pathway takes place in mitochondrial membranes, although it is now increasingly clear that this pathway can also be triggered at the level of other cellular organelles such as the endoplasmic reticulum, nucleus, and lysosomes. However, the involvement of these other organelles requires that extra-mitochondrial triggers first converge towards mitochondria (Danial and Korsmeyer, 2004). The intrinsic pathway is initiated by mitochondrial outer membrane permeabilization (MOMP) and the subsequent release of pro-apoptotic mitochondrial proteins into the cytoplasm.

**Apoptosis-like and necrosis-like PCDs**

Apoptosis-like and necrosis-like PCDs are often considered as alternative death programs, mainly because of their independence from caspases. Indeed, it is assumed that these PCDs can still occur when caspases are inhibited (Leist and Jäättelä, 2001; Jäättelä and Tschopp, 2003). Caspase inhibition can result, among other factors, from energy depletion (Leist et al., 1997), a mutation in the *Apaf-1* gene (Chautan et al., 1999) and nitrative/oxidative stress (Leist et al., 1997; Leist et al., 1999). Neurons are particularly sensitive to both energy depletion and oxidative stress. They depend entirely on the aerobic metabolism of glucose, which generates adenosine triphosphate (ATP) and reactive oxygen species (ROS) as by-products of incomplete oxygen reduction to water in the course of oxidative phosphorylation (Klein and Ackerman, 2003; Lang-Rollin et al., 2003; Rego and Oliveira, 2003). Considerable progress has recently been made in understanding the molecular mechanisms involved in apoptosis-like PCD, whereas much less is known about necrosis-like PCDs such as deaths involving autophagy and paraptosis. It has been reported that oncogenic *Ras* gene can trigger autophagic PCD (Chi et al., 1999). However, it appears that in addition to its involvement in a particular form of PCD (characterized
autophagy also plays a role in cell survival. It thus mediates a cytoprotective response in adverse conditions (e.g., low-nutrient states, pathological conditions) by catabolising intracellular substrates to produce energy, and by removing damaged organelles such as mitochondria to prevent apoptosis. Therefore, although autophagy by itself does not induce cell death, it is associated with PCD in particular conditions (e.g., when pro-apoptotic proteins Bax and Bak are down-regulated). The criteria for identifying this type of PCD were exclusively morphological. A similar death phenotype to autophagy is initiated after activation of neurokinin-1 receptor by its ligand substance-P (Castro-Obreón et al., 2002). This latter PCD, characterized by insensitivity to broad-range caspase inhibitors and extensive cytoplasmic vacuolation, have been designed as paraptosis (Castro-Obreón et al., 2002; Sperandio et al., 2004).

**Naturally occurring apoptosis**

The role of apoptosis in normal physiology is as significant as that of its counterpart, mitosis. It demonstrates a complementary but opposite role to mitosis and cell proliferation in the regulation of various cell populations. It is estimated that to maintain homeostasis in the adult animal body, around 10 billion cells are made each day just to balance those dying by apoptosis (Renehan et al., 2001). That number can be increased significantly when there is increased apoptosis during normal development and aging or during disease. Apoptosis is critically important during various developmental processes. As examples, both the nervous system and the immune system arise through the overproduction of cells. This initial overproduction is then followed by the death of those cells that fail to establish functional synaptic connections or productive antigen specificities, respectively (Nijhawan et al., 2000; Opferman and Korsmeyer, 2003). Apoptosis is also necessary to rid the body of pathogen
invaded cells and is a vital component of wound healing in that it is involved in the removal of inflammatory cells and the evolution of granulation tissue into scar tissue (Greenhalgh, 1998). Dysregulation of apoptosis during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis. Apoptosis is also needed to eliminate activated or auto-aggressive immune cells either during maturation in the central lymphoid organs (bone marrow and thymus) or in peripheral tissues (Osborne, 1996). Furthermore, with age some cells begin to deteriorate at a faster rate and are eliminated via apoptosis. One theory is that oxidative stress plays a primary role in the pathophysiology of age-induced apoptosis via accumulated free-radical damage to mitochondrial DNA (Harman, 1992; Ozawa, 1995). It is clear that apoptosis has to be tightly regulated since too little or too much cell death may lead to pathology, including developmental defects, autoimmune diseases, neurodegeneration, or cancer.

**Apoptosis in pathological conditions**

Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune lymphoproliferative syndrome, ischemia, and neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease. Previous, as well as recent studies relate also hypothyroidism with programmed cell death during brain development (Patel and Rabie, 1980; Rabie et al., 1980; Xiao and Nikodem, 1998; Ambrogini et al., 2005; Huang et al., 2005a; Huang et al., 2005b).

Some conditions feature insufficient apoptosis whereas others feature excessive apoptosis. Cancer is an example where the normal mechanisms of cell cycle regulation are dysfunctional, with either an overproliferation of cells and/or decreased removal of cells (King and Cidlowski, 1998). In fact, suppression of apoptosis during carcinogenesis is thought to play a central role
in the development and progression of some cancers (Kerr et al., 1994). There are varieties of molecular mechanisms that tumor cells use to suppress apoptosis. Tumor cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins such as Bcl-2 or by the down-regulation or mutation of pro-apoptotic proteins such as Bcl-2-associated-X-protein (Bax).

**Mechanisms of apoptosis**

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events (Fig. 2). To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). An additional pathway involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway.

This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent (apoptosis-like) cell death pathway via single stranded DNA damage (Martinvalet et al., 2005).

Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition that together result in the distinctive structural pathology described previously (Hengartner, 2000). Caspase (cysteine-dependent aspartate specific proteases) are the key players in apoptosis. Caspase-3 is considered the most important of
the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD. In proliferating cells, CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Sakahira et al., 1998). Caspases are widely expressed in an inactive proenzyme form in most cells and each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase-8, -9, and -10, which in turn will activate the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages, once activated can often activate other pro-caspases, allowing initiation of a protease cascade. Some pro-caspases can also aggregate and autoactivate. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signalling pathway and thus leads to rapid cell death. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighbouring amino acids. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. To date, ten major caspases have been identified and broadly categorized
Fig. 2. Schematic representation of apoptotic events. The main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (Elmore, 2007).

into: initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997; Rai et al., 2005). The other caspases that have been identified include caspase-11, which is reported to regulate apoptosis and cytokine maturation during septic shock, caspase-12, which mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β (Ab), caspase-13, which is suggested to be a bovine gene, and caspase-14, which is highly expressed in embryonic tissues but not in adult tissues (Hu et al., 1998; Nakagawa et al., 2000; Koenig et al., 2001; Kang et al., 2002). Extensive protein cross-linking is another characteristic of apoptotic cells...
and is achieved through the expression and activation of tissue transglutaminase (Nemes Jr et al., 1996). DNA breakdown by Ca2+- and Mg2+-dependent endonucleases also occurs, resulting in DNA fragments of 180 to 200 base pairs (Bortner et al., 1995).

**Extrinsic Pathways**

The extrinsic signalling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain” (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways. In these pathways, there is clustering of receptors and binding with the homologous trimeric ligand. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors.

**Intrinsic Pathways**

The intrinsic signalling pathways that initiate apoptosis involve a diverse array of non-receptor mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals include the absence of certain growth factors, hormones, and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. In other words, there is the withdrawal of factors, loss of apoptotic suppression, and subsequent activation of apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition
MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens et al., 2004). The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (Cory and Adams, 2002). The members of the Bcl-2 family of proteins govern mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic (Li et al., 1998; Esposti, 2002).

**Execution Pathway**

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution caspases that begins this phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effectors or “executioner” caspases, cleaving various substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee et al., 2001). Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD (Sakahira et al., 1998).

**Apoptosis in the central nervous system**

Apoptosis plays a major role in the differentiation and development of CNS (Oppenheim et al., 1992; Johnson and Deckwerth, 1993; Raff et al., 1993). Data from basal forebrain, striatum, and dorsal lateral geniculate nucleus showed that apoptotic cells were present mainly during the first two weeks of postnatal development, when neurons accomplish their morphological and neurochemical differentiation and establish their connections. Apoptosis in all these areas is caused by activation of active caspase-3. These findings suggest that apoptosis is
temporally coordinated with neuronal maturation and may play a critical role in the establishment of the cytoarchitecture and connectivity in the CNS (Sophou et al., 2006; Mellios et al., 2009; Zacharakia et al., 2010). Apoptosis in other areas such as the cerebral cortex, the thalamus, and the substantia nigra occurs within the first month of postnatal development, being more intense during the first postnatal week (Ferrer et al., 1994; Spreafico et al., 1995; Burke, 2004; Mellios et al., 2009). In the septum, the loss of cells due to apoptosis, which is partially caused by activation of caspase-3, occurs during postnatal development, with regional differences (Venero et al., 1999). Programmed death of supernumerary neurons occurring around the time when the neurons are making functional connections, abrogated by mRNA and protein synthesis inhibitors, is indicative of an activation of a specific genetic program (Martin et al., 1988).

The dependence of developing neurons on afferent and efferent connections for survival and phenotypic maturation was investigated in several models of lesion-induced apoptotic cell death in the basal forebrain (BF), the striatum and the dorsal lateral geniculate nucleus (dLGN) (Sophou et al., 2006; Mellios et al., 2009; Zacharakia et al., 2010). The lesions were preformed at different postnatal ages within the time window of naturally occurring apoptosis and involved ablations of the cerebral cortex, which is a major source of trophic factors, destruction of monoaminergic afferent pathways with the selective neurotoxin 6-OHDA and deprivation of the dorsal lateral geniculate nucleus from its retinal afferents by eye enucleation. The results showed that while lesions of the cerebral cortex did not induced significant changes in the frequency of apoptotic neurons in the BF, they resulted in phenotypic changes of BF cholinergic neurons, as atrophy and down regulation of choline acetyltransferase (ChAT) expression (Sophou et al., 2006). On the contrary, in the striatum, lesions of the cerebral cortex resulted in a significant increase in
the frequency of apoptotic neurons with a peak seven days postlesion. 6-OHDA lesions of the monoaminergic afferents elicited a similar response with respect to the magnitude and peak stage of apoptosis of striatal neurons. These responses were attributed mainly to the loss of striatal neurons expressing GABA or ChAT, since their frequency was significantly lowered in lesioned compared to control animals (Mellios et al., 2009). In the dLGN, lesions of the visual cortex resulted in massive apoptosis one day postlesion, that led to the elimination of the nucleus. Following eye enucleation, apoptosis in the dLGN also showed a peak one day postlesion, but was relatively restricted (Zacharaki et al., 2010).

On the other hand, many studies suggest that apoptosis is involved in the pathogenesis of acute and chronic neurodegenerative disorders such as Alzheimer, Parkinson, and Huntington disease (Barinaga, 1998; Burke and Kholodilov, 1998; Ekshyyan and Aw, 2004). Data showed that the neurotoxic deposition of the protein beta-amyloid (Ab) leading to Alzheimer’s disease within specific areas of the brain including the septum causes apoptosis of neurons (Schliebs, 2005; Jarvis et al., 2007). Evidence suggests that gene expression is required for neuronal death (Oppenheim et al., 1990; Comella et al., 1994).

The lack of THs in early life has a marked effect on the development of the rat cerebellum (Legrand, 1979, 1986). In the cerebellum, the magnitude of apoptosis of hypothyroid rats reached a peak at 8 days postnatal, but was four times higher than in control animals (Xiao and Nikodem, 1998). In humans, the lack of adequate levels of THs during the critical period of development, results in cretinism, a syndrome of severe mental retardation often accompanied by growth retardation and/or neurological deficits (Chen and Xiao, 1992).

Finally, apoptosis occurs in the striatum and the cerebral cortex after transient ischemia (Li and Zuo, 2009). A gradual increase in the density of
apoptotic TUNEL+ nuclei was also observed in the anterior cingulate and retrosplenial areas of the cortex, the striatum, and the CA1 area and dentate gyrus of the hippocampus over the first 24h post-N-methyl-D-aspartate (NMDA) or kainate injection (Van Lookeren Campagne et al., 1995).

**Anatomy of the brain areas selected for investigation**

**The striatum**

The striatum (STR) is part of the basal ganglia. It receives its name from its striated-like appearance created by the dense axon bundles of the internal capsule, which pass through the nucleus. The striatum comprises the caudate nucleus, the putamen and the nucleus accumbens (Fig. 3) (Gerfen, 2004). In primates, the striatum is structurally divided into the caudate and putamen nuclei by the fibres of the internal capsule, whereas in rodents these structures are not differentiated and are collectively referred to as the caudate-putamen (CPu) or neostriatum (Kandel et al., 2000). The striatum of both primates and rodents contains a rostroventral extension called the nucleus accumbens (Nauta, 1979). The components of the basal ganglia include also the globus pallidus (lateral segment), the subthalamic nucleus, the medial globus pallidus and the substantia nigra (Braus et al., 2001). They are a group of interconnected subcortical nuclei that play a major role in the control of voluntary movement (Alexander et al., 1986; Albin et al., 1989), but are also involved in a variety of non-motor functions including cognition, memory and emotion (Middleton and Strick, 2000; Packard and Cahill, 2001). It has primary roles in reward mechanisms, subserving, addiction, and craving as well as involvement in motor planning (Sullivan et al., 2005).
Neuronal types

Neurons of the striatum, are distinguished in the medium spiny projection neurons and the interneurons (Kawaguchi et al., 1995). The vast majority of the striatal neurons are the medium spiny projection neurons, which represent the 90-95% of the total neuronal population of the nucleus (Kemp and Powell, 1971; Groves, 1983; Graveland and Difiglia, 1985). The remaining 5-10% of the total population of neurons are interneurons of the aspiny type (Graveland and Difiglia, 1985; Kawaguchi et al., 1995).

The medium spiny projection neurons take their name from their morphological appearance, with a cell body approximately 20-25 μm in diameter, from which radiate 7-10 moderately branched dendrites that are densely laden with spines. They use γ-aminobutyric acid (GABA) as their major neurotransmitter and are divided into two major subpopulations, based on their primary sites of axonal projection and neuropeptide synthesis (Gerfen, 1992b; Smith et al., 1998; Bolam et al., 2000). One subpopulation (referred to as striatonigral), which projects preferentially to the output nuclei of the basal ganglia (the substantia nigra pars reticulata and the entopeduncular nucleus/globus pallidus internal segment), expresses, in addition to GABA, the neuropeptides substance P and dynorphin, bears the D1 subtype of dopamine receptors, and give rise to the “direct” output pathway of the basal ganglia. Striatonigral neurons also send projections to the dopaminergic neurons of the substantia nigra pars compacta (Nauta and Domesick, 1984; Gerfen, 1992b; Mendez et al., 1993). The second subpopulation (striatopallidal), which projects almost exclusively to the globus pallidus external portion, expresses the neuropeptides enkephalin and the D2 dopamine receptor subtype (Anderson and Reiner, 1990; Gerfen et al., 1990), and forms the “indirect” output pathway of the basal ganglia. Medium spiny projection neurons receive inputs from the cortex, thalamus, and amygdala, which make asymmetric synapses on dendritic
spines and, to a lesser degree, dendritic shafts. These glutamate inputs provide the major excitatory input to medium spiny neurons. In addition, medium spiny neurons receive a number of inputs from outside of the striatum and from within the striatum. These include dopamine inputs from the substantia nigra, inhibitory GABA inputs from the axon collaterals of other spiny neurons, inhibitory inputs from GABA and peptide containing striatal interneurons, and inputs from cholinergic striatal interneurons. Other inputs to medium spiny neurons, comprise GABAergic inputs from the lateral globus pallidus, inputs from the subthalamic nucleus, serotonergic inputs from the dorsal raphe and noradrenergic inputs from the locus coeruleus (for more details see Gerfen, 2004).

γ-Aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the mammalian central nervous system. It plays an important role in regulating neuronal excitability throughout the nervous system (Watanabe et al., 2002). In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neuronal processes. In contrast, GABA exhibits excitatory actions in insects, mediating muscle activation at synapses between nerves and muscle cells, and also the stimulation of certain glands. In mammals, some GABAergic neurons, such as chandelier cells, are also able to excite their glutamatergic counterparts (Szabadics et al., 2006). GABA regulates the proliferation of neural progenitor cells (LoTurco et al., 1995; Antonopoulos et al., 1997; Haydar et al., 2000), the migration (Behar et al., 1998) and differentiation (Barbin et al., 1993; Ganguly et al., 2001) the elongation of neuraxon and the formation of synapses. GABA can influence the development of neural progenitor cells via brain-derived neurotrophic factor (BDNF) expression (Obrietan et al., 2002).
Fig. 3. Drawing of a selected coronal brain section taken from Paxinos and Watson Atlas of the Rat Brain. Abbreviations: Aca, anterior commissure, anterior part; AcbC, accumbens nucleus, core; AcbSh, accumbens nucleus, shell; acer, anterior cerebral artery; AID, agranular insular cortex, dorsal part; AIV, agranular insular cortex, ventral part; cc, corpus callosum; Cg, cingulate cortex; Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2; Cpu, caudate putamen (striatum); DCI, dorsal part of claustrum; Den, dorsal endopiriform nucleus; DI, dysgranular insular cortex; E, ependyma and subependymal layer; ec, external capsule; GI, granular insular cortex; IC; inferior colliculus; ICj, islands of Calleja; ICjM, islands of Calleja, major island; IEn, intermediate endopiriform nucleus; IG, indusium griseum; lo, lateral olfactory tract; LacbSh, lateral accumbens shell; LSD, lateral septal nucleus, dorsal part; LSI, lateral septal nucleus, intermediate part; LSS, lateral stripe of the striatum; LSV, lateral septal nucleus, ventral part; LV, lateral ventricle; M1, primary motor cortex; M2, secondary motor cortex; mfb, medial forebrain bundle; MS, medial septal nucleus; Pir1, piriform cortex, layer 1; rf, rhinal fissure; S1DZ, primary somatosensory cortex; dysgranular zone; S1DZO, primary somatosensory cortex, oral dysgranular zone; S1FL, primary somatosensory cortex, forelimb region; SIJ, primary somatosensory cortex, jaw region; S1ULp, primary somatosensory cortex, upper lip region; Shi, septohippocampal nucleus; Tu1, olfactory tubercle, layer 1; VCI, ventral part of claustrum; VDB, nucleus of the vertical limb of the diagonal band; VP, ventral pallidum. (Paxinos and Watson, 1986).

Striatal interneurons, which extend axons within the striatum, have been divided into two major classes according to cytochemical, physiological and morphological criteria (Kawaguchi et al., 1995; Kawaguchi, 1997): (1) the large aspiny cholinergic interneurons, identifiable by the presence of choline acetyltransferase and (2) the medium aspiny GABAergic interneurons, of which there are several varieties.

Striatal cholinergic interneurons have a very large cell body, up to 40 μm in diameter from which extend long aspiny dendrites, which may split into secondary and tertiary branches. Cholinergic neurons extend an extremely fine and extensive axon. These neurons receive asymmetric glutamatergic inputs
from the cortex and the thalamus (Wilson et al., 1990; Lapper and Bolam, 1992). They receive also symmetric inputs from axons containing substance P (Bolam et al., 1986). Acetylcholine is important to striatal function, as it affects the information flux from the cerebral cortex to the medium spiny neurons.

Medium aspiny GABAergic interneurons are subdivided on the patterns of coexpression of neuropeptides into three subclasses: (1) Parvalbumin striatal interneurons. This is the largest population of GABAergic striatal interneurons as they represent the 1% of all striatal neurons (Kawaguchi et al., 1995). This type of neurons is often referred to as a fast-spiking interneuron. These neurons receive inputs from the cerebral cortex and provide inputs to medium spiny projection neurons. Although, distributed throughout the striatum parvalbumin neurons are more frequent in the dorsolateral region of the striatum (Kawaguchi et al., 1995). (2) Calretinin/GABA interneurons. This type of striatal GABAergic interneurons coexpresses the calcium-binding protein calretinin (Bennett and Bolam, 1993). Calretinin protects striatal neurons from the overproduction of calcium, which is involved in the pathogenesis of Huntington disease (Cicchetti et al., 2000; Lévesque et al., 2003). Calretinin GABA interneurons are distributed mainly in the dorsal medial striatum. (3) Somatostatin/NPY striatal interneurons. This type of GABAergic interneurons coexpresses somatosatatin, neuropeptide Y, or nitric oxide synthetase (NOS)(Vincent et al., 1983a; Vincent et al., 1983b; Chesselet and Robbins, 1989; Dawson et al., 1991). These neurons may be visualized by NADPH-diaphorase histochemical staining. Nitric oxide synthase is responsible for nitric oxide synthesis, which plays role in the development and plasticity of the CNS (Murata and Masuko, 2003). These interneurons are distributed in the ventral part of the striatum; a subset of these neurons (about 20%) expresses the calcium-binding protein calbindin (Gerfen, 2004).
Despite the low number of interneurons in striatum, their important functional role in striatal physiology has become increasingly apparent in recent years. It has been shown that GABA interneurons receive cortical inputs (sensory and motor), and have as principal target striatal medium spiny projection neurons. They are therefore the principal source of local inhibition in the striatum. Possible roles of this inhibition, although relatively unknown, may be to modulate the cortical excitation of spiny neurons, and/or to synchronize the activity of these cells (Bolam et al., 2000).

Aside from being classified as part of the “direct” or “indirect” pathway, the striatal medium spiny neurons can also be classified as being part of the “patch” (also referred to as the striosomes) or “matrix” compartment. This classification originates from earlier studies, which have divided the striatum into two compartments, with different neurochemical markers and levels of μ-opioid binding (Graybiel et al., 1990; Gerfen, 1992a). For instance, neurons which lie in the striatal matrix have high acetylcholinesterase activity, low μ-opioid receptor binding, contain the calcium binding protein calbindin, and have rich somatostatin-immunoreactivity (Gerfen, 1992a). On the other hand, neurons in the patch compartment have high μ-opioid receptor binding and low acetylcholinesterase activity (Herkenham and Pert, 1982; Gerfen, 1992a). There is no preferential distribution of medium spiny projection neurons and interneurons in any of the two compartments, however, the patch and the matrix differ in their afferents and efferents (Gerfen, 1992b). For example, although striatonigral neurons from both compartments project to the substantia nigra, those originating in the patches, innervate the dopaminergic (DA) neurons of the ventral tier of the substantia nigra pars compacta and DA cell islands in the substantia nigra pars reticulata, whereas striatonigral neurons originating in the matrix, target GABA-ergic neurons in the substantia nigra pars reticulata (Gerfen, 1984, 1992b).
**Connections of the striatum**

**Afferents**

The major afferent inputs to the striatum arise from the cerebral cortex. All areas of the cortex project to the striatum in a topographical manner, and all striatal areas receive glutamatergic cortical innervation (Smith et al., 1998; Bolam et al., 2000; Gerfen, 2004). All areas of the striatum also receive DA inputs. In general it is agreed that DA neurons from the substantia nigra pars compacta innervate the caudate-putamen (neostriatum), while the nucleus accumbens receives projections from DA cells in the ventral tegmental area, although there seem to be areas (medial striatum) where the two inputs overlap. The nigrostriatal dopaminergic input is also compartmentalized. DA neurons from the ventral tier of the substantia nigra pars compacta project heavily to patch compartments, whereas, DA neurons in the ventral tegmental area and the dorsal tier of substantia nigra pars compacta innervate the matrix compartments (Gerfen, 1992b). A third major source of striatal afferents arises from the thalamus. The caudate-putamen receives glutamatergic projections from the intralaminar thalamic nuclei (centromedian-parafascicular and rostral nuclei). The striatum is also innervated by serotonergic fibers from the dorsal raphe nucleus (Geyer et al., 1976), which are mostly concentrated in the caudal aspects of the striatum (Ternaux et al., 1977), and noradrenergic inputs from the locus coeruleus (for more details see Gerfen, 2004). Cortical and thalamic afferents to the striatum follow the patch/matrix boundaries, while the serotonergic innervation does not (Okumura et al., 2000).

**Efferents**

A first subpopulation of the medium spiny projection neurons, the striatonigral neurons, preferentially projects to the output nuclei of the basal ganglia, which are the substantia nigra pars reticulata and the entopeduncular nucleus/globus pallidus internal segment. These neurons give rise to the “direct” output pathway of the basal ganglia. Striatonigral neurons also send projections to the
A second subpopulation of the medium spiny projection neurons, the striatopallidal neurons, projects almost exclusively to the globus pallidus external portion (Anderson and Reiner, 1990; Gerfen et al., 1990). These neurons form the “indirect” output pathway of the basal ganglia.

**Striatal receptors**
The most abundant and widespread dopamine receptor subtype is the D1 receptor (Jaber et al., 1996). D1 receptors are heavily expressed in the striatum, as evidenced by mRNA detection and binding studies (Jaber et al., 1996; Vallone et al., 2000). In the rat striatum, D1 receptor-immunoreactivity is present at birth, and is concentrated in the striosomes. The density of D1 receptors in the matrix increases with age (beginning at postnatal day 2), and at two weeks postnatally the labelling has a homogeneous distribution between the two compartments, similar to that seen in the adults (Murrin and Zeng, 1989; Caille et al., 1995). The D2 receptor is concentrated in the striatum (Jaber et al., 1996; Vallone et al., 2000).

**Development of the striatum**
Cells of the striatum arise from the lateral ganglionic eminence of the telencephalic neuroepithelium (Deacon et al., 1994; Bhide 1996). Neurogenesis in the striatum begins on E12 and continues throughout embryonic life (Lauder et al., 1986). Large cholinergic neurons in the striatum are produced between E12 and E17, with the majority being born between E13 and E15. Cholinergic neurons appear to be among the earliest cells produced in the striatum. Their generation peak is at E13 caudally, and at E15 rostrally (Phelps et al., 1989). The medium-spiny neurons in the neostriatum are generated mainly from E12 to
E21-E22. There are several neurogenetic gradients among these neurons (Bayer, 1984). The most prominent gradient is that the ventrolateral neurons are older than the dorsomedial neurons. There are also divergent neurogenetic gradients between anterior and posterior parts of the striatum. There is also a neurogenetic gradient between patch neurons and matrix neurons throughout the striatum (Bayer, 1984). Striatal neurons show compartmental preferences, depending on the time that they leave the mitotic cycle. The earliest born striatal neurons (between E12-E16) are restricted mainly to the patch compartment, while neurons that are born later (between E18-E22) end up primarily in the matrix compartment (Fishell and Van der Kooy 1990; Marchand and Lajoie, 1986; Van der Kooy and Fishell, 1987). Striatal GABAergic interneurons that express parvalbumin or calretinin are born between E13 and E20, with the majority between E14 and E17 (Sadikot and Sasseville, 1997; Rymar et al., 2004). The somatostatin interneurons are born between E15 and E16 (Semba et al., 1988).

The maturation of striatal neurons undergoes a prolonged period, which lasts until the end of the fourth postnatal week, with patch neurons to precede and matrix neurons to follow (Phelps et al., 1989; Tepper et al., 1998). All these cells are the main targets of dopaminergic afferents, which are present in the striatum from E18. The dopaminergic innervation of the nucleus is completed by P4 (Kalsbeek et al., 1992). The postsynaptic targets of dopaminergic inputs in the neostriatum during the first two postnatal weeks are mainly dendrites and cell somata, while during the third postnatal week, period in which medium spiny neurons show the highest density of spines, the axospinous dopaminergic synapses increase too. These findings suggest that the development of the dopaminergic system in the striatum is an active process that parallels the morphological alterations of striatal neurons. In addition, the fact that dopaminergic axons contact different parts of the postsynaptic neurons,
depending on age, implies that dopamine may contribute to the development and maturation of the striatum (Tepper et al., 1998; Antonopoulos et al., 2002).

The septum

The septum (Fig. 3) is determined as an area which is located between the anterior horns of the lateral ventricles (septum = from Latin, saeptum: a dividing medial interventricular wall of the telencephalon). The first detailed description of the anatomy of the septum was provided in 1901 by Cajal, contrasting to the earlier notion that it is a specialized part of the cerebral cortex. Until present, a general agreement about the classification or the exact boundaries of the septum has not been reached. What is generally accepted is that the septum can be viewed as an interface or a relay station between diencephalon and telencephalon. It is assumed to maintain the balance between the endocrine and emotional components of the central nervous system.

The septum lies beneath the genu of the corpus callosum, and separates the right and left lateral ventricles. It is involved in the control of a variety of physiological and behavioral processes related to higher cognitive functions, emotions, fear, aggression, and stress, as well as autonomic regulations (Jakab and Leranth, 1995). On the basis of topography, cytoarchitecture, and connections, this area of the basal forebrain is parceled into four major groups: (1) the lateral septal nucleus (LS) and the septofimbrial and septohippocampal nuclei, (2) the medial septum-diagonal band of Broca (MSDB), (3) the bed nuclei of the anterior commissure and of the stria medullaris and the triangular nucleus and (4) the bed nuclei of the stria terminalis (Risold, 2004). The LS primarily mediates descending limbic cortical pathways to diencephalic areas, whereas the MSDB mainly relays ascending pathways to telencephalic areas (Swanson and Cowan, 1979; Jakab and Leranth, 1995; Antonopoulos et al.,
The LS is the largest nucleus of the septum. It is subdivided into three main parts: the dorsal (LSD), the intermediate (LSI) and the ventral (LSV) part. The MSDB is subdivided into the medial septal nucleus (MS) and the nucleus of the diagonal band of Broca (DB). DB is further composed of two parts: the horizontal limb of DB (hDB) and vertical limb of DB (vDB) (Swanson and Cowan, 1979). (Fig. 3).

The LS, which is involved in limbic activity, including learning and memory (Urban et al., 1995; Jaffard et al., 1996), contains neurons producing various neurotransmitters and neuropeptides, such as opioid peptides, excitatory aminoacids and GABA (Jakab and Leranth, 1995; Risold and Swanson, 1997; Tsukahara and Yamanouchi, 2003). It also contains high levels of brain-derived neurotrophic factor protein, suggesting that this neurotrophin is transported into the lateral septum via afferent nerve fibres arising from neurons elsewhere in the brain (Conner et al., 1997). Induced dysfunction of the rat lateral septum causes hyperirritability and hyperaggressiveness (Albert and Wong, 1978; Albert, 1980).

**Neuronal types**

Several cell types have been described in the LS after Golgi impregnation (Alonso and Frotscher, 1989a, 1989b; Jakab and Leranth, 1995). The dendrites of all cell types are covered with spines. Some of them also exhibit somatic spines and they have been called “somatospiny neurons” (Jakab and Leranth, 1990a, 1990b). No strictly local interneurons have been described in the lateral septum, but axons of some neurons in dorsal parts of the nucleus exhibit local collaterals, suggesting their involvement in local inhibition circuits (Phelan et al., 1989). At least five cell types have been described in the MSDB (Brauer et al., 1988; Dinopoulos et al., 1988; Jakab and Leranth, 1995). Most of them are
dendritic spine free. Some of these cells, characterized by their large size and dark thionine stain, correspond to the cholinergic neurons.

All nuclei of the septum are rich in GABAergic neurons, that express glutamic acid decarboxylase (Kohler and Chan-Palay, 1983; Panula et al., 1984; Onteniente et al., 1986; Feldblum et al., 1993; Risold et al., 1997). GABAergic neurons do not form a homogeneous population and non-GABAergic neurons containing other neurotransmitters are found in the septal region. GABAergic neurons in the LS have been found to contain excitatory aminoacids and many neuropeptides mainly somatostatin, encephalin, and neurotensin (Jakab and Leranth, 1995). Neurons in the MSDB contain a large variety of neurotransmitters, including glutamate (Manns et al., 2001), enkephalin (Kivipelto and Panula, 1986), vasoactive intestinal peptide, dynorphyn-B, substance P, neurotensin, somatostatin, neuropeptide Y and calcitonin-gene-related peptide (Köhler and Eriksson, 1984). However, the cholinergic and GABA-ergic neurons represent the largest neuronal populations in the MSDB, and both send numerous projections to hippocampus. It was estimated that in the MSDB 40-78% of the neurons are cholinergic (Amaral and Kurz, 1985; Kiss et al., 1999), and 30-50% are GABA-ergic (Köhler and Eriksson, 1984; Kiss et al., 1990).

**Connections of the septum**

**Afferents**

Nuclei of the lateral group are characterized by massive glutamatergic afferents from the pyramidal cell layers of the Ammon’s horn and subiculum and by massive bidirectional connections with the hypothalamus and the ventral midbrain (Swanson and Cowan, 1979; Swanson et al., 1987; Risold and Swanson, 1997). One of the major sources of septal afferents is the supramammillary area (Leranth and Kiss, 1996). The LS receives also a wealth
of serotonergic, dopaminergic and noradrenergic afferents. These afferents are distributed in all subdivisions of the LS, with the LSI and LSV being more densely innervated (Dinopoulos et al., 1993; Antonopoulos et al., 1997; Antonopoulos et al., 2004). Finally, LS receives abundant vasopressin and galanin projections from the medial amygdala and the bed nuclei of the stria terminalis (for review see the article of Risold, 2004). On the other hand, the lateral septal nucleus is viewed as a medial component of the striatum, as it receives abundant glutamatergic afferents from the cortex (hippocampal formation) that innervate GABAergic spiny neurons in this structure (Risold et al., 1997; Swanson and Petrovich, 1998; Swanson, 2000; Swanson and Risold, 2000).

**Efferents**

Axons from the LS innervate several nuclei of the thalamus the hypothalamus, and the ventral midbrain (for review see the article of Risold, 2004). Most of the projections to the thalamus are found in midline nuclei, in particular the nucleus reuniens, the thalamic paraventricular nucleus, and the paratenial nucleus (Staiger and Nurnberger, 1991; Risold and Swanson, 1997). Lateral septal nucleus also projects heavily in the entorhinal cortex and the hippocampal formation (temporal CA1 field, and ventral subiculum) (Risold and Swanson, 1997). Finally, a small input from the LS has been described in the lateral habenular nucleus (Staiger and Nurnberger, 1991; Risold and Swanson, 1997).

**Development of the septum**

The lateral and medial septal nuclei evolve from the septal ridge, which forms the rostroventromedial wall of the telencephalic vesicle (Alvarez-Bolado et al., 1995; Swanson and Risold, 2000). The fusion of the right and left septal regions appears at E15 (Bayer, 1979a, 1979b). The source of most neurons in the septum is the neuroepithelium lining the ventromedial wall of the lateral ventricle.
Septal neurons are generated from E12 to E18, with the neurons of the lateral septal nucleus being born between E14 and E18 (Swanson and Cowan 1976; Bayer, 1979a; Alvarez-Bolado et al., 1995). The fiber tracts that originate in the hippocampus and innervate the lateral septum appear at E16 (Linke et al., 1995); the serotonergic fibers appear at E15/16 (Wallace and Lauder, 1983, 1992) and develop later, from E18 onwards, following a medial to lateral gradient (Dinopoulos et al., 1993) and the dopaminergic and noradrenergic fibers, which are sparse at birth, complete their distribution and density within the first three postnatal weeks (Antonopoulos et al., 1997, 2004). The differentiation and maturation of lateral septal neurons begin as hippocampal afferents enter the nucleus (Bayer, 1979b) and continue until P6 (Verney et al., 1987). Albeit, monoamine systems, which innervate early the lateral septum are likely to influence its development before they assume a neurotransmitter role (Osterheld-Haas et al., 1994; Levitt et al., 1997; Weis et al., 1998; Kulkarni et al., 2002).
Materials and methods

In this work, we studied the effect of hypothyroidism on apoptosis in forebrain structures. We used normal and hypothyroid animals and we followed the TUNEL method (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling) to detect dying cells; then we examined their apoptotic morphology with light microscopy. Moreover, we used immunohistochemistry to detect the expression of the apoptotic marker active caspase-3. Immunohistochemistry for GABA was used in the striatum to examine the effect of hypothyroidism on the survival and phenotype of the majority of striatal neurons. For quantitative analysis, we also used cresyl violet staining (Nissl staining technique) to determine the number of “live” cells in the striatum and the lateral septum of normal and hypothyroid animals.

Induction of hypothyroidism

Inhibition of thyroid hormone synthesis occurred by a group of thioureylene antithyroid drugs [2-mercapto-1-methylimidazole, methimazole (MMI); propylthiouracil (PTU); thiourea or carbimazole]. MMI was originally reported to have a three times greater placental transfer than PTU (Marchant et al., 1977). These drugs interfere with the conversion of iodide to iodine and consequently the iodination of tyrosyl groups. The tyrosyl groups are also inhibited from joining to form T3 and T4.

Congenital hypothyroidism in a first group of animals was pharmacologically-induced by giving dams 0.025% w/v MMI in their drinking water from the 9th gestational day and afterwards until the neonatal rats were sacrificed. The day on which a vaginal plug was found in maternal rats was
considered as gestational day 1. After weaning, postnatal day (P) 20, the neonates continued to receive MMI directly through drinking water until sacrifice, on P60. Acquired hypothyroidism in a second group of animals was pharmacologically induced by 4.5 weeks of treatment with 0.025% w/v MMI in their drinking water, from P35 until sacrifice, on P70, according to the protocol of Constantinou and colleagues (Constantinou et al. 2005).

The effectiveness of MMI treatment was confirmed by the determination of the serum T3 and T4 with radioimmunoassay in both male and female animals by Constantinou and colleagues. The specific radioimmunoassay kits (T3-RIA and T4-RIA) were supplied from the Hellenic Centre of Natural Research “Demokritos”.

**Animals**

Wistar albino rats of both sexes (n = 101) were used in this study. All animals were bred in the laboratory of Anatomy, Histology, and Embryology of the Faculty of Veterinary Medicine. Animals were treated according to the standards of the international statutes on animal handling (86/609/EEC), exposed to regular light–dark cycle (light period: 7:00 a.m. to 7:00 p.m.; dark period: 7:00 p.m. to 7:00 a.m.; at 22±1°C), housed four per cage and given laboratory chow and water *ad libitum*. The breeding was approved by the Prefecture of Thessaloniki, Veterinary Directorate.

Fifty-one animals with congenital pharmacologically induced hypothyroidism were used for the study of apoptosis, active caspase-3 immunohistochemistry and GABA immunohistochemistry. Six animals (n = 6) were used in each of the following ages: P0 (day of birth), P3, P5, P7; three animals (n = 3) in each of ages P10, P14 and P21, and eighteen adult animals (n = 18) at P60.
Fourteen adult animals (n = 14) with acquired pharmacologically induced hypothyroidism at P70 were used for the same purpose.

Thirty-six normal animals were used as control. Three animals (n = 3) were used in each of the following ages: P0, P3, P5, P7, P10, P14, P21 and fifteen adult animals at P60 (n=7) and at P70 (n=8).

All animal groups used for the different experimental procedures are summarized in Table 2.
Table 2. Animal groups used for different experimental procedures

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>TUNEL method (paraffin sections 10 μm)</th>
<th>Active caspase-3 immunohistochemistry (paraffin sections 10 μm)</th>
<th>GABA immunohistochemistry (paraffin sections 10 μm)</th>
<th>Nissl staining (paraffin sections 10 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animals</td>
<td></td>
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<td></td>
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<tr>
<td>(n = 36)</td>
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<tr>
<td>P 0</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>P 3</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P 5</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>P 7</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>P 10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P 14</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P 21</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P 60 &amp; P70 (adult)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Congenital hypothyroidism</td>
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<tr>
<td>(n = 51)</td>
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</tr>
<tr>
<td>P0</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P3</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P5</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>P7</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>P10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P14</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P21</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P60 (adult)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Acquired hypothyroidism</td>
<td></td>
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<tr>
<td>(n = 14)</td>
<td></td>
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<td></td>
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<tr>
<td>P70 (adult)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Tissue preparation

Animals were deeply anaesthetized with ether and then were fixed with 4% w/v paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, by transcardiac perfusion using a “Harvard apparatus” pump. The pump flow rate, syringe needle size and fixative volume for each animal age are shown in Table 3.

Table 3. Overview of pump flow rate, syringe needle size and fixative volume for each animal age

<table>
<thead>
<tr>
<th>Age</th>
<th>Pump flow rate (ml/min)</th>
<th>Syringe needle size (gauge)</th>
<th>Fixative volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>26</td>
<td>24</td>
<td>200-250</td>
</tr>
<tr>
<td>P3</td>
<td>30</td>
<td>24</td>
<td>200-250</td>
</tr>
<tr>
<td>P5</td>
<td>40</td>
<td>24</td>
<td>250</td>
</tr>
<tr>
<td>P7</td>
<td>45</td>
<td>24</td>
<td>250</td>
</tr>
<tr>
<td>P10</td>
<td>28</td>
<td>21</td>
<td>300</td>
</tr>
<tr>
<td>P14</td>
<td>28</td>
<td>21</td>
<td>300</td>
</tr>
<tr>
<td>P21</td>
<td>40</td>
<td>19</td>
<td>300</td>
</tr>
<tr>
<td>P60</td>
<td>60 blunt tip</td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>P70</td>
<td>60 blunt tip</td>
<td></td>
<td>400</td>
</tr>
</tbody>
</table>

Brains destined for TUNEL processing, active caspase-3 and GABA immunohistochemistry were removed, postfixed in the same fixative for 2 hours, and then transferred into PB at 4° C. They were then dehydrated through an ascending series of ethanol from 50% v/v to 100% v/v, followed by chloroform, a mixture of chloroform and paraffin 1:1, and finally embedded in paraffin wax. The procedures and the time needed for brain samples to remain in dilutions during their dehydration are shown in Table 4.
Table 4. Overview of procedures and time needed brain samples to remain in dilutions during their dehydration

<table>
<thead>
<tr>
<th>Age</th>
<th>Alcohol 50% (min)</th>
<th>Alcohol 70% (min)</th>
<th>Alcohol 95% (min)</th>
<th>Alcohol 100% (min)</th>
<th>Chloroform 100% (min) 1</th>
<th>Chloroform 100% (min) 2</th>
<th>Mixture of chloroform &amp; paraffin 1:1 (min)</th>
<th>Paraffin wax (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>45</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>180</td>
</tr>
<tr>
<td>P3</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>45</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>180</td>
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<tr>
<td>P5</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>45</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>180</td>
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<tr>
<td>P7</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>45</td>
<td>60</td>
<td>22</td>
<td>15</td>
<td>210</td>
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<tr>
<td>P10</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>45</td>
<td>60</td>
<td>30</td>
<td>15</td>
<td>210</td>
</tr>
<tr>
<td>P14</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>50</td>
<td>60</td>
<td>30</td>
<td>15</td>
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</tr>
<tr>
<td>P21</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>90</td>
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<tr>
<td>P60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>90</td>
<td>90</td>
<td>30</td>
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</tr>
<tr>
<td>P70</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>90</td>
<td>90</td>
<td>30</td>
<td>20</td>
<td>240</td>
</tr>
</tbody>
</table>

Consecutive coronal paraffin sections, 10 µm thick, were cut from each brain. Sections were collected on “superfrost plus”® microscope slides and were dried at room temperature overnight. Sections were first deparaffinized in xylene and then rehydrated through a descending series of ethanol. Selected sections were stained with Cresyl Violet (Nissl stain) for the assessment of the delineation of the contours and rostral and/or caudal borders of the nuclei striatum and lateral septum and for the quantitative analysis of “live cells”. The atlases of Paxinos and Watson (Paxinos and Watson, 2005 2007) and Paxinos and colleagues (Paxinos et al. 1991) as well as our earlier material were used as reference.
Apoptotic cells detected with the TUNEL method

TUNEL method

The hallmark for apoptosis is the chromatin condensation and DNA fragmentation into internucleosomal fragments of multiples of approximately 180 basepairs (Wyllie, 1980; Wyllie et al., 1984) and/or high molecular weight fragments (HMW) of 50 or 300 kilobasepairs (Oberhammer et al., 1993). The TUNEL method is used to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks (Kressel and Groscurth, 1994; Ito and Otsuki, 1998). This method considered reliable, as it has high sensitivity and also labels a large number of cells in situ.

The TUNEL method is based on the activity of the enzyme Terminal deoxynucleotidyl Transferase (TdT) enzyme to transfer and incorporates nucleotides to the 3’-OH ends of the fragmented DNA. Normal nuclei have very low levels of 3’-OH ends and do not produce any appreciable signal as opposed to apoptotic nuclei. The activity of the TdT enzyme transfers and incorporates the biotinylated nucleotide dUTP (b-dUTP) to the 3’-OH ends of the fragmented DNA (Gavrieli et al., 1992). Then, the detection of b-dUTP was achieved with avidin-biotin method.

The avidin-biotin method uses the avidin-biotin-peroxidase complex (ABC). This complex contains avidin, which is a glycoprotein with an extraordinarily high affinity for the small molecular weight vitamin, biotin. Because this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin is essentially irreversible. In addition to this high affinity, the avidin-biotin system can be effectively exploited because avidin has four binding sites for biotin. One of them is bound the biotin of the nucleotide dUTP (b-dUTP), which is incorporated to the 3’-OH
ends of the fragmented DNA. The reaction product was visualized with the chomogen diaminobenzidine (DAB), which is oxidized by the $\text{H}_2\text{O}_2$ in the presence of the peroxidase of ABC. The reaction product is a reddish brown precipitate, superposed in the nuclei where the fragmented DNA is present.

**Application and modification of the TUNEL method**

The method used in the present study for the detection of DNA fragmentation in the nuclei of dying cells was a modification of the TUNEL method described by Gavrieli et al. (1992). The detailed protocol has previously been described by Sophou et al. (2006). Briefly, paraffin sections were preincubated for 15 min in terminal transferase (TdT) buffer (25 mM Tris buffer, pH 6.6, 200 mM sodium cacodylate and 0.25 mg/ml bovine serum albumin), followed by incubation in a mixture of 1.25 mM cobalt chloride, 2.4 U/µl recombinant TdT and 14 µM biotinylated-d-UTP (Roche Diagnostics, Basel, Switzerland), in a humid chamber at 37° C, for 90 min. The reaction was terminated by rinsing the slides in DW, followed by 0.1 M phosphate buffered-saline (PBS) for 10 min. Nonspecific binding sites were blocked with 1% normal goat serum (NGS) for 15 min. The sections were then incubated for 2 h with the ABC (Vector Laboratories) diluted 1: 100 in PBS. The reaction product was visualized with 0.075% DAB (Sigma) and 0.01% $\text{H}_2\text{O}_2$ in 0.1 M Tris buffer (TB), pH 7.4, for 15 min. Sections were lightly counterstained with 10% toluidine blue, dehydrated through an ascending series of ethanol and xylene and mounted with DPX.

In sections incubated with 5% DNAase in buffer (sodium acetate, 0.1 M and MgSO$4$, 5 mM, pH 5) for 20 min at 37° C, prior to the incubation in the biotinylated-d-UTP mixture (positive control), all cells were labeled. In sections processed with the TUNEL method, omitting the TdT (negative control), no labeled cells were present.
For using semithin sections processed with the TUNEL method in the light microscope level, animals were perfused with 4% w/v paraformaldehyde and 0.2% v/v glutaraldehyde in 0.1M PB. Brains were removed, postfixed in the same fixative for 2 hours, and then transferred into PB at 4°C. Coronal sections were cut at 50 µm using a Vibroslice. Free-floating sections processed with the TUNEL method, were then postfixed in 1% v/v OsO₄ for 30 min, rinsed in 0.1 M acetate buffer, stained in 1% w/v aqueous uranyl acetate for 30 min, dehydrated in ethanol and flat-embedded in Araldite. The flat-embedded specimens were first examined with the light microscope, and areas with TUNEL+ cells were removed and remounted on Araldite stubs for thin sectioning. Semithin (1µm) sections were stained with toluidine blue and screened for cells with the apoptotic morphology.

**Active caspase-3 immunohistochemistry**

For the detection of active caspase-3-ir, paraffin sections were first processed for microwave-mediated antigen retrieval, in 0.01M citrate buffer (pH 6). Sections were first heated at 750 W for 3 min and subsequently at 350 W for 9 min. They were then cooled for 35 min at room temperature. Sections were rinsed in 0.1 M PBS, pH 7.4, blocked with 10% NGS for 15 min, and then incubated in primary antibody to active caspase-3 (polyclonal rabbit anti-active caspase-3, 1:100; Chemicon International) in PBS containing 0.5% Triton X-100 for 24 hours at 4°C. This antibody detects only the cleaved p17 fragment of caspase-3 and does not detect the precursor form in cells undergoing apoptosis. Sections were subsequently washed in PBS and transferred into biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) in PBS, for 2 hours. They were washed again in PBS and placed in the ABC complex (1:100 in PBS) for 1 hour. After washing in PBS and TB, sections were incubated in a solution of 0.075% diaminobenzidine containing 0.01% H₂O₂ in TB, for 10 min. After several rinses
in TB and PB, sections were counterstained with 10% toluidine blue, dehydrated and coverslipped with DPX. Specific immunoreactivity was eliminated in control sections in which primary or secondary antibodies were omitted.

**GABA immunohistochemistry**

For the detection of GABA-ir, paraffin sections were first processed for microwave-mediated antigen retrieval, in 0.01 M citrate buffer (pH 6), as described above. Sections were rinsed in 0.1 M PBS, pH 7.4, blocked with 10% NGS for 15 min and then incubated in primary antibody to GABA (polyclonal rabbit anti-GABA, 1:500; Sigma) in PBS containing 0.5% Triton-X 100 for 24 hours at 4°C. After washes in PBS, sections were transferred into biotinylated horse anti-rabbit or goat anti-rabbit IgG (1:200; Vector Laboratories) in PBS for 2 hours. They were then placed in the ABC complex (1:100 in PBS) for 1 hour and incubated in a solution of 0.075% diaminobenzidine containing 0.01% H2O2 in TB, for 10 min, counterstained with 10% toluidine blue, dehydrated and coverslipped with DPX. Specific immunoreactivity was eliminated in control sections in which primary or secondary antibodies were omitted.

**Nissl staining technique**

We used cresyl violet solution to stain the extranuclear RNA granules, which are most abundant in the rough endoplasmic reticulum of neurons (Nissl bodies). This staining method is useful to localize the pericaryon of the neuronal cells. For this purpose paraffin sections, 10 μm thick, collected on “superfrost plus®” microscope slides, were dried at room temperature overnight. Samples were deparaffinized in xylene bath (two changes) 5 min each and then rehydrated through a descending series of ethanol for 5 min each. Samples were then placed in DW for 1 min, and then were incubated for 5 min at 42°C in 0.1% cresyl violet solution (1g crystal violet, 10 ml glacial acetic acid and 990 ml DW at 35°C.
C for 12 hours and filtered through 3mm filter paper). Samples were then washed in DW to remove excess stain and were exposed to acid ethanol (100% ethanol with trace acetic acid) for 5-10 min until contrast was the appropriate. Samples were dehydrated to an ascending series of ethanol gradient for 1 min in each, cleaned in xylene and coverslipped with DPX.

**Quantitative analysis**

The striatum and the lateral septum were used in this study for quantitative analysis. For animals of early postnatal ages (P0–P7), sections for analysis were selected within the area located between planes 79 (rostral) and 88 (caudal) from the atlas of Paxinos and colleagues (Paxinos et al., 1991) and collected at 50 to 100 μm intervals. For animals of later postnatal ages (P14–P60), the sections were selected between planes 15 (rostral) and 36 (caudal) from the atlas of Paxinos & Watson (2007) and collected at 100 to 200 μm intervals. In these sections the contours of lateral septum and striatum were delineated with the aid of a camera lucida, according to the atlases of Paxinos & Watson (2005; 2007) and Paxinos et al. (1991), using adjacent cresyl violet-stained sections as guides. A plethora of sections from our previous studies on the striatum and the lateral septum of neonate, developing and adult rat brains were also used as reference (Dinopoulos et al., 1993; Antonopoulos et al., 1997; Antonopoulos et al., 2002; Sophou et al., 2006; Mellios et al., 2009). The positions of TUNEL-labeled cells were plotted on the drawings, using a calibrated grid of an area 0.1 mm². The numerical density of TUNEL+ cells in each section was determined as the mean number of TUNEL+ profiles counted in 0.1mm². Having confirmed that TUNEL+ cells were homogenously distributed in the striatum of normal and hypothyroid animals we systematically randomly superimposed the counting grid of 0.1mm² on the field of view in each sampling section of the striatum. Based on a dissector method, it has been suggested that apoptotic nuclei (~5 μm
in diameter) are rarely split by the microtome blade (Clarke and Oppenheim, 1995; Oo and Burke, 1997). Considering the section thickness (10 μm) and the distance between consecutive sections (not less than 50 μm), it is assumed that the present evaluation of TUNEL+ profiles is unbiased and no correction factor for double counting was used. “Live” cells were defined as cresyl violet-stained cells with an evident nucleus and a nucleolus in the plane of section and were estimated as the mean number of “live” cells observed in an area of 0.1mm². The numerical density of active caspase-3- and GABA-ir neurons, in each section, was also estimated in an area of 0.1mm². The counting grid of 0.1mm² was systematically randomly superimposed on the field of view in each sampling section of the striatum. In the lateral septum the counting grid of 0.1mm² was superimposed in the ventral part of the nucleus as TUNEL+ and active caspase-ir profiles were clustered in its ventral part on the field of view in each sampling section. All reported values are expressed as mean ± SEM, for the different ages examined. To avoid nonspecific slice shrinkage deriving from experimental variability, the analysis was performed on tissue from control and experimental animals processed together. The optical equipments used in this study were an optical Zeiss Axioplan photomicroscope equipped with a camera lucida and a Nikon DS-Fi 1-L2 digital camera connected to a Nikon Eclipse 80 I microscope for acquiring images of labeled neurons.

**Statistical analysis**

Both parametric and nonparametric statistical methods were applied for the statistical evaluation of the experimental results. As all forms of parametric tests are based on the assumptions that the within-groups data are samples drawn from normally distributed populations with equal variances, both formal tests (Shapiro-Wilk and Lilliefors tests) and graphical displays were performed for assessing departures from Gaussian distribution, while variances were tested for
homogeneity using the Levene’s test. For accessing the assumptions of normality and stability of variances, data were transformed to log$_{10}$ or sqrt (Zolman, 1993). More particularly, a fixed-effect hypothesis model of analysis of variance was employed with two factors, namely (1) “age” and (2) “treatment”, to determine possible significant interaction effects between these factors on the mean number of TUNEL+ cells, as well as on the mean number of Nissl cells. Furthermore, in case of normality and variances’ homogeneity, one-way analysis of variance (ONE-WAY ANOVA) was performed, to evaluate possible significant effects of age on the number of TUNEL+ cells and of Nissl cells. Differences between mean values of specific age groups were evaluated using the Duncan's new multiple range test. Where assumptions about either variability or the form of the populations’ distribution were seriously violated, with or no transformed data, the Kruskal-Wallis nonparametric test was applied to evaluate age depended differences, while differences between mean values of specific age groups were evaluated using the nonparametric Wilcoxon rank sum test (Mann-Whitney U-test). The t-test was also used to evaluate treatment depended differences, while where assumptions about normality were seriously violated the nonparametric Wilcoxon rank sum test (Mann-Whitney U-test) was used. In addition, one-way analysis of variance was also used to evaluate possible significant effects of treatment on the numerical density of GABA-ir cells or on the Cross-sectional area of the striatum, as well as of age on the mean number of Caspase-3-ir cells. Differences between mean values of specific groups were evaluated using the Duncan's new multiple range test. Where assumptions about either variability or the form of the populations’ distribution were seriously violated, the nonparametric Kruskal-Wallis test and the Mann-Whitney U-test were used, respectively. All analyses were conducted using the statistical software program SPSS for Windows (v. 15.0). Significance was
declared at \( P \leq 0.05 \), unless otherwise noted. Back-transformed mean values are reported in the results.

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin–Biotin–peroxidase Complex</td>
</tr>
<tr>
<td>Ab</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way Analysis of Variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma/leukaemia 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase Activated Dnase</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-dependent aspartate specific proteases</td>
</tr>
<tr>
<td>Caspase-3-ir</td>
<td>Caspase-3-immunoreactivity</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus Callosum</td>
</tr>
<tr>
<td>Cc</td>
<td>Cubic centimeters</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline Acetyltransferase</td>
</tr>
<tr>
<td>Cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate-putamen</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DA-Rs</td>
<td>Dopamine Receptors</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DB</td>
<td>Diagonal band of Broca</td>
</tr>
<tr>
<td>DCRs</td>
<td>DeCoy receptors</td>
</tr>
<tr>
<td>DD</td>
<td>Death Domains</td>
</tr>
<tr>
<td>DED</td>
<td>Death Effector Domains</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAPbinding protein with low PI</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-Inducing Signaling Complex</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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</table>
DR  Death Receptors
DW  Distilled Water

EGF  Epidermal Growth Factor
EGFR  EGF Receptor
EP  Entopeduncular nucleus

FasL  Fas Ligand
FT4  Free Thyroxine

g  gram
GABA  Gammaaminobutyric Acid
GP  Globus Pallidus
GPe  Globus Pallidus, external segment
GPi  Globus Pallidus, internal segment
GPCRs  G-protein Coupled Receptors

hDB  horizontal limb of DB
HMW  High Molecular Weight fragments
hr(s)  hour(s)
HRP  Horseradish Peroxidase
HtrA2  High temperature requirement protein A2

IAPs  Inhibitors of Apoptosis Proteins
ICAD  Inhibitor of CAD
IDD  Iodine Deficiency Disorders
IGFR-I  Insulin-like Growth Factor Receptor-I

LC  Live Cell
LS  Lateral Septum
LSD  Dorsal part of Lateral Septum
LSI  Intermediate part of Lateral Septum
LSV  Ventral part of Lateral Septum

M-CSF  Macrophage Colony-Stimulating Factor
mg/kg  milligram per kilogram
min(s)  minute (s)
ml  milliliter
mm  millimeter
MMI  2-Mercapto-1- methylimidazole
MOMP  Mitochondrial Outer Membrane permeabilization
MPT  Mitochondrial Permeability Transition
MS  Medial Septum
MSDB  Medial Septum-Diagonal Band of Broca

MSvDB  Medial Septum / vertical Band of the diagonal band of Broca

μm  micrometers

NA  Nucleus Accumbens
NGS  Normal Goat Serum.
NK1  Neurokinin1
NMDA  N-methyl-D-aspartate
NOS  Nitric Oxide Synthase
NuMA  Nuclear protein
NSE  neuron specific enolase

OPG  Osteoprotegerin

PARP  Poly (ADP-ribose) Polymerase
PB  Phosphate Buffer
PBS  Phosphate Buffer Saline.
PCD  Programmed Cell Death
PCP2  Purkinje cell specific gene,
PTU  Propylthiouracil

ROS  Reactive oxygen species
RT  Room Temperature

s  seconds
SD  Standard Deviation
NIS  Sodium Iodide Symporter
Smac  Second mitochondria-derived activator
SN  Substantia Nigra
SNC  Substantia Nigra pars compacta
SNR  Substantia Nigra pars reticulata
SP  Substance P
SPSS  Stochastic Package for Social Sciences
STN  Subthalamic Nucleus
STR  Striatum

T3  Tri-iodo-thyronine
T4  Thyroxine
TB  Tris Buffer.
TBS  Tris-buffered Saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>TC</td>
<td>TUNEL+ Cell.</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl Transferase</td>
</tr>
<tr>
<td>Tg</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>THs</td>
<td>Thyroid Hormones</td>
</tr>
<tr>
<td>Th2</td>
<td>T cell expansion of type 2 helper T</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thyroperoxidase</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer Ribosomal Nucleic Acid</td>
</tr>
<tr>
<td>TRs</td>
<td>Thyroid Receptors</td>
</tr>
<tr>
<td>TRα1</td>
<td>Thyroid Receptor α1</td>
</tr>
<tr>
<td>TRβ1</td>
<td>Thyroid Receptor β1</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid Stimulating Hormone</td>
</tr>
<tr>
<td>TSHR</td>
<td>TSH Receptor</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-like WEAK inducer of apoptosis</td>
</tr>
<tr>
<td>vDB</td>
<td>vertical limb of DB</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
</tbody>
</table>
Results

For studying, the effects of hypothyroidism on cell survival in the striatum and the lateral septum during development and in various forebrain structures in the adult, animals with congenital or acquired hypothyroidism were used. Sections stained with cresyl violet were used for evaluating the effect of hypothyroidism on the density of “live” neurons; sections stained with the TUNEL method were used for the in situ detection of DNA fragmentation, for evaluating dying cells. It is well documented that the TUNEL method provides a useful means for the study of cell death in normal and pathological nervous tissue (Wilcox et al., 1995). Immunohistochemical localization of active caspase-3 was also used, in order to investigate the type of cell death in the rat forebrain structures. The immunohistochemical localization of the apoptotic marker activated caspase-3, in combination with morphological criteria allows for the reliable identification of dying cells as apoptotic (Goping et al., 1999; Stadelmann and Lassmann, 2000). Consistent with the literature (Wyllie et al., 1980; Clarke, 1990; Clarke and Oppenheim, 1995; Clarke, 1999; Häcker, 2000), the morphological features of TUNEL+ cells in the present material were:

(i) the round, or irregularly-shaped pyknotic nucleus containing round chromatin clumps and often showing membrane blebbing;

(ii) the reaction product that was dispersed throughout the nucleus, or had a crescent-like appearance at the periphery of the nucleus;

(iii) the shrunken cytoplasm.

Cells with apoptotic morphology were also observed in toluidine blue-stained semithin sections. These cells had a pyknotic nucleus, containing basophilic chromatin clumps and a condensed cytoplasm, characteristic of apoptosis (Clarke and Oppenheim, 1995). The terms TUNEL+ cells, apoptotic cells or labeled cells, are used interchangeably to indicate the dying cells.
“Live” cells in the striatum of hypothyroid animals

Cresyl violet-stained, “live” cells, in the striatum of hypothyroid and normal control animals were examined using a light microscope.

Congenital hypothyroidism
At birth, the mean numerical density of “live” cells in the striatum of animals with congenital hypothyroidism was 368.166 ± 14.720. Statistical analysis revealed that it remained constant until P5, and significantly decreased at the end of the first postnatal week. Thereafter, the mean numerical density of “live” cells remained unchanged until the end of the second postnatal week, significantly decreased again at the end of the third postnatal week, and reached its minimal value at P60, which was significant lower than that at P21 (Table 5; Fig. 4).

Data from statistical analysis also showed that in normal control animals, the corresponding values of “live” cells were significantly higher at P0, P5, P7, P10, P21 and P60 compared to the values of hypothyroid animals, with the exception at P3 and at P14, when the values of the mean numerical density of “live” cells between hypothyroid and normal animals showed no statistically significant difference (Table 5; Fig. 4).

Acquired hypothyroidism
In adult animals with acquired hypothyroidism, the mean numerical density of “live” cells, was significantly higher than the corresponding value of normal control animals at the same age (Table 5; Fig. 4).
Table 5. Numerical density of TUNEL+ and “live” cells in the striatum and the lateral septum of normal and hypothyroid animals.

<table>
<thead>
<tr>
<th>Ages</th>
<th>Treatment</th>
<th>Striatum</th>
<th>Lateral septum</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TUNEL+ cells</td>
<td>“live” (Nissl) cells</td>
<td>TUNEL+ cells</td>
<td>“live” (Nissl) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td>Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>Normal</td>
<td>1.769 ± 0.257</td>
<td>728.666 ± 8.875</td>
<td>1.615 ± 0.289</td>
<td>323.166 ± 5.185</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>4.230 ± 0.302</td>
<td>368.166 ± 14.720</td>
<td>4.384 ± 0.266</td>
<td>253.666 ± 11.921</td>
</tr>
<tr>
<td>P3</td>
<td>Normal</td>
<td>1.625 ± 0.263</td>
<td>350.666 ± 6.936</td>
<td>2.750 ± 0.365</td>
<td>287.166 ± 5.901</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>5.222 ± 0.277</td>
<td>338.666 ± 12.298</td>
<td>5.777 ± 0.277</td>
<td>260.333 ± 2.403</td>
</tr>
<tr>
<td>P5</td>
<td>Normal</td>
<td>4.888 ± 0.789</td>
<td>751.833 ± 9.378</td>
<td>6.222 ± 0.777</td>
<td>290.500 ± 2.109</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>5.000 ± 0.288</td>
<td>322.666 ± 4.386</td>
<td>5.222 ± 0.323</td>
<td>263.500 ± 19.882</td>
</tr>
<tr>
<td>P7</td>
<td>Normal</td>
<td>4.833 ± 0.457</td>
<td>533.500 ± 3.676</td>
<td>4.750 ± 0.616</td>
<td>235.833 ± 8.372</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>5.250 ± 0.250</td>
<td>286.000 ± 6.952</td>
<td>4.125 ± 0.226</td>
<td>225.666 ± 11.514</td>
</tr>
<tr>
<td>P10</td>
<td>Normal</td>
<td>1.888 ± 0.309</td>
<td>377.500 ± 3.640</td>
<td>3.222 ± 0.571</td>
<td>243.500 ± 11.655</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>6.888 ± 0.587</td>
<td>287.333 ± 11.491</td>
<td>7.666 ± 0.408</td>
<td>253.333 ± 7.387</td>
</tr>
<tr>
<td>P14</td>
<td>Normal</td>
<td>1.727 ± 0.272</td>
<td>259.166 ± 7.000</td>
<td>2.727 ± 0.428</td>
<td>259.000 ± 17.576</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>3.900 ± 0.233</td>
<td>273.500 ± 12.164</td>
<td>5.600 ± 0.541</td>
<td>249.833 ± 7.063</td>
</tr>
<tr>
<td>P21</td>
<td>Normal</td>
<td>1.250 ± 0.365</td>
<td>260.000 ± 8.888</td>
<td>1.875 ± 0.226</td>
<td>205.666 ± 13.804</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>4.100 ± 0.233</td>
<td>230.833 ± 3.771</td>
<td>6.300 ± 0.366</td>
<td>211.833 ± 3.239</td>
</tr>
<tr>
<td>P60</td>
<td>Normal</td>
<td>0.000 ± 0.000</td>
<td>198.333 ± 6.550</td>
<td>0.000 ± 0.000</td>
<td>200.833 ± 3.477</td>
</tr>
<tr>
<td></td>
<td>C. Hypothyroid</td>
<td>1.700 ± 0213</td>
<td>176.666 ± 2.472</td>
<td>1.400 ± 0.339</td>
<td>202.500 ± 4.237</td>
</tr>
<tr>
<td>P70*</td>
<td>Normal</td>
<td>0.000 ± 0.000</td>
<td>198.333 ± 6.550</td>
<td>0.000 ± 0.000</td>
<td>200.833 ± 3.477</td>
</tr>
<tr>
<td></td>
<td>A. Hypothyroid*</td>
<td>1.416 ± 0.193</td>
<td>231.000 ± 3.741</td>
<td>0.833 ± 0.166</td>
<td>215.833 ± 2.386</td>
</tr>
</tbody>
</table>

Notes:
* Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).
Values are expressed as Mean number ± SEM of TUNEL+ or Nissl cells / 0.1 mm².

a, b, c, ..: Values in the same column and the same treatment with a different superscript differ significantly (P≤0.05).
A, B, Values in the same column and the same age with a different superscript differ significantly (P≤0.05).
Fig. 4. Histogram showing the numerical density of “live” cells in the striatum of normal and hypothyroid rats at different developmental ages. In hypothyroid animals, the numerical density of live cells showed a significant decrease during development compared to normal animals (*P ≤ 0.05). **Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).

**Apoptotic cells in the striatum of hypothyroid animals**

In the striatum, TUNEL+ cells examined with the light microscope, were readily identified by the intense and selective labeling of their nuclei. Labeled cells were scattered among numerous “live” cells. Apoptotic cells appeared shrunken and detached from the surrounding neuropil, and were often found in tissue “vacuoles”. They showed a homogeneous distribution pattern at all ages examined and displayed all the above described morphological features consistent with apoptotic cell death. In control sections processed with DNAase I, all cells were labeled, whereas sections in which TdT was omitted no labeling was observed.
**Congenital hypothyroidism**

Labeled cells in hypothyroid animals were first observed in the striatum at birth (P0) and were present at all stages of development examined and in the adult (P60) (Fig. 13, 14). Quantitative analysis revealed that their mean numerical density was high at P0 (4.230 ± 0.302), further increased at P3 and remained stable until the end of the first postnatal week; it showed a second significant increase to reach a peak at P10. The numerical density of TUNEL+ cells significantly declined during the second postnatal week, remained unchanged until the end of the third postnatal week, and reached its minimal value in the adult, which was significant lower than that at P21 (Table 5; Fig. 5).

In normal control animals, TUNEL+ cells were also present at all ages examined during development from P0 to P21. However, their mean numerical density was significant lower than that of hypothyroid animals, at all ages examined except at P5 and P7. We never detected apoptotic cells in normal adult animals (Table 5; Fig. 5).

**Acquired hypothyroidism**

In adult animals with acquired hypothyroidism, TUNEL+ cells were present throughout the striatum. In contrast, we never observed apoptotic cells in normal control adult animals (Table 5; Fig. 5).
Fig. 5. Histogram showing the numerical density of TUNEL+ cells in the striatum of normal and hypothyroid rats at different developmental ages. In hypothyroid animals, the numerical density of TUNEL+ cells was significantly different at P0, P3, P10, P14 and P21 compared to normal animals (*P ≤ 0.05). TUNEL+ cells were never found in adult normal animals. **Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).

**Expression of active caspase-3 in the striatum of hypothyroid animals**

It is well established that the activation of caspase-3 is a hallmark of apoptosis and precedes or coincides with DNA fragmentation (Cohen, 1997). We therefore examined the expression of active caspase-3 in the striatum of hypothyroid animals at a time window during which the estimated density of TUNEL+ cells was high, i.e. during the first postnatal week. We also examined the expression of active caspase-3 in adult hypothyroid animals.

The distribution of active caspase-3-ir profiles was spatially correlated with that of TUNEL+ cells at the same ages examined, in hypothyroid animals.
Quantitative analysis in the striatum revealed that the numerical density of caspase-3-ir cells, was higher than the numerical density of TUNEL+ cells at all ages examined (Table 6; Fig. 6).

The numerical density of active caspase-3-ir profiles in animals with congenital hypothyroidism was high at birth, significantly increased at P3, remained unchanged until P5, significantly increased again at the end of the first postnatal week, and finally, decreased significantly in adulthood.

In the adult animals with acquired hypothyroidism, the mean numerical density of active caspase-3-ir profiles was significantly lower compared to the corresponding value in adult animals with congenital hypothyroidism. The mean numerical density of active caspase-3-ir profiles in adult animals with acquired hypothyroidism was similar to the numerical density of TUNEL+ cells (Table 6; Fig. 6).
Table 6. Numerical density of active caspase-3-ir and TUNEL+ cells in the striatum and the lateral septum of hypothyroid animals.

<table>
<thead>
<tr>
<th>Ages</th>
<th>Treatment</th>
<th>Striatum</th>
<th>Lateral septum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caspase-3-ir cells</td>
<td>TUNEL+ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>Hypothyroid</td>
<td>6.000 ± 0.213b</td>
<td>4.230 ± 0.302a</td>
</tr>
<tr>
<td>P3</td>
<td>Hypothyroid</td>
<td>8.750 ± 0.371c</td>
<td>5.222 ± 0.277b</td>
</tr>
<tr>
<td>P5</td>
<td>Hypothyroid</td>
<td>8.000 ± 0.550c</td>
<td>5.000 ± 0.288a,b</td>
</tr>
<tr>
<td>P7</td>
<td>Hypothyroid</td>
<td>14.555 ± 0.603d</td>
<td>5.250 ± 0.250b</td>
</tr>
<tr>
<td>P60</td>
<td>Hypothyroid</td>
<td>8.666 ± 0.484c</td>
<td>1.700 ± 0213c</td>
</tr>
<tr>
<td>P70</td>
<td>Hypothyroid*</td>
<td>1.619 ± 0.233a</td>
<td>1.416 ± 0.193c</td>
</tr>
</tbody>
</table>

**Notes**
- Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).
- Values are expressed as Mean number ± SEM of caspase-3-ir or TUNEL+ cells / 0.1mm².
- a, b, c, ..: Values in the same column with a different superscript differ significantly (P ≤ 0.05).
Effects of hypothyroidism on the survival and phenotype of GABAergic striatal neurons

Based on our data that hypothyroidism resulted in a significant increase of apoptotic cells in the striatum during development and in the adult, we sought to evaluate its effects on chemically defined GABAergic neurons, which constitute approximately the 90% of neurons in the striatum of adult animals. For this purpose, we used adult animals with congenital or acquired hypothyroidism to examine the morphology and number of GABA-ir neurons in the striatum.

GABA-ir neurons showed an accumulation of the reaction product in their cytoplasm and proximal dendrites. In normal animals, GABA-ir neurons displayed a polygonal or ovoid soma shape. In some cases, proximal dendrites
were seen to emanate from the soma poles (Fig. 9). In hypothyroid animals, GABA-ir neurons appeared atrophic with no discernible dendrites. In addition, their staining intensity was lower than that of their normal counterparts. Quantitative analysis revealed that the mean numerical density of GABA-ir cells in adult animals with acquired hypothyroidism was not statistically different from that in animals with congenital hypothyroidism. It is notable that the mean numerical density of GABAergic cells in both hypothyroid animal groups is significantly different from that in normal control animals (reduction about 13.13% in both hypothyroid animal groups) (Table 7; Fig. 7).

**Table 7.** Numerical density of GABA-ir neurons in the striatum of adult normal and hypothyroid animals.

<table>
<thead>
<tr>
<th>Ages</th>
<th>Treatment</th>
<th>GABA-ir neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>P60</td>
<td>Normal</td>
<td>34.666 ± 1.592^a</td>
</tr>
<tr>
<td>P60</td>
<td>Hypothyroid</td>
<td>30.111 ± 0.866^b</td>
</tr>
<tr>
<td>P70</td>
<td>Hypothyroid^*</td>
<td>30.166 ± 0.658^b</td>
</tr>
</tbody>
</table>

**Notes**
- ^*Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).
- Values are expressed as the Mean number ± SEM of GABA-ir cells / 0.1 mm^2.
- a, b: Values in the same column with a different superscript differ significantly (P≤0.05).
Considering that hypothyroidism results in the apoptotic death of cells in the striatum of the adult and in a decrease in the numerical density of GABAergic neurons, we examined whether hypothyroidism also induces an alteration in the size of the adult striatum. Table 8 summarizes the data from adult hypothyroid and normal control animals. The mean cross-sectional area of the striatum in adult animals with congenital hypothyroidism was significantly reduced compared to the mean cross-sectional area of the striatum in normal animals. On the contrary, in animals with acquired hypothyroidism the corresponding cross-sectional area of the striatum was not significantly different from that in normal control animals (Fig. 8).
Table 8. Cross-sectional area of the striatum in adult normal and hypothyroid animals.

<table>
<thead>
<tr>
<th>Ages</th>
<th>Treatment</th>
<th>Cross-sectional area of the striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>P60</td>
<td>Normal</td>
<td>6164571.3 ± 149292.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P60</td>
<td>Hypothyroid</td>
<td>4264655.0 ± 169207.68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P70</td>
<td>Hypothyroid*</td>
<td>6094610.6 ± 140784.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes
*Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).
Values represent the Mean ± SEM cross-sectional area of the striatum (caudate-putamen), in µm², measured in 3 corresponding coronal paraffin sections, 10µm thick, at successive 200µm intervals rostro-caudally and in 3 animals.
a, b: Values in the same column with a different superscript differ significantly (P≤0.05).

Fig. 8. Histograms showing the cross-sectional area (x10⁶ µm²) of the striatum measured in 3 corresponding coronal sections per animal, 10µm thick, at successive 200µm intervals rostro-caudally and in 3 animals. *Value significant different from normal P60 and hypothyroid P70 animals (P≤0.05). **Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).
Fig. 9. Photomicrographs showing the morphological features of GABA-ir neurons in the striatum of normal adult rat (A) and congenital hypothyroid adult rat (B). In hypothyroid animals, GABA-ir neurons (arrows) appeared atrophic with shorter dendrites. Their staining intensity was also reduced. Scale bar, 50 μm.
“Live” cells in the lateral septum of hypothyroid animals

Cresyl violet-stained, “live” cells, in the lateral septum of hypothyroid and normal animals were examined using a light microscope.

Congenital hypothyroidism
Quantitative analysis in the lateral septum showed that the mean numerical density of “live” cells in animals with congenital hypothyroidism was $253.666 \pm 11.921$ at birth and remained constant until P5; it decreased significantly at the end of the first postnatal week, but significantly increased again at P10. The mean numerical density of “live” cells remained unchanged until the end of the second postnatal week, significantly decreased at the end of the third postnatal week and remained unchanged until adulthood (Table 5; Fig. 10).

Quantitative analysis in normal control animals revealed that the mean numerical density of “live” cells was significantly higher than the corresponding value of hypothyroid animals only at P0 and P3. No significant differences were estimated in the values of “live” cells between normal and hypothyroid animals at all other ages examined during development and in the adult (Table 5; Fig. 10).

Acquired hypothyroidism
In adult animals with acquired hypothyroidism, the mean numerical density of “live” cells was not significantly different from the corresponding value of normal control animals (Table 5; Fig. 10).
Fig. 10. Histogram showing the numerical density of “live” cells in the lateral septum of normal and hypothyroid rats at different developmental ages. In hypothyroid animals, the numerical density of live cells showed a significant decrease at P0 and P3 compared to normal animals (*P≤0.05). **Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).

**Apoptotic cells in the lateral septum of hypothyroid animals**

TUNEL+ cells in the developing lateral septum, which were readily identified by the intense and selective labeling of their nuclei, displayed all morphological features of apoptotic cells. These cells were scattered among numerous “live” cells in the lateral septum, although they were preferentially concentrated in its ventral part, especially in adult animals. Labeled cells were first observed at birth and were present during the first three weeks of postnatal development.
Labeled cells were also observed in adult animals with congenital or acquired hypothyroidism (Fig. 11, 13, 14). In control sections processed with DNAase I, all cells were labeled, whereas sections in which TdT was omitted no labeling was observed.

**Congenital hypothyroidism**

Quantitative analysis in the ventral part of the lateral septum of hypothyroid animals revealed that the mean numerical density of TUNEL+ cells, was high at birth. It increased significantly at P3, remained stable at P5, declined significantly at P7 and increased again to reach a peak at P10 (Table 5; Fig. 11). Thereafter, the numerical density of TUNEL+ cells significantly decreased at the end of the second postnatal week, remained steady during the third postnatal week and declined again to its minimal value at P60 (Table 5; Fig. 11).

In the lateral septum of normal control animals TUNEL+ cells were present at all corresponding ages during development, from P0 to P21. Quantitative analysis revealed that the mean numerical density of TUNEL+ cells in normal control animals was significantly lower than that of hypothyroid animals at all ages examined, except at P5 and P7. We have not detected TUNEL+ cells in normal adult animals (Fig. 11).

**Acquired hypothyroidism**

In adult animals with acquired hypothyroidism TUNEL+ cells were present in the lateral septum. Labeled cells were never found in normal adult animals (Table 5; Fig. 11).
Fig. 11. Histogram showing the numerical density of TUNEL+ cells in the lateral septum of normal and hypothyroid rats at different developmental ages. In hypothyroid animals, the numerical density of TUNEL+ cells showed a significant increase at P0, P3, P10, P14 and P21 compared to normal animals (*P ≤ 0.05). ** Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).

Expression of active caspase-3 in the lateral septum of hypothyroid animals

We examined the expression of active caspase-3 in the lateral septum of hypothyroid animals at a time window during which the estimated density of TUNEL+ cells was high, i.e. during the first postnatal week. We also examined the expression of active caspase-3 in adult hypothyroid animals. The distribution of active caspase-3-ir profiles was spatially correlated with that of TUNEL+ cells at the same ages examined. Quantitative analysis in the ventral part of the lateral septum revealed that the numerical density of caspase-3-ir cells, was
higher than the numerical density of TUNEL+ cells at corresponding time points.

The numerical density of active caspase-3-ir profiles in animals with congenital hypothyroidism was high at P0, and significantly increased at P3; then, significantly declined at P5, before it showed a peak at P7. The numerical density of active caspase-3-ir profiles significantly decreased in adulthood (Table 6; Fig. 12).

In adult animals with acquired hypothyroidism, the corresponding value was significantly lower than that at animals with congenital hypothyroidism and approximated the value of the numerical density of TUNEL+ cells (Fig. 12).

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**Fig. 12.** Histogram showing the numerical density of active caspase-3-ir and TUNEL+ cells in the lateral septum of hypothyroid rats at different developmental ages and in the adult. **Methimazole-treated animals for 4.5 weeks (acquired hypothyroidism).**
Fig. 13. Photomicrographs showing TUNEL+ cells (arrows) in the striatum (A) and the lateral septum (B, C) of rats with congenital hypothyroidism at P7 (A, B) and P14 (C). The schematic representations show the area which is depicted in photographs A-C. lv: lateral ventricle. Scale bar, 100 μm.
Fig. 14. Photomicrographs showing TUNEL+ cells (arrows) in the cerebral cortex (A), the striatum (B, F), the corpus callosum (C, D) and the lateral septum (E) of rats with congenital hypothyroidism at P7 (B), P14 (C) and P21 (A, D, F). cc, corpus callosum; STR, striatum. Scale bar, 80 μm (A), 100 μm (B-D), 25 μm (E) and 15 μm (D).
Fig. 15. Photomicrographs showing active caspase-3-ir cells (arrows) in the lateral septum (A, C) and the striatum (B, D), of rats with congenital (A, B) or acquired hypothyroidism (C, D) at P5 (A, B) and P70 (C, D). Scale bar, 100 μm.
Apoptotic cells in forebrain structures of adult animals with congenital or acquired hypothyroidism

In addition to the striatum and the lateral septum, other forebrain structures were found to have TUNEL+ cells in adult animals. The qualitative examination showed that TUNEL+ and active caspase-3-ir cells were present in almost every forebrain structure of adult animals with congenital or acquired hypothyroidism. Apoptotic cells displayed all various morphological features consistent with apoptotic cell death (Fig. 16). TUNEL+ and active caspase-3-ir cells were identified in: anterior cingulate cortex (sparse cells), motor and somatosensory areas of the frontoparietal cortex (sparse cells), primary olfactory cortex, corpus callosum, nucleus accumbens, vertical and horizontal limbs of the diagonal band of Broca and islands of Calleja (Figs 16, 17). The distribution pattern of TUNEL+ cells in animals with congenital and acquired hypothyroidism appeared in three representative sections from the rat forebrain (Fig. 18). TUNEL+ cells appeared more densely distributed in animals with congenital than in animals with acquired hypothyroidism in all forebrain structures (Fig. 19).
Fig. 16. Photomicrographs showing TUNEL+ cells (arrows) in the striatum (A) and the corpus callosum (C) of an adult rat with congenital hypothyroidism. The framed area is shown in (B) at a higher magnification with the TUNEL+ cells displaying typical morphological features of apoptosis. Scale bar, 100 μm (A, C), 25 μm (B).
Fig. 17. Photomicrographs showing TUNEL+ cells (arrows) in the cortex (A-E) and the lateral septum (F) of an adult rat with congenital (A-C) or acquired hypothyroidism (D-F). Scale bar, 80 μm.
Fig. 18. Distribution pattern of TUNEL+ cells in forebrain structures of adult hypothyroid rats. TUNEL+ cells are depicted in red dots and in three representative sections at successive 500μm intervals. The sections were taken from Paxinos and Watson Atlas of the Rat Brain (from up to bottom: A, Bregma 1.7mm, B, Bregma 1.2mm, and C, Bregma 0.7mm).
Fig. 19. Histogram illustrating the numerical density of TUNEL+ cells in forebrain structures of adult hypothyroid rats. The numerical density was determined as the mean ± SEM number of TUNEL+ cells/section in animals with acquired hypothyroidism (H1) or in animals with congenital hypothyroidism (H2). *Significant different values (P≤0.05). The mean numbers of TUNEL+ cells were counted in 8 coronal paraffin sections, 10μm thick, at successive 200μm intervals.
Discussion

Thyroid hormones are essential for normal brain development in all vertebrates (Howdeshell, 2002) and the consequences of thyroid hormone insufficiency during development are permanent (Zoeller and Rovet, 2004). Deficiency of thyroid hormones during critical periods of brain development leads to a dramatic impairment of mental performance (Porterfield and Hendrich, 1993). Hypothyroidism in the developing brain results in progressive intraneuronal accumulation of neurofilament proteins in the proximal hillock region of axons, which has also been documented in neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease (Chinnakkaruppan et al., 2009). Such intra-axonal protein inclusions affect axonal transport leading to degeneration and death of neurons (Xu et al., 1993; Lariviere and Julien, 2004; Sarkar, 2005). Apoptosis is a cardinal feature of brain development; however, the role of apoptosis in TH deficiency in the central nervous system remains to be elucidated. In the present study, we identified dying cells in the striatum and the lateral septum of hypothyroid animals by using the TUNEL method for the \textit{in situ} detection of DNA fragmentation. This method has been widely used for the study of apoptosis under light microscopy. The apoptotic type of cell death should be confirmed by the detection of specific apoptotic hallmarks such as caspase-3 activation (Hernlund, 2009). According to the pioneering studies of Eayrs, the rat is a viable model for examining the effects of thyroid hormone deficiency on the developing mammalian brain (Eayrs, 1960, 1971). In the present thesis we studied the effects of thyroid hormone deficiency on the developing and adult brain of pharmacologically-induced hypothyroid rats.
Technical considerations

Our data together with evidence from other studies (Martinez et al., 2001; Kumar et al., 2006; Sinha et al., 2009) showed that congenital hypothyroidism can be induced by providing dams with methimazole in their drinking water from the 9th gestational day onwards until sacrifice of the neonatal rats. Congenital hypothyroidism is induced because drug has access to foetuses by placental transfer and to neonates through lactation (Hasebe et al., 2008). After weaning (P20), the neonates continued to receive MMI directly through drinking water until sacrifice. The acquired hypothyroidism was induced in our experiments according to the checked protocol by Constantinou and colleagues (Constantinou et al., 2005), in which rats rendered pharmacologically hypothyroid by 4.5 weeks of treatment with MMI in their drinking water, from P35 until the day of sacrifice.

Apoptotic mechanisms play a crucial role during postnatal and adult neurogenesis (Biebl et al., 2000; Dayer et al., 2003; Kuhn, 2008; Zhang et al., 2009). In this study, we identified and quantified apoptosis in the striatum and the lateral septum during development and in adult hypothyroid animals using the TUNEL method for the in situ detection of DNA fragmentation in the nuclei of dying cells. Several investigators (Charriaut-Marlangue and Ben-Ari, 1995; Baima and Sticherling, 2002) have questioned this method. However, it has been proposed that this method selectively labels apoptotic cells, as these contain a considerably larger number of nucleotide-binding sites on the fragmented DNA than non-apoptotic cells (Ansari et al., 1993; Christina et al., 2006). It is well documented that the TUNEL method provides a useful means for the study of apoptosis in normal and pathological nervous tissue (Wilcox et al., 1995). Moreover, the TUNEL technique still remains the most favourable method for the evaluation of apoptotic cell death in brain structures (Gawtowicz et al., 2006). Caspase-3 immunocytochemistry is also required for confirming the
apoptotic nature of cell death (Porter et al., 1999; Suurmeijer et al., 1999; Stefanis, 2005; Sophou et al., 2006; Mellios et al., 2009). In our material TUNEL+ cells displayed all morphological criteria of apoptosis (Wyllie et al., 1980; Clarke, 1990; Clarke and Oppenheim, 1995; Clarke, 1999; Häcker, 2000). Semithin sections, counterstained with toluidine blue were also used to identify cells with apoptotic morphology. In the present study the combination of active caspase-3 immunohistochemistry, with morphological criteria allows the reliable identification of dying cells as apoptotic (Stadelmann and Lassmann, 2000; Goping et al., 2003; Mellios et al., 2009).

“Live” and apoptotic cells in the striatum of animals with congenital hypothyroidism during development

In the present study, we showed that the numerical density of “live” cells in the striatum of animals with congenital hypothyroidism was significantly lower, compared to normal animals, in most ages examined during development. The low number of “live” cells already at birth may be due to impaired neurogenesis and/or augmented cell death during gestational life (Sinha et al., 2008; Alva-Sánchez et al., 2009; Saegusa et al., 2010). The synergy of these two implications of embryonic hypothyroidism may efficiently reduce the density of “live” cells in neonatal animals. In particular, at P0, P5 and P7 the numerical density of “live” cells in hypothyroid animals was almost half than that in normal control animals. The significantly decreased numerical density of “live” cells maintained until P10, while high numerical density of TUNEL+ cells in hypothyroid compared to normal animals, was observed early in development (at P0 and P3) and during the second and third postnatal weeks. Although, the numerical density of dying cells in hypothyroid animals was twofold higher than that in normal control animals at P0 and P3, “live” cells in hypothyroid animals showed a peak in reduction of the numerical density, later at P7. Similarly, a
significant reduction of the numerical density of “live” cells in hypothyroid animals was also observed at P21, following a peak in the numerical density of dying cells at P10. TUNEL+ cells represent the dying cells at the time point of sacrifice and the result of cell death on the numerical density of “live” cell population in hypothyroid animals appears few days later.

Recent data show that maternal TH deficiency during the early gestational period causes massive premature elevation in the expression of neuronal nitric oxide with an associated neuronal death in embryonic rat neocortex (Sinha et al., 2008). THs are essential for foetal neocortical cytoarchitecture in rat, since TH deficiency results in the decreased number and length of radial glia, loss of neuronal bipolarity, and impaired neuronal migration (Pathak et al., 2010). Perinatal hypothyroidism-induced neuronal cell loss involves down regulation of neurotrophic survival signalling and increased truncation of the receptor p75(NTR) in the developing rat cerebellum. The increased proteolysis of p75(NTR) in vivo and its association with death of granule cerebellar neurons brings forward hitherto a p75(NTR) dependent signalling, which along with compromised survival signalling could provide a neurotrophic basis of understanding the cause of enhanced cell death in developing cerebellum (Sinha et al., 2009). At weaning, the neuroblast-producing subgranular zone of the dentate gyrus in hypothyroid animals showed increased apoptosis and decreased cell proliferation suggestive of impaired neurogenesis (Saegusa et al., 2010). All these studies indicate that embryonic hypothyroidism may result in increased cell death or reduced neurogenesis in the neuronal population during embryonic life and the early stages of postnatal development.

The results from normal control animals during development are in agreement with findings from other investigations, which have shown that in the normal rat striatum apoptosis occurs within the first month of postnatal
development, being more intense during the first postnatal week (Maciejewska et al., 1998; Mellios et al., 2009).

The apoptotic type of cell death was confirmed by the detection of active caspase-3, which is considered as a reliable apoptotic marker in developing and diseased brain (Cohen, 1997; D’Mello et al., 2000) and was shown to be either cytoplasmic or nuclear (Beer et al., 2000). Active caspase-3 was localized in profiles with apoptotic morphology. Specifically, it was localized in pyknotic nuclei or in the cytoplasm of shrunken cells, which appeared detached from the surrounding neuropil. The detection of active caspase-3-ir cells in the developing and adult striatum of hypothyroid animals confirms that congenital hypothyroidism induces caspase-dependent apoptosis. Activation of caspase-3 has been shown to precede the appearance of DNA fragmentation (Namura et al., 1998). However, caspase-3 activity has been observed 5-6 days after the appearance of TUNEL+ cells in hippocampal neurons (Cho et al., 2003). In the basal forebrain, the distribution of active caspase-3-ir profiles was spatially correlated with that of TUNEL+ cells at the same ages examined (Sophou et al., 2006). The numerical density of active caspase-3-ir cells in the striatum and the nuclei of the basal forebrain during developing was lower than that of TUNEL+ nuclei in the same ages examined (Tanaka et al., 2000; Mellios et al., 2009). Lower numerical density of active caspase-3-ir cells than that of TUNEL+ nuclei was also reported in studies following lesions of connections during development (Sophou et al., 2006; Mellios et al., 2009). In the present study, the peak in the numerical density of the caspase-3-ir cells preceded by 3 days (at P7) the maximum numerical density of TUNEL+ profiles (at P10). The numerical density of active caspase-3-ir cells, in animals with congenital hypothyroidism was found significantly higher than the numerical density of TUNEL+ cells in the same ages during development and in the adult. Our results are in line with findings of a recent investigation, which has shown that the
cleaved caspase-3-positive nuclei outnumbered those showing TUNEL+ labeling at all times examined in excitotoxically-injured cortex of postnatal rats. This study also showed that, although some cells showed both cleaved caspase-3 and TUNEL staining, many cleaved caspase-3-positive cells did not colocalize with TUNEL and, furthermore, many TUNEL+ cells didn’t display cleaved caspase-3 labeling (Acarin et al., 2007). It is difficult in the present study to interpret the increased numerical density of active caspase-3-ir cells compared to the numerical density of TUNEL+ cells in hypothyroid animals. A rational could be that hypothyroidism increases the extent of apoptosis and its duration by down-regulating the anti-apoptotic genes \textit{Bcl-2} and \textit{Bcl-xL} and maintaining a high level of the pro-apoptotic gene \textit{Bax} in mitochondrial fraction, compared to a limited presence in euthyroid state (Muller et al., 1995; Singh et al., 2003a; Singh et al., 2003b). In the excitotoxic study of Acarin et al. (2007), the cleaved caspase-3 is not only associated with apoptotic cell death, but its activation is important for astroglial cytoskeleton remodelling following cellular injury. Furthermore, a recent work support the excitotoxic effects of hypothyroidism on cytoskeleton in the cerebral cortex of rats (Zamoner et al., 2008). These hyperphosphorylated subunits would accumulate in the cell body (Grant and Pant, 2000) contributing to the damage of the hypothyroid brain. The results of the action of cleaved caspase-3 on astroglial cytoskeleton could give an explanation (Acarin et al., 2007). The requirement for caspase-3 in DNA fragmentation is not restricted to neurons but may apply to all cell types (D’Mello et al., 2000). Thus, the possibility in our study that the outnumbered active caspase-3-ir cells are apoptotic cells of neuronal or glial origin and non-apoptotic cells cannot be excluded.

The reduced numerical density of “live” cells postnatally in combination with the widespread loss of cells in the hypothyroid animals until adulthood could be explained by the impaired neurogenesis and /or augmented cell death
during gestational and postnatal life of the congenital hypothyroidism. The present study demonstrates that congenital hypothyroidism significantly reduces the mean cross-sectional area of the striatum in adult animals. On the contrary, acquired hypothyroidism does not affect the cross-sectional area of the adult striatum. These data show that only the prolonged action of thyroid hormone deficiency on cell survival and/or differentiation results in the decrease of the size of the striatum in hypothyroid animals. Reduced cross-sectional area of the striatum has been observed in case of placental insufficiency, which accompanies significantly larger cerebral ventricles, reduced cross-sectional area of the cerebral cortex and reduced hippocampal volume; anatomical changes that resemble those found in some individuals with schizophrenia (Mallard et al., 1999). Although, it is not clear which factors could be responsible for these effects, the reduced levels of thyroid hormones may play a significant role in this reduction (Jones et al., 1990).

“Live” and apoptotic cells in the lateral septum of animals with congenital hypothyroidism during development

Based on the action of thyroid hormones, earlier studies demonstrated that thyroid hormones play an important role for the harmonious structural development of the CNS. The critical role of THs in neuronal cell proliferation, migration, differentiation, and maturation has been well established. Specifically, T3 promotes survival and differentiation of septal neurons in vitro (Filipcik et al., 1992). Although, the direct action of the THs on the lateral septum in the available literature is not elucidated, this study demonstrated that the numerical density of “live” cells in the lateral septum of hypothyroid animals was significantly decreased early in development at P0 and P3, compared to normal animals. The present study also showed increased numerical density of TUNEL+ cells in the developing lateral septum of hypothyroid animals, which
was more than twofold of those in normal control animals, at birth, at P3, at P10 (first peak), and at P21 (second peak). Although, the numerical density of dying cells in hypothyroid animals was more than double of those in normal control animals at P0 and P3, and high at P5, “live” cells in hypothyroid animals showed a significant reduction of their numerical density later, at P7. Similarly, a significant reduction of the numerical density of “live” cells in hypothyroid animals also observed at P21, after a peak in the numerical density of dying cells at P10. Thus, as in the striatum, the “live” cell population in hypothyroid animals showed a significant reduction a few days after the peak in the density of TUNEL+ cells. We assume that in the lateral septum embryonic hypothyroidism affect the numerical density of “live” cells after birth. However, congenital hypothyroidism did not cause a profound reduction of “live” septal neurons, as in the striatum. Therefore, it appears that hypothyroidism affects in different ways, different areas in the developing brain.

The results from normal control animals during development are in agreement with findings from other investigations, which have shown that in the normal rat lateral septum apoptosis occurs within the first month of postnatal development, being more intense during the first postnatal week (Tsukahara and Yamanouchi, 2003; Kouki et al., 2006). Several experimental manipulations have been shown to induce apoptosis in the lateral septum. In chronic neurotoxicity with sublethal or subclinical doses of organophosphorus ester apoptotic neuronal cell death has been observed in the lateral septum (Abou-Donia, 2003). Moreover, the lateral septum, among other brain areas (endopiriform and entorinal cortices, dorsal thalamus, hippocampus, and amygdala) is affected in soman (O-2,2 trimethypropil methylposphono-fluoridate) treated mice, with numerous TUNEL+ cells observed within the above mentioned areas (Baille et al., 2001).
The apoptotic morphology of dying cells was confirmed by the detection of active caspase-3, which is considered as a reliable apoptotic marker in developing and diseased brain (Cohen, 1997; Beer et al., 2000; D’Mello et al., 2000). The distribution of active caspase-3-ir profiles was spatially correlated with that of TUNEL+ cells at corresponding ages examined in hypothyroid animals. Active caspase-3-ir cells appeared more numerous in the ventral part of the developing lateral septum than in the dorsal or intermediate part. These results are in agreement with the results of Tsukahara and colleagues (2004), and Kouki and colleagues (2006), who also detected the greater number of active caspase-3-ir cells in the ventral part of the developing lateral septum. Another interesting observation in the present study was that the numerical density of caspase-3-ir cells was much higher than the numerical density of TUNEL+ cells at all corresponding time points, as observed in the striatum.

The numerical density of active caspase-3-ir profiles in animals with congenital hypothyroidism was found high at P0, increased markedly at P3, declined slightly at P5 and showed a peak at P7. The numerical density of active caspase-3-ir profiles was found significantly decreased at P60 compared to that at P7. In adult animals with acquired hypothyroidism the value of the numerical density of active caspase-3-ir cells was significantly lower than that of adult animals with congenital hypothyroidism. Similar results concerning the number of active caspase-3-ir cells were described in the ventral part of the developing lateral septum of the rat by Tsukahara et al. (2004).

According to our findings, apoptotic cells in the developing lateral septum are caspase-dependent and hypothyroidism affects in different ways different areas in the developing brain.
“Live” and apoptotic cells in the striatum and the lateral septum of adult hypothyroid animals

Evidence suggests that thyroid function abnormalities in adulthood may have profound behavioural consequences, such as anxiety, depressive symptoms and impaired memory (Davis and Tremont, 2007). Although the role of thyroid hormones in the adult brain is not yet specified, investigations in recent years have provided the molecular basis for thyroid hormone action in mature neurons. Concerning the effects of hypothyroidism on the striatum, it has been suggested that thyroid hormones might participate in regulating the muscarinic cholinergic neurotransmission in the striatum of adult rat (Iriuchijima et al., 1991). It has also been proposed that adult-onset hypothyroidism results in the hypoexpression of synaptic plasticity target genes in the striatum, which plays a key role in motor behaviour and procedural learning (Vallortigara et al., 2008).

In the present study we found that congenital hypothyroidism induce more apoptotic cell death in the striatum and the lateral septum than the acquired in the adult rat, and also the two types of hypothyroidism have differential effects on the final number of surviving cells in these areas. Specifically, apoptotic cells were found in both areas and in both animal models, in contrast to normal control animals, which never contained apoptotic cells in adulthood. These results are consistent with data from earlier studies which showed hypothyroidism-induced cell death in the granular layer of the dentate gyrus and in the cerebellum of the adult rat (Koibuchi and Chin, 2000), but no evidence of apoptosis in the striatum (Mellios et al., 2009), or other areas (Sophou et al., 2006; Zacharaki et al., 2010) of the normal adult rat brain.

In the striatum of congenital hypothyroid adult rats, the numerical density of “live” cells was significantly lower than that of normal control animals. In the lateral septum of congenital hypothyroid adult rats, the numerical density of “live” cells was similar to that of normal control animals. On the contrary, in
both the striatum and the lateral septum of adult animals with acquired hypothyroidism the numerical density of “live” cells was significantly higher than that of normal control animals. It therefore appears that although both types of hypothyroidism can elicit an apoptotic response of the adult brain, the long-lasting effects on the final cell number differ between developmentally-induced and adult-onset hypothyroidism.

The type of TUNEL+ cells observed in the striatum and the lateral septum during development and in the adult was not identified; however, our observations suggest that most dying cells were of neuronal origin. This assumption is based primarily on morphological criteria of active caspase-3-ir cells, in which we can better recognise the neuronal morphology than in TUNEL+ cells. A typical neuron is characterized by the morphology of the cell body, the presence of a well discernible nucleolus and the dendrites emanating from the cell body.

Evidence supports a role for THs in the regulation of adult neurogenesis and that the adult-onset of hypothyroidism significantly decreases the hippocampal neurogenesis (Ambrogini et al., 2005; Desouza et al., 2005). During postnatal propylthiouracil-induced hypothyroidism, pro-apoptotic Bax was up-regulated and anti-apoptotic Bcl-2 was down-regulated, both of which were normalized in the adult brain; in contrast apoptosis-inducing factor was down-regulated in the adult (Zhang et al., 2009). It has been suggested that thyroid hormones participate in the maintenance of the adult hippocampal neuronal population, and that they have different physiological roles as neuronal survival factors: T4 prevents the activation of apoptotic pathways, whereas T3 activates cell differentiation and proliferation mechanisms (Alva-Sánchez et al., 2009).

Considering data from the available literature in conjunction with the present findings, we suggest that in congenital hypothyroid animals the size of
the adult cell population in the striatum and the lateral septum is regulated through impaired neurogenesis and apoptosis that occur throughout development. In contrast, mechanisms in the adult-onset hypothyroid brain attempt to compensate for decreased neurogenesis and apoptotic death.

Apoptotic cells in forebrain structures of adult animals with congenital or acquired hypothyroidism

The present study showed that in adult animals with congenital or acquired hypothyroidism TUNEL+ cells and active caspase-3-ir cells were present, apart from the striatum and the lateral septum, in almost every forebrain structure. TUNEL+ cells and active caspase-3-ir cells were identified in the: anterior cingulate cortex (sparse cells), motor and somatosensory areas of the frontoparietal cortex (sparse cells), primary olfactory cortex, corpus callosum, nucleus accumbens, vertical and horizontal limbs of the diagonal band of Broca and islands of Calleja. The TUNEL+ cells and the caspase-3-ir cells appeared more densely distributed in the adult rats with congenital hypothyroidism, compared to animals with acquired hypothyroidism. Several experimental manipulations have been shown to induce apoptosis in the adult brain. Data from other studies showed that TUNEL+ cells were present in the penumbral cerebral cortex of adult rats with middle cerebral arterial occlusion. (Li and Zuo, 2009). In addition, radiation induced apoptosis in the subependyma and corpus callosum of the young adult rat brain (Bellinzona et al., 1996).

Thyroid hormones exert a crucial role in trophic events of the CNS during development, adulthood, and aging. Deficiency of thyroid hormones during critical periods of brain development leads to a dramatic impairment of mental performance (Porterfield and Hendrich, 1993), whilst in the adult human it results in various neurological symptoms including affective abnormalities and cognitive deficits, which can be so severe as to be defined “reversible dementia”
Moreover, a history of thyroid dysfunction has been cited as a possible risk factor for Alzheimer’s disease (Heyman et al., 1983). Neuronal cell migration is also, even more subtly, affected by hypothyroidism in the cerebral cortex. Deficiency of TH at the stage of the “inside–out migration” process causes alterations in the final structure of the adult neocortex, which displays a blurred layering and altered distribution of callosal connections (Gravel and Hawkes, 1990; Berbel et al., 1993; Lucio et al., 1997; Berbel et al., 2001). Hypothyroidism causes reduction of the neuropil in the olfactory bulb, the granular layers of the hippocampus and the cerebellum, which has as result an increment in cell density. However, a significant reduction in the number of nigral dopamine neurons, associated with a spontaneous unilateral circling behavior, was observed in a genetically hypothyroid mouse (Kincaid, 2001). Moreover, in birds, hyperthyroidism augmented neuronal death, which was not compensated by neuronal replacement (Tekumalla et al., 2002). Adult-onset hypothyroidism induces morphological alterations in CA3 pyramidal cells as seen by changes in cellular volume, cytoplasmic hyperchromasia, picnotic nuclei and nuclear material loss (Alva-Sánchez et al., 2004). In addition, the neuronal damage induced by hypothyroidism in adult hippocampus is related to an apoptotic process of neuronal death (Alva-Sánchez et al., 2007). The mechanism by which adult-onset hypothyroidism induces atrophy in pyramidal cells is still unknown, but it could be related to thyroid hormones regulating apoptosis, as has been demonstrated in other tissues (Alisi et al., 2005).
Effects of hypothyroidism on the survival and phenotype of GABAergic striatal neurons

Gamma-aminobutyric acid is the major inhibitory neurotransmitter in the striatum. The majority of GABA-containing neurons are projection neurons and represent the source of the direct striatonigral and indirect striatopallidal neural pathways of the basal ganglia (Kawaguchi et al., 1995; Cicchetti et al., 2000). These neurons, also called medium-sized spiny neurons, are estimated to constitute the 90% of the total neuronal population within the rat striatum (Wilson and Groves, 1980; Somogyi et al., 1981; Tepper et al., 2007). GABAergic neurons in the striatum of normal animals, display a polygonal or ovoid soma shape with one or more branched dendrites emanating from the soma poles (Mellios et al., 2009).

In the present study, we observed that in hypothyroid animals GABA-ir neurons were atrophic with shorter dendrites and reduced staining intensity. We also found that the numerical density of GABA-ir cells in adult animals with congenital or acquired hypothyroidism was reduced by 13.13% compared to normal control animals. The numerical density of GABA-ir cells in adult animals with congenital hypothyroidism was similar to that in animals with acquired hypothyroidism. These findings are in line with findings in the striatum of rats with cortical lesions or with 6-OHDA lesions of the catecholaminergic afferent systems. Both lesions induced a similar reduction in number, as well as phenotypic changes of GABAergic striatal neurons, such as atrophy and down-regulation of transmitter expression (Mellios et al., 2009).

The effects of thyroid dysfunction, i.e. hyperthyroidism or hypothyroidism, on GABAergic systems derive from clinical evidence in human nervous disorders. There is also experimental evidence in rats and mice indicating that thyroid hormones have effects on multiple components of the brain GABAergic system. These include effects on enzyme activities
responsible for the synthesis and degradation of GABA, levels of glutamate and GABA, GABA release and reuptake by neurons, and GABA\(_A\) receptor expression and function. There is also good evidence that GABA regulates the function of the thyroid system inhibiting TRS release from the pituitary (Wiens and Trudeau, 2006). Taken together, these data may explain the morphological alterations and the reduced number of GABA-ergic neurons found in this study and support the reciprocal regulation of the thyroid and GABA systems in vertebrates.

**Clinical implications**

Thyroid hormones have a multitude of effects on the central nervous system, and it is widely accepted that disturbances of mood and cognition often emerge in association with putative impairment of thyroid metabolism in the brain. The association between congenital hypothyroidism and profound mental retardation has been recognized and the extraordinary influence of thyroid hormones on the developing nervous system has been extensively studied. It is also accepted that THs continue to play a critical role in the adult brain, influencing mood and cognition. In patients with hypothyroidism, all behavioral and intellectual abnormalities are generally reversible following return to the euthyroid status, although some defects may persist in a subset of patients (Yen, 2001; Bauer et al., 2008; Williams, 2008).

The present study showed that congenital hypothyroidism augments apoptotic cell death in the developing striatum and lateral septum, and affects the number of “live” cells, through the reduction of their normal density after birth. Congenital and acquired hypothyroidisms induce apoptotic cell death in the adult forebrain, which never occurs in normal animals, and affect the survival and phenotype of GABAergic striatal neurons. These effects result in a significant shrinkage of the size of the adult striatum. Finally, the duration of
hypothyroidism (congenital or acquired) appears to differentially affect cell viability, with congenital having more profound effects.

These data provide the anatomical basis of thyroid deficiency, suggesting that the active process of apoptosis during brain development for the establishment of neuronal connections is also involved in aging in neurodegenerative disorders and in hormone disturbances.

**Conclusions**

The results of the present study lead to the following conclusions:

- Congenital hypothyroidism augments apoptotic cell death in the developing striatum and lateral septum.
- Congenital hypothyroidism affects the number of “live” cells, through the reduction of their normal density after birth in the striatum and the lateral septum.
- Congenital and acquired hypothyroidism induce apoptotic cell death in the adult forebrain, which never occurs in normal animals.
- Apoptotic cell death in the developing and adult forebrain is mediated by the activation of caspase-3.
- Congenital and acquired hypothyroidism affect the survival and phenotype of GABAergic striatal neurons.
- Congenital hypothyroidism induces a significant shrinkage of the size of the striatum in the adult.
- The duration of hypothyroidism (congenital or acquired) appears to differentially affect cell viability, with congenital having more profound effects.
Thyroid hormones (thyroxine, T4 and triiodothyronine, T3) play a pivotal role in differentiation, growth, and metabolism of nearly all tissues. In the brain, they have multiple actions on neurogenesis, neuronal cell migration and differentiation, synaptogenesis and myelination. Hypothyroidism during development results in profound mental retardation, deaf-mutism, and spastic diplegia, known in humans as cretinism. Adult thyroid dysfunction is also associated with both neurological and behavioral abnormalities. Recent studies relate hypothyroidism with programmed cell death (apoptosis) during brain development. This study aimed to evaluate the effect of hypothyroidism on cell survival in developing and adult rat forebrain structures in two experimental models of pharmacologically induced hypothyroidism; one with congenital and the other with acquired hypothyroidism. The selected brain areas for studying hypothyroidism were the striatum and the lateral septum.

Congenital hypothyroidism was pharmacologically induced by giving dams methimazole in their drinking water from the 9th gestational day and afterwards until the neonatal rats were sacrificed. Acquired hypothyroidism was pharmacologically induced by 4.5 weeks of treatment with methimazole in their drinking water, from postnatal day 35 until sacrifice. We used the TUNEL method for the *in situ* labeling of DNA fragmentation in dying cells. In addition, we used immunohistochemistry to detect the expression of the reliable apoptotic marker active caspase-3. Immunohistochemistry for GABA was used to examine the effect of hypothyroidism on the survival and phenotype of striatal neurons. For quantitative analysis, we also used cresyl violet staining (Nissl staining method) to determine the number of “live” cells in the striatum and the lateral septum of hypothyroid and normal animals.
The results of the present study showed that, hypothyroidism induces apoptotic cell death in the rat forebrain structures during development and in the adult. The apoptotic cell death is mediated by the activation of active caspase-3. Specifically, congenital hypothyroidism significantly increases the density of apoptotic cells in the developing striatum and lateral septum, compared to normal control animals. Our observations based on morphological criteria suggested that most dying cells were of neuronal origin. Congenital hypothyroidism also affects the number of “live” cells, reducing their normal density after birth in the two brain areas examined. On the contrary, acquired hypothyroidism increases the normal density of “live” cells in the above brain areas. Apoptotic neurons, which were never detected in normal adult animals, are present in almost all forebrain structures of adult hypothyroid animals. Our findings also revealed that the density of GABAergic cells in both groups of hypothyroid animals is significantly reduced (about 13.13%) compared to the density of normal control animals. The size of the striatum in adult animals with congenital hypothyroidism is also significantly reduced, compared to normal control animals. On the contrary, in animals with acquired hypothyroidism the corresponding size of the striatum was not affected. Finally, cell survival is dependent on the onset and duration of hypothyroidism.

The results of the present study lead to the following conclusions: 1) congenital hypothyroidism augments apoptotic cell death in the developing striatum and lateral septum; 2) congenital hypothyroidism affects the number of “live” cells, through the reduction of their normal density after birth in the striatum and the lateral septum; 3) congenital and acquired hypothyroidism induce apoptotic cell death in the adult forebrain, which never occurs in normal animals; 4) apoptotic cell death in the developing and adult forebrain is mediated by the activation of caspase-3; 5) congenital and acquired hypothyroidism affect the survival and phenotype of the GABAergic striatal
neurons; 6) congenital hypothyroidism induces a significant shrinkage of the size of the striatum in the adult; 7) the duration of hypothyroidism (congenital or acquired) appears to differentially affect cell viability, with congenital having more profound effects.
Περίληψη

Είναι γνωστό ότι οι ορμόνες του θυρεοειδούς αδένα (θυροξίνη, T4 και τριωδιοθυρονίνη, T3) παίζουν σημαντικό ρόλο στην ανάπτυξη, στη διαφοροποίηση και στον μεταβολισμό όλων σχεδόν των ιστών του σώματος. Στον εγκέφαλο εμφανίζουν πολλαπλή δράση αφού επηρεάζουν τη νευρογένεση, τη μετανάστευση και τη διαφοροποίηση των νευρικών κυττάρων, τον σχηματισμό συνάψεων και την εμμυέλωση των νευρικών νιόν. Ο υποθυρεοειδισμός κατά τη διάρκεια της ανάπτυξης έχει ως αποτέλεσμα πνευματική καθυστέρηση, κινητικές δυσλειτουργίες (αταξία, σπαστικότητα) και κώφωση, εκδηλώσεις γνωστές στον ανθρώπο ως κρετινισμός. Η Τ3 και η Τ4 προέρχονται από ιωδίωση της τυροσίνης του μορίου της θυρεοσφαιρίνης. Ο κύριος μηχανισμός δράσης των ορμονών αυτών ασκείται κυρίως μέσω των πυρηνικών υποδοχών τους, οι οποίοι ανιχνεύονται σε όλους τους ιστούς-στόχους. Πρόσφατα, έχουν βρεθεί ειδικές θέσεις δέσμευσης μεγάλης χημικής συγγένειας της Τ3 στα συναπτοσώματα και τις συναπτικές μεμβράνες και έχει αποδειχθεί ότι η Τ3 και όχι η Τ4 μπορεί να απελευθερωθεί στη συναπτική σχισμή με ασβεστιο-εξαρτώμενο τρόπο. Η διεργασία αυτή χαρακτηρίζει τους κλασικούς νευροδιαβιβαστές και προσδίδει στην Τ3 έναν πιθανό νευροδιαβιβαστικό-νευροτροποποιητικό ρόλο. Έχει αποδειχθεί ότι η έλλειψη των θυρεοειδικών ορμονών προκαλεί μείωση του μεγέθους και του αριθμού των πυραμιδοειδών νευρών του νεόφλοιου, των κοκκοειδών και των πυραμιδοειδών νευρών του υποκάμπου, των κυττάρων Purkinje της παρεγκεφαλίδας και των χολινεργικών κυττάρων του βασικού πρόσθιου εγκεφάλου, αλλά διαταράσσει και τη μορφολογία και την ανάπτυξη των δενδριτών σε ολόκληρο τον εγκέφαλο. Εντούτοις, οι ακριβείς μηχανισμοί μέσω των οποίων οι θυρεοειδικές ορμόνες ασκούν τη δράση τους παραμένουν αδιευκρίνιστοι. Πρόσφατες μελέτες εμπλέκουν τις θυρεοειδικές ορμόνες σε
αποπτωτικές διεργασίες, οι οποίες έχουν ελάχιστα μελετηθεί. Ειδικότερα, είναι γνωστό ότι η T3 επάγει την απόπτωση σε αρκετούς ιστούς κατά την οργανογένεση και κατά τη διαδικασία της διαφοροποίησης. Αντιθέτως, στον νευρικό ιστό και συγκεκριμένα σε καλλιέργειες κοκκοειδών κυττάρων παραγκεφαλίδας οι ορμόνες του θυρεοειδούς αδένα προάγουν την έκφραση του Bcl-2 αντιαποπτωτικού παράγοντα. Οι παρατηρήσεις αυτές δείχνουν ότι οι θυρεοειδικές ορμόνες εμπλέκονται στο μηχανισμό της απόπτωσης και πιθανότατα η δράση τους είναι ιστοειδική. Είναι πλέον αποδεκτό ότι οι μηχανισμοί επιβίωσης και απόπτωσης των νευρικών κυττάρων κατά την ανάπτυξη του εγκεφάλου σχετίζονται με τους μηχανισμούς του οδηγού σε απώλεια κυττάρων στο γήρας, σε νευροεγκεφαλικές ασθένειες, μετά από κρανιοεγκεφαλικές κακώσεις και σε ορμονικές διαταραχές. Από τα ανωτέρω γίνεται προφανές ότι δεν έχει διευκρινιστεί πλήρως ο ρόλος των ορμονών του θυρεοειδούς και η εμπλοκή τους στους μηχανισμούς της απόπτωσης και δεν έχει μελετηθεί η απόπτωση σε περιοχές του εγκεφάλου του ευμίου, πλην της παραγκεφαλίδας, κατά την ανάπτυξη και στο ενήλικο σε κατάσταση υποθυρεοειδισμού. Στην έρευνα αυτή μελετήθηκαν η επίδραση του νεογνικού υποθυρεοειδισμού στην απόπτωση των νευρικών κυττάρων σε περιοχές του τελικού πρόσθιου εγκεφάλου, όπως είναι το νεοαραβιδοτό σώμα και ο έξω πυρήνας του διαφράγματος κατά την ανάπτυξη και στο ενήλικο επίμυο, αλλά και η επίδραση του επίκτητου υποθυρεοειδισμού στην απόπτωση νευρώνων στις ίδιες περιοχές ενήλικων επιμύων. Για τη μελέτη αυτή χρησιμοποιήθηκαν ιστοχημικές και ανοσοϊστοχημικές μέθοδοι, για να διερευνηθούν η κατανομή και η πυκνότητα των αποπτωτικών κυττάρων στις παραστάσεις περιοχές του εγκεφάλου και τα αποτελέσματα συγκρίθηκαν με αντίστοιχα αποτελέσματα από φυσιολογικός αναπτυσσόμενα αλλά και ενήλικα ζώα, τα οποία χρησιμοποιήθηκαν ως μάρτυρες. Ελέγχθηκε επίσης η έκφραση της ενεργοποιημένης κασπάσης-3, η οποία αποτελεί αξιόπιστο δείκτη της
απόπτωσης, καθώς και η επίδραση του νεογνικού και επίκτητου υποθυρεοειδισμού στην επιβίωση των GABAεργικών νευρώνων του νεοραβδωτού σώματος.

Τα πειραματόζωα που χρησιμοποιήθηκαν προέρχονται από εκτροφή του Εργαστηρίου Ανατομικής, Ιστολογίας και Εμβρυολογίας της Κτηνιατρικής Σχολής του Α.Π.Θ., στην οποία τηρούνται όλες οι προδιαγραφές που προβλέπονται στις οδηγίες της Ευρωπαϊκής Ένωσης για την προστασία των πειραματόζωων (Οδηγία της 24ης Νοεμβρίου 1986, με αριθμό 86/609/EEC). Οι υποθυρεοειδικοί αναπτυσσόμενοι επίμυες προέρχονται από μητέρες στις οποίες χορηγήθηκε 0,025% w/v 1-μεθυλ-2-μερκαπτοϊμιδαζόλη (μεθιμαζόλη, ΜΜΙ) στο πόσιμο νερό ad libitum από την 9η μέρα της κύησης. Ο υποθυρεοειδισμός σε ενήλικα ζώα προκλήθηκε με χορήγηση σε επίμυες ηλικίας 35 ημερών της ίδιας ποσότητας ΜΜΙ στο πόσιμο νερό για 4,5 εβδομάδες. Η ΜΜΙ εμποδίζει τη σύνθεση των ορμονών του υποθυρεοειδούς αδένα, αποτρέποντας την ιωδίωση της θυρεοσφαιρίνης, τόσο στη μητέρα όσο και στο έμβρυο, αφού περνά εύκολα τον πλακούντα και εκκρίνεται με το γάλα της μητέρας, διατηρώντας έτσι τον υποθυρεοειδισμό και στους νεογέννητους επίμυες. Στην έρευνα αυτή χρησιμοποιήθηκαν υποθυρεοειδικοί και φυσιολογικοί επίμυες (μάρτυρες) ηλικίας 0 ημερών (ημέρα γέννησης, μεταγεννητική ημέρα M0), M1, M3, M5, M7, M10, M14, M21, M60 και M70, οι οποίοι διαχωρίστηκαν τυχαία στις δύο πειραματικές ομάδες. Τα επίπεδα των θυρεοειδικών ορμονών προσδιορίστηκαν με ραδιοανοσολογική μέθοδο (RIA) στο πλάσμα του αίματος, που συλλέχτηκε κατά την ευθανασία των πειραματόζωων. Για τη σήμανση των αποπτωτικών κυττάρων στις τομές παραφίνης από τους παραπάνω εγκεφάλους χρησιμοποιήθηκε ένα τροποποιημένο πρωτόκολλο της μεθόδου TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labelling). Αντιγεύτηκε, επίσης, ανοσοϊστοχημικά η ενεργός κασπάση-3, δείκτης που σχετίζεται με το φαινόμενο της απόπτωσης. Για την ταυτοποίηση
του είδους των αποπτωτικών κυττάρων (νευρικά-νευρογλοιακά) ελέγχθηκαν τα μορφολογικά χαρακτηριστικά των αποπτωτικών κυττάρων σε ημίλεπτες τομές που είχαν αντιχρωματισθεί με κυανό της τολουϊδίνης, και των κασπάση-3-ανοσοδραστικών κυττάρων. Επίσης μελετήθηκε η επίδραση του υποθυρεοειδίσμου στην επιβίωση των ΓΑΒΑεργικών νευρώνων του ραβδώτου σώματος, οι οποίοι αποτελούν ποσοστό πάνω από 90% των νευρώνων του. Τα αποτελέσματα συγκρίθηκαν με αντίστοιχα αποτελέσματα από φυσιολογικός αναπτυσσόμενα και ενήλικα ζώα. Για τη στατιστική ανάλυση και αξιολόγηση των πειραματικών δεδομένων χρησιμοποιήθηκε η μεθοδολογία της ανάλυσης των διακυμάνσεων, σύμφωνα με το παραγωγικό σχέδιο ανάλυσης και τις κατάλληλες στατιστικές δοκιμασίες.

Τα αποτελέσματα έδειξαν ότι ο νεογνικός υποθυρεοειδίσμος αύξησε την πυκνότητα των αποπτωτικών κυττάρων κατά τη διάρκεια της ανάπτυξης στο νεοραβδώτο σώμα και στον έξω πυρήνα του διαφράγματος, σε σύγκριση με την αποτελέσματα των αποπτωτικών κυττάρων στα φυσιολογικά ζώα. Απόψεις, επίσης, παρατηρήθηκε στα ενήλικα υποθυρεοειδικά ζώα σε όλους τους πυρήνες του τελικού εγκεφάλου, εύρημα το οποίο ουδέποτε έχει παρατηρηθεί σε φυσιολογικά ενήλικα ζώα. Ο επίκτητος υποθυρεοειδίσμος προκάλεσε επίσης αποπτωτικό κυτταρικό θάνατο σε ενήλικα ζώα σε όλους τους πυρήνες του τελικού εγκεφάλου, αν και η πυκνότητα κατανομής των κυττάρων αυτών ήταν μικρότερη της πυκνότητας κατανομής των αποπτωτικών κυττάρων σε ενήλικα ζώα με νεογνικό υποθυρεοειδίσμο. Τα αποπτωτικά κύτταρα εμφανίζονταν στην πλειοψηφία τους μορφολογικά χαρακτηριστικά νευρικών κυττάρων.

Η δράση του νεογνικού υποθυρεοειδίσμου είχε ως αποτέλεσμα την μείωση της πυκνότητας των «ζωντανών» κυττάρων στο νεοραβδώτο σώμα και στον έξω πυρήνα του διαφράγματος κατά τη διάρκεια της ανάπτυξης. Επίσης, ο νεογνικός αλλά και ο επίκτητος υποθυρεοειδίσμος μείωσαν την πυκνότητα των
Δημιουργία κυττάρων του νεοραβδωτού σώματος σε ενήλικα ζώα σε σύγκριση με την πυκνότητα των ίδιων κυττάρων στα φυσιολογικά ζώα. Τέλος, ο νεογνικός υποθυρεοειδισμός είχε ως αποτέλεσμα τη συρρίκνωση του νεοραβδωτού σώματος στα ενήλικα ζώα. Αντιθέτως, παρόμοια δράση δεν παρατηρήθηκε σε ζώα με επίκτητο υποθυρεοειδισμό.

Από την παρούσα μελέτη προκύπτουν τα εξής συμπεράσματα: 1) ο νεογνικός υποθυρεοειδισμός αύξησε τον αποπτωτικό κυτταρικό θάνατο στο αναπτυσσόμενο νεοραβδωτό σώμα και στον εξω πυρήνα του διαφράγματος, 2) ο νεογνικός υποθυρεοειδισμός μείωσε τη φυσιολογική πυκνότητα των "ζωντανών" κυττάρων μετά τη γέννηση στο νεοραβδωτό σώμα και στον εξω πυρήνα του διαφράγματος, 3) ο νεογνικός και ο επίκτητος υποθυρεοειδισμός προκάλεσαν αποπτωτικό κυτταρικό θάνατο στους πυρήνες του τελικού εγκέφαλου, ο οποίος ουδέποτε έχει παρατηρηθεί σε φυσιολογικά ζώα, 4) ο αποπτωτικός κυτταρικός θάνατος στους πυρήνες του τελικού εγκέφαλου προκαλείται από την ενεργοποίηση της κασπάσης-3 τόσο κατά την ανάπτυξη όσο και στα ενήλικα ζώα, 5) ο νεογνικός και ο επίκτητος υποθυρεοειδισμός επηρεάζουν την επιβίωση και τον φαινότυπο των GABAεργικών νευρώνων του νεοραβδωτού σώματος, 6) ο νεογνικός υποθυρεοειδισμός προκαλεί συρρίκνωση του μεγέθους του νεοραβδωτού σώματος σε ενήλικα ζώα, 7) η διάρκεια του υποθυρεοειδισμού (νεογνικός ή επίκτητος) φαίνεται να επηρεάζει με διαφορετικό τρόπο την επιβίωση των κυττάρων στο τελικό εγκέφαλο, με τον νεογνικό να προκαλεί επιδείνωση στο φαινόμενο του αποπτωτικού κυτταρικού θανάτου.  

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تأثر نقص نشاط الغدة الدرقية على موت الخلايا الطبيعي في تراكيب الفص الأمامي للمخ أثناء المرحلة الجنينية للجرذان والجرذان البالغة.

تلعب هرمونات الغدة الدرقية (هرمون التايروكسيسين T4 وهرمون التراي أيودوثيرونين T3) دوراً محورياً في عملية التحور والنمو والأيض في معظم نسخة الجسم؛ ففي المخ توجد لها تأثيرات عديدة على عمله تكوين الخلايا العصبية وتجهيزها وتجويرها وتكون المادا الشحمية التي تسمى بالمياليين أو النخاعيين (myelination) بالإضافة إلى تأثيرها على تخليق الشباك والمياليين. ومن ثم فإن نقص هرمونات الغدة الدرقية أثناء مراحل تطور الجنين يؤدي إلى تأخر عصبي كبير ومضاعفات عصبية، والذي يعرف في الإنسان بمرض القصامة (cretinism).

ووجد أن إعتلال الغدة الدرقية في الحيوانات البالغة يرتبط بحالات الخلل العصبي والخلل السلوكي، أما الدراسات الحديثة فقد تناولت العلاقة بين نقص هرمونات الغدة الدرقية وإزداد حدوث موت طبيعي للخلايا (apoptosis) أو ما يعرف بالاستماتة أثناء تطور المخ.

وتهدف الدراسة الحالية إلى رصد تأثير نقص هرمونات الغدة الدرقية على بقاء الخلايا في الفص الأمامي للمخ لأجنة الجرذان وفي الجرذان البالغة، وقد احتضنت الدراسة منطقتين في الفص الأمامي للمخ لدراسة هذا النقص الهرموني وآلا وهو الجسم المخاط والجزء الوحشي من الحاجز الجانبي من خلال نموذجين تجريبيين حيث يتم إحداث نقص مستحث في هرمونات الغدة الدرقية الأول في الأجنة يمثل النقص الموروث أو الخلقي والآخر في الجرذان بعد الولادة والذي يمثل النقص المكتسب، وفي النموذج الأول تم إعطاء الإدمامات عطار الميثيمزول في مياه الشرب من اليوم التاسع للحمل، مع استمرار إضافة هذا العطار بعد الولادة أيضًا لكي تتمكن الجرذان الوليدة من تناوله لحين إعدادها، أما بالنسبة للنموذج الثاني فقد تم إعطاء العطار ذاته في مياه الشرب من اليوم الخامس والثلاثون بعد الولادة ولمدة أربعة أسابيع ونصف أي حتى عمر سبعون يوماً للحصول على نقص هرمونات الغدة الدرقية المكتسب.

كما استندت الدراسة على استخدام طريقة اختبار التحليل العصبي لتسلسل تدمير الخلايا الكيميائي في الخلايا المستماة. هذا بالإضافة إلى استخدام مجموعة مماثلة من التبادلات الكيميائية (Immunohistochemistry for Caspase-3) للكشف عن تدمير الخلايا المتكسية. وإضافة إلى استخدام نوعية من التبادلات الكيميائية للكشف عن تأثير نقص هرمونات الغدة الدرقية (Immunohistochemistry for GABA) في الخلايا المستماة.
الغدة الدرقية على النمط الظاهري لخلايا العصبية في الجسم المخطط وبيقاها. كما استخدمنا أيضاً
طريقة صبغة النيس (Nissl staining method) للتحليل الكمي لحصر عدد الخلايا الحية الموجودة
في الجسم المخطط، والحاجز الجانبي في كل من الحيوانات الصلبة و الحيوانات التي تعاني من نقص في
هرمونات الغدة الدرقية.

وأظهرت نتائج الدراسة الحالية أن نقص هرمونات الغدة الدرقية يسبب موت طبيعي للخلايا الفص
الأمامي للمخ (إثمتانة) في الجرذان أثناء نموها، وفي البالغ منها. إضافة إلى ذلك فإن هذه الإستمتانة
الخلوية تحدث بواسطة تنشيط الكاسبيز-3 النشط. ومن الملاحظ ويشمل في حالات نقص
هرمونات الغدة الدرقية الخلفي أن كثافة الخلايا المستمتانة تزداد بشكل ملحوظ في الجسم المخطط والحاجز
الجانبي في الحيوانات النامية مقارنة بخلايا المجموعة الضابطة الطبية، كما ولاحظ أن أكثر الخلايا
المحضرة كانت من أصل عصبي وذلك استنادا على المعايير المورفولوجية، وأن النقص الخلفي لهذه
الهرمونات يؤثر على عدد الخلايا الحية في نفس المناطق سابقة الذكر مما يخفض كثافتهم الطبيعية بعد
الولادة. وعلى النقيض فإن النقص المكتمب لهرمونات الغدة الدرقية يزيد الكثافة الطبيعية للخلايا الحية
في الفص الأمامي للمخ.

كما أوضحنا الدراسة أيضاً أن هذه الخلايا العصبية المستمتانة موجودة تقريبا في كل تركيب
الفص الأمامي للمخ للحيوانات المصابة بنقص هرمونات الغدة الدرقية البالغة، التي لم يشار إليها في
الدراسات المتاحة التي تناولت دراسة الاستمتانة في الحيوانات البالغة الضابطة. و كذلك تبين أن كثافة
الخلايا الناقلة للنمط الظاهري في كلتا المجموعتين للحيوانات المصابة بنقص الهرمونات الدرقية تنخفض
بشكل ملحوظ بمقدار 13.13% بمقارنة بخلايا المجموعة الضابطة، وحول العلاقة بين حجم الجسم
المخطط ونقص هرمونات الغدة الدرقية؛ فقد تبين من خلال الدراسة إنخفاض الحجم بشكل ملحوظ في
النموذج الموروث أو الخلفي مقارنة بمجموعة الحيوانات الضابطة، في حين أن المجموعة التي تعاني من
نقص الهرمونات المكتسبة لم تؤثر على الحجم المطلب للجسم المخطط.

وأخيرا فإن بقاء الخلايا العصبية يعتمد على توقيت ومدى نقص هرمون الغدة الدرقي.

- ومن خلال هذه الدراسة نستنتج أن:
  1- النقص الموروث أو الخلفي لهرمونات الغدة الدرقية يحفز موت الخلايا المبرمج (الإثمتانة) في
  الجسم المخطط والحاجز الجانبي للحيوانات النامية.
النقص الهرموني الموروث أو الخلقي يؤثر على عدد الخلايا العصبية الحية خلال فجوات كثافتها الطبيعية بعد الولادة في الجسم المخطط والحاجز الجانب.

3- نقص هرمونات الغدة الدرقية بنوع واحد يؤدي إلى موت الخلايا العصبية الطبيعي في الفص الأمامي للدماغ للحيوانات البالغة، والذي لم يحدث في الحيوانات الطبيعية.

4- موت الخلايا الإسماتي في الفص الأمامي للدماغ للحيوانات البالغة والبالغة يحدث بواسطة تنشيط الكاسبيز-3.

5- نقص هرمونات الغدة الدرقية بنوع واحد يؤثر على النمط الظاهري وفاة الخلايا العصبية الناقصة للنهايات الظاهرية الموجودة في الجسم المخطط.

6- نقص هرمونات الغدة الدرقية الولد أو الخلق يسبب إنكماشاً محظياً في حجم الجسم المخطط للحيوانات البالغة.

7- الفترة الزمنية لنقص هرمونات الغدة الدرقية التي يتعرض لها الحيوان تلعب دوراً هاماً في التأثير على حبيبة الخلايا العصبية مع الأخذ في الاعتبار أن النقص الموروث أو الخلقي له تأثير أكثر حدة.
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