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Oral-examination:: .....

A transgenic strategy to define SLCO1C1-expressing structures during brain development

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Prof. Dr. Markus Schwaninger

Dedicated to my parents

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbst verfaßt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Des Weiteren erkläre ich, daß ich an keiner anderen Stelle ein Prüfungsverfahren beantragt oder die Dissertation in dieser oder einer anderen Form bereits anderweitig als Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt habe.

Heidelberg, _____ _____

Ming-Fei Lang

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Summary

Thyroid hormones (THs) are important for brain development. Maternal hypothyroxinemia, low maternal thyroxine levels, is a major cause of defects in fetal brain development. In adults, thyroxine enters brain through the thyroxine transporter SLCO1C1, which is expressed in endothelial cells of the blood-brain barrier (BBB) and in epithelial cells of the choroid plexus. Till now, SLCO1C1 is the only known transporter that mediates the entry of thyroxine into the adult brain. However, the expression pattern of SLCO1C1 during development was unknown.

To characterize the expression of SLCO1C1 in development, a transgenic approach was employed by bacterial artificial chromosome (BAC) recombineering. Sequences of Cre recombinase (constitutive) or Cre recombinase fused to a mutated estrogen receptor ligand binding domain (inducible) were inserted into the Slco1c1 locus on a BAC by homologous recombination. The modified BACs were subsequently injected into pronuclei to generate mice carrying the Slco1c1-driven Cre, which is expressed either constitutively or in an inducible manner.

In the transgenic mice, both Cre and Slco1c1 transcripts could be detected by RT-PCR only in the brain. Mice from the constitutive line showed Cre expression in BBB and choroid plexus according to immunohistochemistry. This localization was confirmed by crossing mice with a reporter mouse line. In addition, neurons in many brain regions (such as cortical layer 2/3, the hippocampus and olfactory bulbs) expressed Cre during brain development, since Cre activity could not be detected in adult neurons but Cre-mediated recombination was found in the neurogenic zones at E14 and E18. In the inducible line, no Cre activity in the adult neurons was found, which also confirmed an early neuronal expression of Slco1c1-Cre. The neuronal expression of Cre in development indicates a role of thyroxine in the SLCO1C1-expressing neurons. Hypothyroxinemia induced by propyl-thio-uracil (PTU) decreased the number of cortical layer 2/3 SLCO1C1-expressing neurons.

Furthermore, a role of SLCO1C1 in adult neurogenesis was also investigated. By RT-PCR, Slco1c1 transcripts could be detected in cultured neural stem cells (NSC). In adults, Cre colocalized with a neural stem cell marker, GFAP, in the subventricular zone. Both under normal conditions and in a stroke model, Cre was found to colocalize with an immature neuronal marker, Dcx, near the subventricular zone.

In conclusion, Slco1c1-Cre transgenic mouse lines were generated and were used to identify Slco1c1-expressing brain structures during development. SLCO1C1 may be involved in the effects of thyroid hormones on neurogenesis.

Zusammenfassung

Schilddrüsenhormone sind wichtig für die Entwicklung des Gehirns. Ein zu geringer Thyroxinspiegel der Mutter ist die Hauptursache für Fehlbildungen des fötalen Gehirns. Bei Erwachsenen gelangt das Hormon Thyroxin über den Thyroxintransporter SLCO1C1, der auf Endothelzellen der Blut-Hirn-Schranke und Epithelzellen des Plexus choroideus exprimiert wird, ins Gehirn. SLCO1C1 ist bisher der einzig bekannte Transporter, der die Aufnahme von Thyroxin ins adulte Gehirn vermittelt. Das Expressionsprofil von SLCO1C1 während der Entwicklung war bisher allerdings unbekannt. Um die Expression von SLCO1C1 während der Entwicklung zu untersuchen, wurde ein transgener Ansatz mittels BAC Rekombination gewählt. Die Nukleotidsequenz der Cre Rekombinase (konstitutiv) oder der Cre Rekombinase, die an die mutierte Ligandenbindungsstelle des Östrogenrezeptors fusioniert wurde (induzierbar), wurde auf dem BAC mittels homologer Rekombination in den Genlokus von Slco1c1 eingefügt. Die modifizierten BACS wurden anschließend in den Pronukleus von Mäusen injiziert um transgene Tiere zu generieren, welche die Cre Rekombinase im Slco1c1 Locus entweder konstitutiv oder induzierbar exprimieren.

In diesen transgenen Mäusen konnten sowohl die Transkripte der Cre also auch von Slco1c1 exklusiv im Gehirn mittels RT-PCR nachgewiesen werden. In der Immunhistochemie wiesen Mäuse aus der konstitutiven Linie Cre Expression in den Endothelzellen der Blut-Hirn-Schranke und im Plexus choroideus auf. Dieses Expressionsmuster wurde durch die Verpaarung der Cre-Linien mit einer Reportermauslinie bestätigt.

Außerdem konnte gezeigt werden, dass Cre während der Entwicklung in Neuronen vieler Hirnregionen (wie in der kortikalen Ebene 2/3, dem Hippokampus und dem Bulbus olfactorius) aktiv ist. Cre-vermittelte Rekombination wurde in den neurogenen Zonen an Tag E14 und E18 nachgewiesen, während keine Cre Aktivität in adulten Neuronen vorhanden war.

In der induzierbaren Cre Linie konnte nach Tamoxifen-Injektion keine Cre Aktivität in adulten Neuronen gefunden werden passend zu einer embryonalen transkriptionellen Aktivität des Slco1c1-Locus in neuronalen Progenitorzellen. Die neuronale Expression von Cre während der Entwicklung lässt vermuten, dass Thyroxin eine Rolle in der Expression von SLCO1C1 spielt. Hypothyroxinämie, die durch die Gabe von Propylthiouracil (PTU) induziert wurde, reduzierte die Anzahl der Neuronen in den kortikalen Schichten 2/3, die von SLCO1C1 exprimierenden Progenitorzellen abstammen.

Weiterhin wurde die Rolle von SLCO1C1 in der adulten Neurogenese untersucht. Durch RT-PCR konnten Slco1c1 Transkripte in kultivierten neuronalen Stammzellen (NSC) nachgewiesen werden. In adulten

Slco1c1-Cre Tieren waren Cre und der neuronale Stammzellmarker GFAP in der Subventrikulärzone kolokalisiert. Sowohl unter Kontrollbedingungen als auch nach einer zerebralen Ischämie konnte gezeigt werden, dass die Lokalisation von Cre mit dem Marker für unreife Neuronen Dcx nahe der Subventrikulärzone übereinstimmt .

Zusammenfassend wurde die Slco1c1-Cre transgene Mauslinie generiert und genutzt, um Areale des Gehirns zu identifizieren, in denen Slco1c1 während der Entwicklung exprimiert wird. SLCO1C1 ist möglicherweise an den Effekten von Schilddrüsenhormonen bei der Neurogenese beteiligt.

Contents

1. Introduction

Thyroid hormones (THs) are synthesized and secreted from the thyroid gland. When reaching different tissues through blood, the active form, 3,5,3'-triiodothyronine (T3), can be produced locally from its precursor, thyroxine (T4). The local production of T3 is achieved by deiodinases, which belong to selenoenzymes (Bianco et al. 2002; Bianco and Kim 2006; Kohrle 2007). T3 is believed to contribute to most of the actions of thyroid hormones. Since T4 composes most of the secreted THs, its local conversion to T3 is very important for cellular development, growth and maintenance. T3 functions as a ligand of thyroid hormone receptors (TRs). TRs are nuclear hormone receptors. Upon T3 binding, they form heterodimers with retinoid X receptors (RXRs). The TR/RXR complex is important for binding to the TH-responsive elements (TREs) in the promoter region of the target genes (Fig. 1.1A). By this nuclear receptor-mediated mechanism, THs act on metabolism, growth and differentiation of cells (Wu and Koenig 2000; Yen et al. 2006). In addition to the regulation of gene transcription in the nucleus, THs also exert non-genomic effects through binding to cytoplasmic TRs or other proteins that can bind THs (Yen 2001; Oetting and Yen 2007; Leonard 2008). The amount of THs secreted from the thyroid gland is tightly controlled by a hypothalamic-pituitary-thyroid axis (Fig. 1.1B).

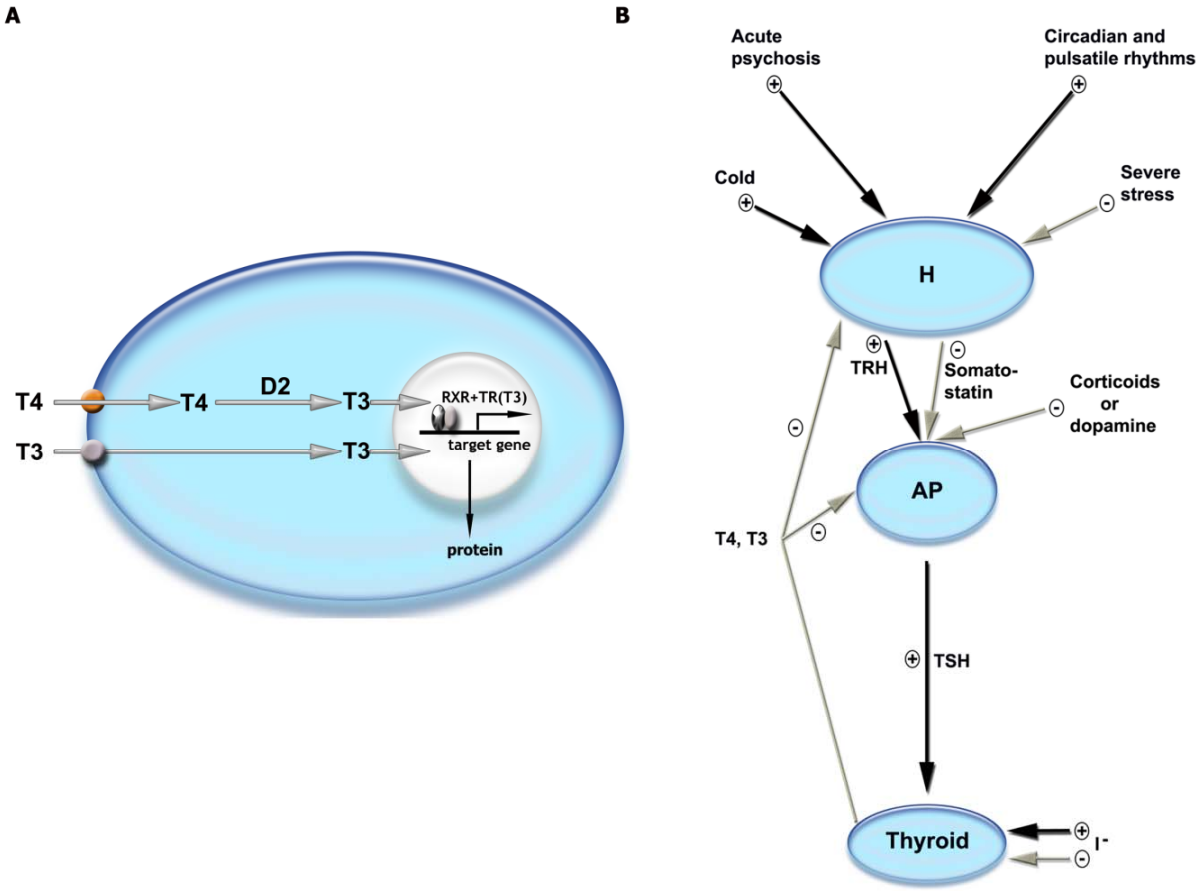

Fig.1.1 Thyroid hormone action and the hypothalamic-pituitary-thyroid axis. A. Both thyroid hormones (T4 and T3) enter cells, where T4 is converted to the active form T3 by deiodinase 2 (D2). T3 binds to the nuclear receptor TR. Together with the retinoid X receptor (RXR), T3-bound TR interacts with the promoter of the target genes to regulate their expression. B. Acute psychosis or prolonged exposure to cold may activate the axis. Hypothalamic TRH stimulates pituitary TSH release, while somatostatin and dopamine inhibit it. TSH stimulates T4 and T3 synthesis and release from the thyroid, and they in turn inhibit both TRH and TSH synthesis and release. Small amounts of iodide are necessary for hormone production, but large amounts inhibit T3 and T4 production and release. Abbreviations: H, hypothalamus; AP, anterior pituitary; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; I^-, iodide. (Black arrows, stimulatory influence; grey arrows, inhibitory influence) Adapted from (Basic & Clinical Pharmacology 1998).

Current models of TH metabolism in the brain

There are two models of TH metabolism in the brain. The most prevailing model is shown in Fig. 1.2. This model is based on the expression of TH transporters and deiodinases. TH transporters are important for the cellular relocation and deiodinases are important for the metabolism. Recently a number of reviews discussed TH utilization by the brain in the context of this model (Heuer 2007; Schwartz and Stevenson 2007; Visser et al. 2007).

SLCO1C1 is a T4 transporter and its expression is found specifically in brain capillary endothelial cells (BBB) and choroid plexus. Thus, SLCO1C1 mediates the entry of T4 into the brain parenchyma. What thyroid hormone transporters are expressed in astrocytes is less well known. There is controversy about whether SLCO1C1 is expressed in astrocytes. The GENSAT project shows possible Slco1c1 expression in glia-like cells by transgenically expressing EGFP under the control of the Slco1c1 locus (GENSAT). Another study examined the expression of Slco1c1 in glia by Northern blotting (Chu et al. 2008), but did not find Slco1c1 in either a cultured glial cell line or glial tumor. Therefore, it is unknown which transporter brings T4 into astrocytes. In astrocytes, deiodinase 2 (D2) is expressed, which converts T4 into T3 (Bianco et al. 2002; Bianco and Kim 2006). Locally converted T3 is released from astrocytes by another unknown transporter and is taken up by neighboring neurons through MCT8. The TH inactivating enzyme D3 is primarily expressed in neurons.

Altogether the BBB expression of the T4 transporter SLCO1C1, the astrocyte expression of D2, and the neuron expression of MCT8 and D3 form a chain of TH transportation and metabolism in the CNS. The severe neurological dysfunction in Allan–Hernon–Dudley syndrome due to genetic MCT8 inactivation in human strongly supports that neurons cannot directly use T4 to compensate for their deficiency in T3 uptake during development. This model is also supported in pathological conditions. T4 infusion, instead of T3, improves neurological outcome after cardiac arrest induced global cerebral ischemia (D'Alecy 1997). This effect is mediated by an upregulation of D2 activity in astrocytes (Margaill et al. 2005).

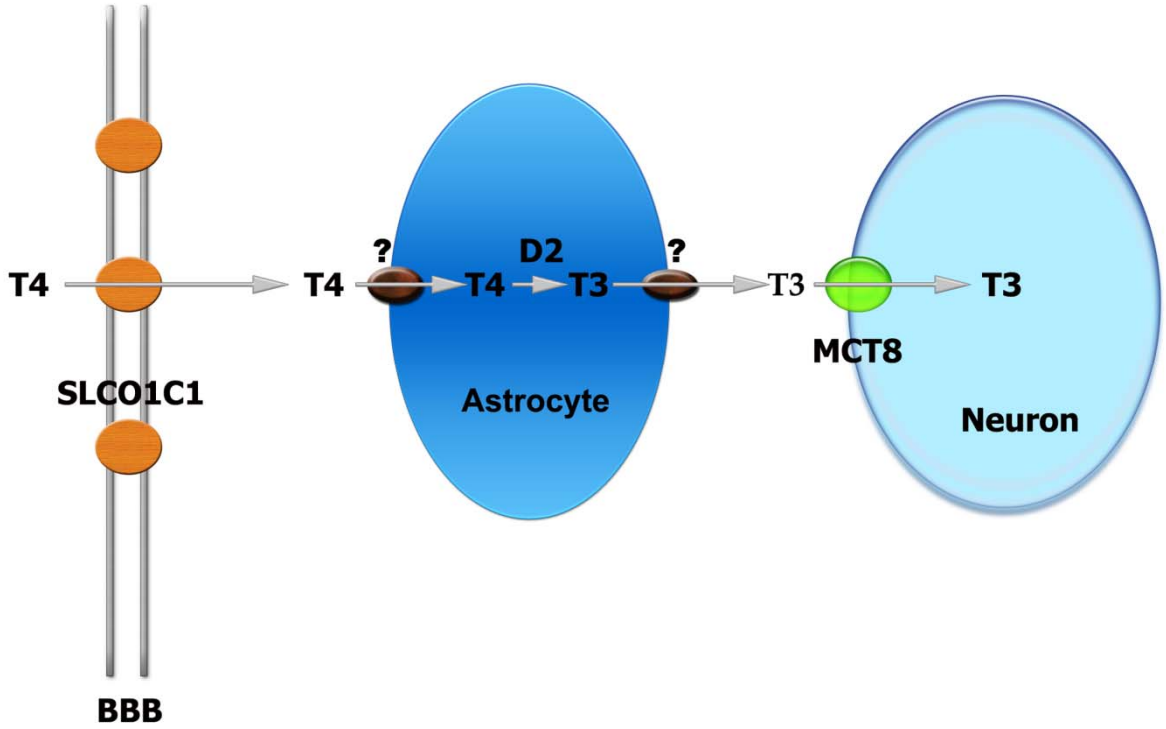

Fig. 1.2 The most popular model of T4 transport in the brain. Thyroxine (T4) from blood gets its entry into the brain parenchyma through a thyroid hormone transporter SLCO1C1 expressed in the endothelial cells of the BBB. Then by an unknown transporter, T4 is transported into astrocytes, where it is converted to T3 by deiodinase 2 (D2). Another unknown transporter released T3 from the astrocytes, which enters neurons by neuronal expressed MCT8.

The second model is just emerging (Fig. 1.3). This model challenges the first by proposing that both locally generated and serum/CSF-derived T3 may be used by neurons. D2 activity is considered in the first model to be the only source of brain T3. But the D2 knockout mice show very mild defect in neural development (Galton et al. 2007). Thus, an alternative explanation is that serum/CSF-derived T3 could enter the brain in an amount that mitigates partially the T3 deficiency in brain development. Brain T3 concentration is only reduced by 50% in D2KO mice, while T4 increases as expected. The T4 increase corresponds well to the decrease in T3, which is also about 50%. This may indicate that serum/CSF-derived T3 accounts for half of the total brain T3.

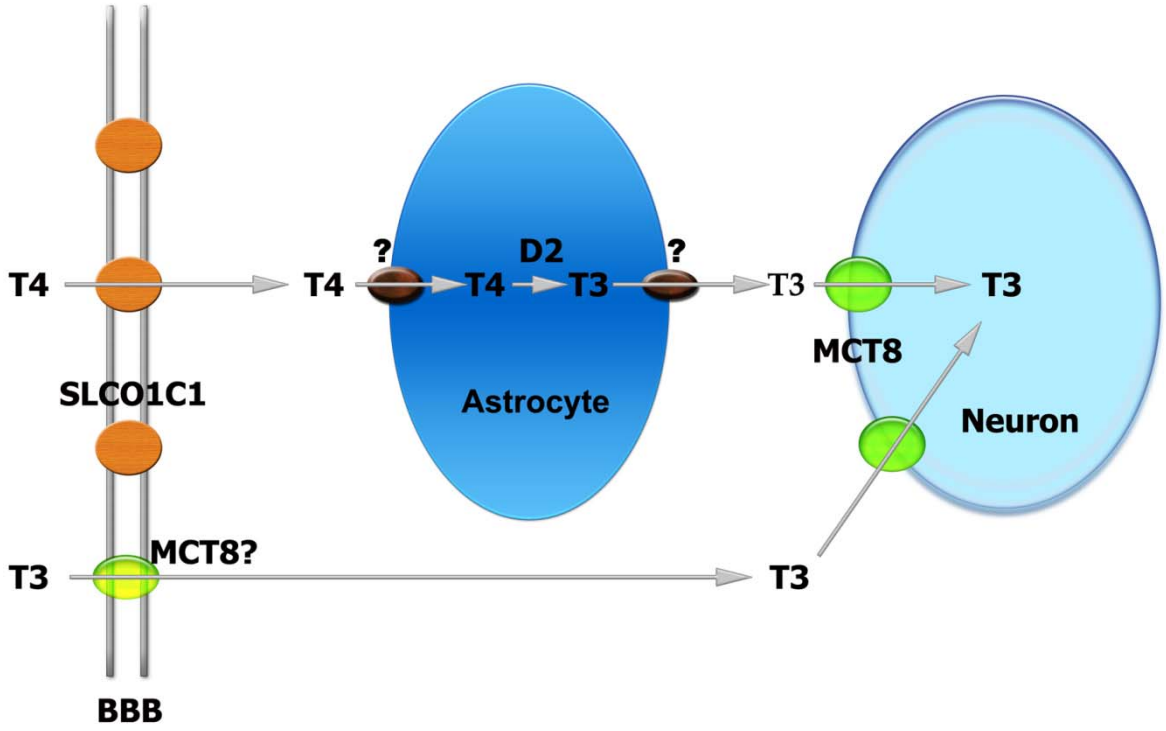

Fig. 1.3 The alternative model of thyroid hormones transport in the brain. In addition to the model in Fig. 1.2, T3, transported likely by MCT8 through the BBB into the brain parenchyma, enters neurons directly and plays an important role in brain development.

1.1 Thyroid hormones and brain development

Thyroid hormones are well known for its importance in brain development. Hypothyroidism during pregnancy leads to many neurological disorders, such as mental retardation, ataxia, spasticity, and deafness (Cao et al. 1994; Thompson and Potter 2000; Forrest et al. 2002; de Escobar et al. 2004; Perez-Lopez 2007). Studies have shown that hypothyroxinemia, reduced maternal thyroxine (T4) levels, is a major cause of fetal brain developmental defects (Calvo et al. 1990; de Escobar et al. 2004; Morreale de Escobar et al. 2004; Cuevas et al. 2005). Despite the existence of utero-placental barrier, a certain amount of maternal T4 can enter the fetal tissues. This amount is sufficient to provide the fetus with T3 for its development after local conversion. Mothers with hypothyroxinemia during pregnancy gave birth to children with neurological alterations, as surveyed in the Netherlands, Italy, and the USA (Haddow et al. 1999; Vermiglio et al. 2004; Kooistra et al. 2006). Fig. 1.4 shows the relationship between iodine deficiency (the major cause of maternal hypothyroxinemia) and neurodevelopment.

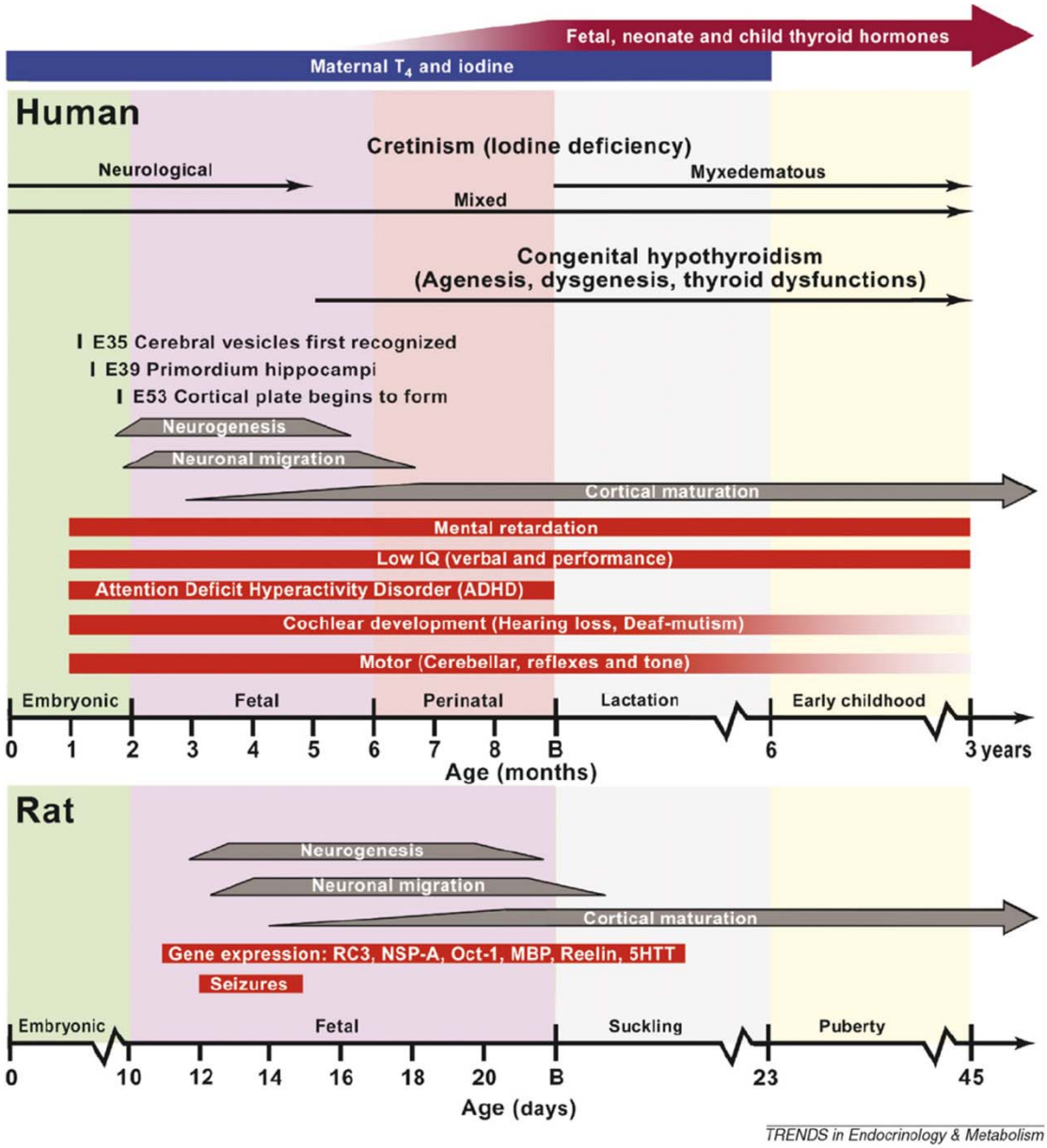

Fig. 1.4 Main neurodevelopmental events and neurological alterations associated with iodine deficiency during fetal and neonatal life. Time scales and the respective developmental periods are indicated for humans (upper) and rats (lower) panels. The developmental periods are highlighted with different background colors. The period in which the child's thyroid hormone is secreted is indicated by the upper brown arrow. The gradation indicates the period in which the production of fetal thyroid hormones increases to reach neonate values. The period in which both T4 and iodine are transferred from the mother to the fetus is indicated by a blue bar. Some major developmental events of the cerebral cortex are also indicated for both humans and rats. Thyroid hormone deficiencies and their etiologies, which are indicated in parentheses, are shown in the upper part of the human panel. Thin black arrows represent the crucial periods related to these disorders. Some neurological alterations

associated with maternal/fetal T4 and iodine deficiency and crucial vulnerability periods are shown by red bars. In the lower panel (rat), red bars indicate genes that are regulated by thyroid hormones and behavioral alterations associated with maternal and/or fetal T4 and iodine deficiency. For cochlear and motor disorders, heavy gradation indicates the period in which the brain is more sensitive to iodine deficiency. From (Berbel et al. 2007).

When talking about the dependence of the fetal brain development on the maternal thyroid status, two examples are worth mentioning. One is the congenital hypothyroidism (CH). In this disease, the fetus has a dysfunctional thyroid gland, while the mother produces normally thyroid hormones (de Vijlder 2003; Park and Chatterjee 2005). The other is iodine-deficiency disorders (IDDs). In this situation, both the mother and the fetus have a problem in producing thyroid hormones. In CH thyroid hormone replacement therapy can be applied after birth to the infant, while in IDDs correction of thyroid hormones has to be initiated early in and throughout pregnancy (Morreale de Escobar et al. 2004). These two examples demonstrate the essential role of maternal thyroid hormones in fetal brain development.

1.1.1 Thyroid hormone and neuron

Generally thyroid hormones can influence all aspects of neuronal development, including morphological changes, migration, differentiation, and myelination. Though not fatal, these effects lead to various degrees of neurological disorders in the neonates. Often the defects are irreversible.

1.1.1.1 Morphological changes

Most of the studies on the influences of hypothyroidism on the morphological changes in brain development were performed in rat. However, results from these studies have relevance for mouse and human development. Hypothyroidism affects neurons throughout the developing brain. In hippocampus, smaller cell bodies and less dendritic arborization were observed in the apical and the basal region of pyramidal neurons (Rami et al. 1986). In cerebral cortical pyramidal neurons, dendritic structures were altered especially on the upper region of these cells (Bass and Young 1973; Ipina and Ruiz-Marcos 1986; Ruiz-Marcos and Ipina 1986). Similar changes were also observed in other neurons, such as cholinergic neurons (Gould and Butcher 1989) and cerebellar Purkinje cells (Nicholson and

Altman 1972). The fine structure of dendritic spines was also impaired by hypothyroidism (Berbel et al. 1985). The dendrites of neurons form a network in the brain for communication between cells. Thus, alterations in the dendrite arborization and spine formation can directly change the function of neurons (Hoffmann and Dietzel 2004).

1.1.1.2 Migration

The migration of new-born neurons from the neurogenic zones into their final residence is part of the process of neurogenesis. Two major routes have been studied for neuronal migration, the radial and the tangential (Lambert de Rouvroit and Goffinet 2001; Ayala et al. 2007). Many diseases are caused by defects in neuronal migration during development, such as human lissencephaly with cerebellar hypoplasia due to autosomal recessive Reelin mutation (Hong et al. 2000) and human X-linked lissencephaly due to mutation of Doublecortin (Gleeson et al. 1998), and human X-linked periventricular heterotopia due to mutation of Filamin 1 (Fox et al. 1998).

Migration defects were also found in animals with hypothyroidism during development. Medial ganglionic eminence-derived neurons of fetus from a transiently hypothyroxinemic mother lost their preferential direction of tangential migration in an *in vitro* study (Cuevas et al. 2005). Concerning radial migration, ectopic BrdU-labeled neurons were found in the cerebral cortex and in the hippocampus of rats born from mothers with transient hypothyroxinemia during pregnancy (Lavado-Autric et al. 2003; Auso et al. 2004). The migration impairment could be corrected by either applying T4 or I^- supplementation to the pregnant mothers. These studies clearly demonstrated the importance of maternal T4 in fetal brain development.

1.1.1.3 Differentiation

In addition to delayed migration and ectopic localization of neurons, neurons that do migrate to their final residence show alterations in phenotype. Maternal hypothyroxinemia causes a reduction in hippocampal parvalbumin (PV)-positive GABAergic interneurons in the offspring, which contributes to suppression of GABA-mediated inhibition. The reduction of PV-positive cells is not due to a loss of GABAergic interneurons (Gilbert et al. 2007). Developmental hypothyroidism can also lead to persistent

changes in adults. A study shows that hypothyroxinemia during fetal development results in impaired synaptic function in adult hippocampus (Sui et al. 2005).

1.1.1.4 Myelination

The axon of a neuron is coated by a myelin sheath, which is formed by oligodendrocyte in the CNS. Any defect in the myelin sheath formation influences functions of the neuronal network. Thyroid hormones are among the factors that control oligodendrocyte development and myelination. Thyroid hormones have been shown to be involved in the proliferation, survival and maturation of oligodendrocytes (Rodriguez-Pena 1999). Maternal hypothyroidism reduces the number of mature oligodendrocytes in anterior commissure (ac) and corpus callosum (cc) (Schoonover et al. 2004). This reduction is accompanied by less myelin basic protein (MBP) production, which is a major building block of myelin sheath. Therefore, lack of thyroid hormones interferes with the action potential (AP) transmission along axons, which is important for neuronal connectivity and network coordination.

1.1.2 Thyroid hormone and astrocyte

Astrocytes are involved in the conversion of T4 to T3. T3 from astrocytes is the main supply for neurons. During development, THs influence astrocytes in their morphology, proliferation and differentiation (Trentin 2006).

Astrocytes undergo a serial of changes in morphology, protein expression, and division during development. Maternal thyroid hormone has been shown to be important for the transition from vimentin- to GFAP-expressing astrocytes in the fetal brain, which marks their maturation (Faivre-Sarrailh et al. 1991; Sampson et al. 2000). Another marker, S100, is also affected by thyroid hormones (Clos et al. 1982). *In vitro*, thyroid hormones induce astrocytes to form more processes (Lima et al. 1997). Depending on the age when astrocytes are cultured, thyroid hormone can also promote proliferation (Trentin and Moura Neto 1995).

Besides, microglia development is also related to thyroid hormones (Lima et al. 2001). Fewer microglia and delayed process extension are found when newborn rats are rendered hypothyroidism pre- or postnatally.

1.2 Thyroid hormone transporters

Substances traffic into and out of cells by the following means, diffusion, facilitated diffusion, active transport, endo/exocytosis and transcytosis. Except diffusion, different membrane proteins are involved, such as transporters, pumps and channels. The importance of membrane transport is manifested in several transport-related diseases. Cystic fibrosis results from dysfunctions of a Cl^- transporter (Rowe et al. 2005; Rowe and Clancy 2006). A subset of human osteopetrosis (over dense bones) is caused by mutations of an H^+ pump (Frattini et al. 2000; Forgac 2007). In neuromyelitis optica (NMO), a serum antibody (NMO-IgG) interacts with the water channel aquaporin 4 (AQP4) and is involved in the pathogenesis (Lennon et al. 2005; Jacob et al. 2007). TRP (Transient Receptor Potential) channels have been found to be the cause of four diseases, ranging from electrolytes disturbance to neurodegeneration (Venkatachalam and Montell 2007). Hypertension could be caused by transporter defects, such as SLC12A1 and SLC12A3 (Simon and Lifton 1998). The thyroid hormone transporter MCT8 was found to link to Allan–Hernon–Dudley syndrome (an X-linked psychomotor retardation) in humans (Dumitrescu et al. 2004; Friesema et al. 2004b).

1.2.1 Cellular uptake of thyroid hormones

It had been long believed that THs could enter cells by diffusion due to their lipophilic properties. In the mid-1970s, evidence emerged that transporters were involved in the cellular uptake of THs (Stitzer and Jacquez 1975; Rao et al. 1976; Krenning et al. 1978). The first TH transporters (Ntcp and Oatp1a1) were discovered about 20 years later (Friesema et al. 1999). Till now TH transporters have been categorized into four groups, Na^+-dependent organic anion transporter, Na^+-independent organic anion transporter, heterodimeric amino acid transporters (HAT), and monocarboxylate transporters (MCT). A majority of the transporters are wildly expressed and multi-specific for substrates. Tissue-specific and TH selective transporters are also found which implies more important functions of these transporters in specific tissues. Most, if not all, of the transporters are bidirectional carriers, meaning they mediate both afflux (flowing in) and efflux (flowing out) of their substrates. This property is in sharp contrast to the multidrug resistance (MDR) pumps, which only empty cells from substances such as ions, carbohydrates, lipids, xenobiotics, and drugs (Borst and Elferink 2002).

1.2.2 Peripheral transporters

Liver – Liver is a major organ for metabolism of bio-products. Therefore, it is not surprising that most TH transports are expressed in hepatocytes. Among them, some transporters are liver-specific.

Ntcp is a member of the Na^+-dependent organic anion transporter family, which also comprises the apical Na^+-dependent bile acid transporter (ASBT, Slc10a2) and the Na^+-dependent organic anion transporter (SOAT, Slc10a6). The Ntcp gene, Slc10a1, is expressed in the basolateral membrane of mammalian differentiated hepatocytes (Boyer et al. 1993; Hagenbuch 1997). In rat, it was recently shown to be expressed also in pancreatic acinar cells (Kim et al. 2002). Its ability to transport THs was identified in Xenopus oocyte with injected rat mRNA coding for Ntcp (Friesema et al. 1999; Jansen et al. 2005). The preference for iodothyronine is T4≈T3>rT3≈3,3'-T2. THs, together with other hormones (growth hormone, estrogen, and glucocorticoids), can regulate its expression (Simon et al. 2004). Other substrates of Ntcp include (un-)conjugated bile salts (Schroeder et al. 1998), sulfated bile salts (Kullak-Ublick et al. 1997; Hata et al. 2003), and steroid conjugates (Schroeder et al. 1998).

OATP1B1 is a Na^+-independent liver-specific transporter. Its expression was found in the basolateral membrane of hepatocytes (Abe et al. 1999; Hsiang et al. 1999; Konig et al. 2000; Tamai et al. 2000). Unlike other OATPs, its rat/mouse homolog has another name, Oatp1b2. On transporting THs, the preference is T4≈T3 (Abe et al. 1998). Other substrates include bile salts, bromosulfophthalein (BSP), steroid conjugates, prostaglandin E2, LTC4, gadoxetate, and so on (Cattori et al. 2000).

There are many other TH transporters in liver, such as OATP1B3 (liver-specific), Oatp1a1, OATP1A2, Oatp1a4, Oatp1a5, members of the OATP2/Oatp2 and the OATP3/Oatp3 subfamily, amino acid transporters, and MCT8 (Jansen et al. 2005; Taylor and Ritchie 2007). MCT8 will be described in a later section in more details.

Kidney – As in liver, many TH transporters are expressed in kidney. This reflects that the kidney is a major excretion organ in the body. Oatp1a3 is a kidney-specific TH transporter (Saito et al. 1996; Masuda et al. 1999; Takeuchi et al. 2001). Its expression is localized in the basolateral membrane of renal tubules. It transports T3 and T4 with similar preference. Other substrates of Oatp1a3 include bile salts, steroids and some cationic compounds. OATP4C1 is kidney-specific in adults. Its rodent ortholog,

Oatp4c1, was also found to be expressed in lung and brain, besides kidney (Mikkaichi et al. 2004). For TH transport, it seems to be uni-directional, only transporting from outside to inside. Other substrates include methotrexate (anti-folate drug) and cAMP. Sitagliptin, a drug for diabetic patients, was shown recently to be transported by OATP4C1 (Chu et al. 2007). Other TH transporters expressed in kidney include OATP1A1/Oatp1a1, Oatp1a5, and MCT8.

Testis – TH transporters in testis are supposed to be involved in spermatogenesis. Oatp6b1 and Oatp6c1 are relatively testis-specific TH transports. They were detected by ISH especially in Sertoli cells, spermatogonia, and Leydig cells. In ovary, a weak expression was also found (Suzuki et al. 2003). Other substrates include bile salts and sulfated androgen. SLCO1C1 is also expressed in testis Leydig cells (Pizzagalli et al. 2002). In a later section, this transporter will be described in more details.

Placenta –TH transport in placenta is important not only for the development of the fetus, but also for the normal function of placenta itself. OATP4A1 was found in various tissues, including heart, lung, brain, placenta, skeletal muscle, liver, and pancreas (Fujiwara et al. 2001). In placenta, it is expressed in apical surface of the syncytiotrophoblasts (Sato et al. 2003). The transport preference for THs is T3≈T4. Other substrates include bile salts and prostaglandin.

HAT is a family of heterodimeric amino acid transporters. This group of transporters consists of a heavy and a light chain. One of the two heavy chains, 4F2hc can form functional dimers with one of the six light chains. Dimers composed of LAT1 and LAT2 have been found to be expressed in placenta and transport THs (Prasad et al. 1999; Friesema et al. 2001; Ritchie and Taylor 2001). LAT1 is localized to the apical membrane of syncytiotrophoblast cells, while LAT2 might be in the basolateral membrane.

The monocarboxylate transporter 8 (MCT8) is expressed even during the first trimester of gestation in both villous cytotrophoblast and syncytiotrophoblasts cells, with increasing expression as gestation advances (Chan et al. 2006). This early expression may imply its importance for transporting maternal THs into the fetus before the fetus can produce them on its own. Another monocarboxylate transporter MCT10 (TAT1) was also implicated in transporting THs across placenta (Friesema et al. 2006).

Other organs – As mentioned earlier, a majority of the transporters are ubiquitously expressed. TH transporter expression has been reported in heart, intestinal tracts, skeletal muscle, eye, and so on. Just

list a few here, MCT8, MCT10 (Kim et al. 2001; Ramadan et al. 2006), and OATP4A1 (Kullak-Ublick et al. 2001).

1.2.3 Central transporters

The importance of THs in brain, especially during brain development was introduced at the beginning of this dissertation. Like in other organs, brain cells utilize THs by first permitting their entry via transporters.

Neuron – MCT8 belongs to the monocarboxylate transporter family. Some members of this family of transporters were first found to carry monocarboxylates (such as lactate, pyruvate, and ketone bodies) across cell membrane together with protons (Garcia et al. 1994a; Halestrap and Meredith 2004; Jansen et al. 2005; Messonnier et al. 2007). They have 12 putative transmembrane domains, with N- and C-termini localized in the cytoplasm. The C-terminal half is supposed to be responsible for substrate selectivity, where the most variations are found (Garcia et al. 1994b; Rahman et al. 1999). Most members of this family are expressed in various tissues. An exception is MCT3. In rodents, it has a restricted expression in the basal membrane of retina pigment epithelia and in the choroid plexus epithelia (Yoon et al. 1997; Philp et al. 1998; Philp et al. 2001). The regulation of MCT expression is highly dependent on body demand for substrates. For example, in rodent brain development, the expression of MCT1 and MCT2 decreased from birth to adult, indicating reduced requirement for lactate as an energy source (Rafiki et al. 2003; Simpson et al. 2007). Pathological conditions such as inflammation (Thibault et al. 2007) and obesity (Metz et al. 2007; Pierre et al. 2007) also regulate MCT expression. Recently there was a study showing that MCT3 could play a role in the development of atherosclerosis by a mechanism involving epigenetic modifications (Zhu et al. 2005). The regulation of MCT8 expression has not been well studied. But there is strong evidence that its expression was not affected by TH levels, since severe congenital hypothyroidism in $Pax8^{-/-}$ mice did not change MCT8 expression (Mansouri et al. 1998; Heuer et al. 2005).

MCT8 was first revealed as a TH transporter in 2003 (Friesema et al. 2003). In this study, rat MCT8 could specifically transport iodothyronines (T4, T3, rT3, and 3,3'-T2). The rate of uptake is very fast (<4min) and has a high capacity (>10 fold of increase). In comparison, other transporters such as rat Ntcp and rat

Oatp1a1 showed slower (>60min) and lower capacity (>2 fold of increase) uptake (Friesema et al. 1999). Human MCT8 is more specific for T3 transport (Friesema et al. 2004a).

Mutations of MCT8 lead to a psychomotor retardation disease, Allan–Hernon–Dudley syndrome (AHDS). AHDS was one of the first X-linked mental retardation (XLMR) syndromes described. First reports of this disease appeared in the literature in 1944 (Allan et al. 1944; Stevenson et al. 1994). In AHDS patients, free serum T3 level is strongly elevated, free or total T4 level is in low normal range, and TSH is in the high normal range. MCT8 mutations have been found in these patients, including truncations, frame shift, and point mutations (missense/nonsense). In a female heterozygous for MCT8, an unfavorable X-inactivation causes AHDS (Frints et al. 2008). However, polymorphism analysis in MCT8 has not been associated with serum levels of T3, T4, or TSH. [More details reviewed elsewhere in (Schwartz and Stevenson 2007; van der Deure et al. 2007)] MCT knockout mice showed similar, though milder, CNS deficits than patients with AHDS (Dumitrescu et al. 2006; Trajkovic et al. 2007).

OATP3A1 was first identified as a prostaglandin transporter with wide tissue distribution. However, a splice variant of OATP3A1, OATP3A1_v2, was found in human brain (Huber et al. 2007). It lacks 18 amino acids at the C-terminus than the other splice variant OATP3A1_v1. Its expression is found predominantly in testis and brain. In brain, immunolocalization was detected in the apical membrane of choroid plexus epithelia and neurons of the frontal cortex. Its substrates include prostaglandin, THs, and vasopressin. The authors suggested that OATP3A1_v2 be involved in oligopeptide transport in brain and be important for neuromodulation of neurotransmission.

Astrocyte – TH transporters on astrocyte have not been identified so far. Beslin et al. showed that in cultured rat astrocyte TH transport was associated with proton transport (Beslin et al. 1995). This implies that members of the MCT family could be candidates for TH transport in astrocytes. But this does not exclude other types of transporters such as OATP2B1, which is also pH sensitive (Nozawa et al. 2004). From GENSAT data, Slco1c1 was also found in astrocyte-like structures. But this needs to be confirmed (http://www.gensat.org/index.html or http://www.ncbi.nlm.nih.gov/projects/gensat).

1.2.4 BBB transporters

Nomenclature – The nomenclature of the thyroxine transporter SLCO1C1 is quite diverse and confusing. In literature, it has been named as Slco1c1, Bsat1, Oatp14, Slc21a14, OATP-F, Oatp2 and SLCO1C1 in different species. The situation is worsened by the fact that the gene and the protein of SLCO1C1 have different symbols. To avoid confusion, in the current study the gene is labeled as Slco1c1, while the protein is SLCO1C1, no matter which species is referred to. In this study the expression of Cre is under the control of the Slco1c1 locus. Therefore, Slco1c1-Cre is used to stress this point.

SLCO1C1 – THs are produced in the thyroid gland and secreted into blood. They must cross the blood-brain barrier to reach brain cells. SLCO1C1 is a member of the Na^+-independent organic anion transporter family. Members in this family are usually multi-specific for substrates and are expressed in various tissues. But SLCO1C1 is an exception, which shows more specific substrate spectrum and more restricted expression. This feature was used to drive tissue-specific expression of Cre recombinase in this dissertation.

Slco1c1 cDNA was first isolated from a human brain cDNA library (Pizzagalli et al. 2002), and was called OATP-F (SLC21A14). In the human genome, the gene Slco1c1 is located on Chromosome 12. It has 15 exons, the first of which encompasses the 5' untranslated region (UTR). The other 14 exons encode a protein of 712 amino acids. SLCO1C1 has 12 putative transmembrane domains, with both N- and C-termini located in the cytoplasm, as the MCTs. There are four postulated N-glycosylation sites. The glycosylation sties at positions 520 and 530 are conserved in other OATPs such as OATP1A2, OATP1B1, and OATP1B3. Its amino acid sequence homology to other OATP members is moderate (30-50% homology).

By Northern blot, Slco1c1 transcript was found in human brain and testis (Pizzagalli et al. 2002). In testis, immunohistochemical staining showed signals in Leydig cells. Expression of SLCO1C1 was also found in eye ciliary body epithelial cells (Gao et al. 2005). Substrates of SLCO1C1 include THs, BSP, and steroid conjugates. It is interesting to note that transport of T4 and rT3 is much higher than the other substrates. Uptake of T3 and other substrates only showed a 2-fold stimulation on expression of SLCO1C1 in CHO cells, while T4 and rT3 had more than 10-fold stimulation (Pizzagalli et al. 2002). This indicates that SLCO1C1 is a relatively selective T4 and rT3 transporter. The very low apparent K_m of T4 (90 nM) and rT3 (128 nM) makes SLCO1C1 important in transporting THs.

The rodent homolog Slco1c1 was first cloned from rat by genomic approaches (Li et al. 2001). It has 84% sequence homology to human Slco1c1. In the rat genome, the gene is on Chromosome 4. As its human homolog, it has 15 exons, the first of which encompasses the 5' untranslated region (UTR). The other 14 exons encode a protein of 716 amino acids. In this study, a gene microarray was hybridized with isolated brain capillary cDNA. Then the expression profile was compared with those from rat liver and kidney by suppression subtractive hybridization (SSH). By this way, a selective brain capillary gene expression profile was identified. Among the identified 50 clones, 8 were found to be part of a 2.6 kb full length cDNA, which was named BSAT1 (BBB-specific anion transporter type 1). Northern blot found Slco1c1 only in the brain and brain capillary had an enriched expression. By expressing Slco1c1 in HEK 293 cells, the authors could show that this transporter mediated bidirectional transport of T4, T3 and other known substrates. The affinity of rat SLCO1C1 to T4 was reported to be much higher than that to the other substrates (Chu et al. 2008).

Sugiyama et al. then followed up on this study (Sugiyama et al. 2003). They generated an antibody and could detect SLCO1C1 (Oatp14 in Sugiyama et al. 2003) in the luminal and abluminal membrane of brain capillary ECs. Additionally, in choroid plexus, SLCO1C1 was also detected by Western blot. Functional analysis was performed by expressing SLCO1C1 in HEK293 cells. Expression of SLCO1C1 greatly stimulated transport of T4, rT3, $E_2$17βG (17β-estradiol-d-17β-glucuronide), and some other drugs. The apparent K_m for T4 was about 180 nM. Transport of T3 was 6-fold lower than that of T4 and rT3. In the same study, they also found that SLCO1C1 expression could be regulated by thyroid hormones. At both transcriptional and translational levels, expression of SLCO1C1 was either up- or down-regulated under hypo- or hyperthyroidism, respectively. This finding point out SLCO1C1 is an important transporter for the homeostasis of brain T4. A similar situation was found for D2 expression under different thyroid hormone conditions (Bianco et al. 2002).

The mouse Slco1c1 was cloned by the same group one year later (Tohyama et al. 2004). In the mouse genome, the gene Slco1c1 is on Chromosome 6 at location 141,472,907-141,518,241. The gene structure is similar to those in rat and in human. The gene product is a protein of 715 amino acids and also has 12 putative transmembrane domains. Its mRNA was only present in brain, not in other tissues examined. In brain capillaries and choroid plexus, SLCO1C1 protein could be detected by Western blot. Immunohistochemical staining located the protein in the basolateral membrane of choroid plexus

epithelial cells. Substrate properties were similar to rat SLCO1C1, more selective to T4 and rT3, but not T3. The apparent K_m for T4 is about 340 nM and for rT3 is 460 nM.

Avian Slco1c1 was cloned from chicken (Nakao et al. 2006). It is on Chromosome 1. The gene lacks the first exon, which is located in the 5'UTR in human and rodent. The gene product is a protein with 710 amino acids. The protein sequence has about 72% homology to the human SLCO1C1. Its expression was found in choroid plexus. T4, but not rT3 and T3, was the confirmed substrate. The apparent K_m for T4 is 6.8 nM. It is supposed to be involved in the photoperiodic response of gonads in birds.

From the above studies, it is safe to say that SLCO1C1 is a specific BBB/choroid plexus transporter with high selectivity for T4. SLCO1C1 has the highest affinity for THs from all the TH transporters reported. The characteristic location and substrate specificity highlight the importance of SLCO1C1 for brain T4 homeostasis.

In contrast to MCT8, till now there has been no human disease discovered that is caused by mutations of SLCO1C1. Polymorphism-function relationship has not been established, though other OATPs show such relationship (Konig et al. 2006; Hagenbuch 2007).

Other transporters. Oatp1a4, Oatp1a5, Oatp1A2, and LAT are expressed in BBB. Their expressions are also found in other tissues and affinity to thyroid hormones is much less than that of SLCO1C1 (Westholm et al. 2008), which supports the idea that SLCO1C1 is the most important transporter for brain thyroxine supply.

1.3 Blood-Brain Barrier (BBB)

Normal functions of the central nervous system (CNS) depend on the maintenance of an extremely stable microenvironment. Neurons are especially sensitive to even slight changes around or inside them. Fluctuations in the CNS are not as well tolerated as in other organs, such as liver and kidney. To protect the homeostasis within the CNS, there are three barriers to regulate substance exchanges between the blood and the CNS. 1. Blood-brain barrier (BBB), formed by the ECs of brain capillaries. 2. Blood-cerebrospinal-fluid barrier (BCSFB), formed by the epithelial cells of the choroid plexus. 3. Blood-

subarachnoid CSF barrier, formed by the epithelial cells of the arachnoid. Among them, the BBB is the most important and has been found to play a role in various neurological diseases. Particularly, the brain capillary ECs behave as a physical barrier (tight junctions), transport barrier (transporters), and metabolic barrier (enzymes).

1.3.1 Brief history

The German microbiologist Paul Ehrlich presented the first evidence of the existence of a barrier between the CNS and other part of the body in 1885. But, unfortunately, he explained his observations wrongly. It was his student Edwin Goldmann who suggested that some barrier prevented the dye they used from entering the CNS, but not that CNS had a different affinity to the dye. The first to use the term Bluthirnschranke (blood-brain barrier) was Lewandowsky in 1900 (Hawkins and Davis 2005). The modern concept of BBB was developed from a series of studies in the 1960s by Reese, Karnovsky, and Brightman (Reese and Karnovsky 1967; Brightman and Reese 1969). Combined with electron microscopy and horseradish peroxidase administration, they could demonstrate that a barrier existed between blood and the CNS and tight junctions between ECs were the most important components of the barrier.

1.3.2 Anatomy

Now we know that the BBB is not only due to ECs. The microenvironment ECs reside in is critical for the induction of the barrier properties (such as tight junctions), which distinguish brain capillaries from those in the other parts of the body. An early study showed tissue-dependent properties of capillaries (Stewart and Wiley 1981). Neural or non-neural embryonic tissues from quail were transplanted into chicken non-neural or neural embryonic tissues, respectively, at the stage when blood vessels were not formed in the donors. The donors then got blood supply from recipient-derived vessels. BBB properties were only found from capillaries in neural tissues in either of the cases. To stress the importance of microenvironment on the development and physiology of BBB, the term ‘neurovascular unit’ was proposed (Cohen et al. 1996; Hawkins and Egleton 2008). Notably, the term ‘neurovascular unit’ is also used in other parts of the body to denote the close interrelation between vessels and neurons (Vinik et al. 2001). Under the concept of neurovascular unit, brain capillary endothelium, astrocytes, pericytes,

neurons, extracellular matrix, and perhaps microglia form a network of interactions and cooperate intimately to maintain the 'barrier', as illustrated in Fig. 1.5.

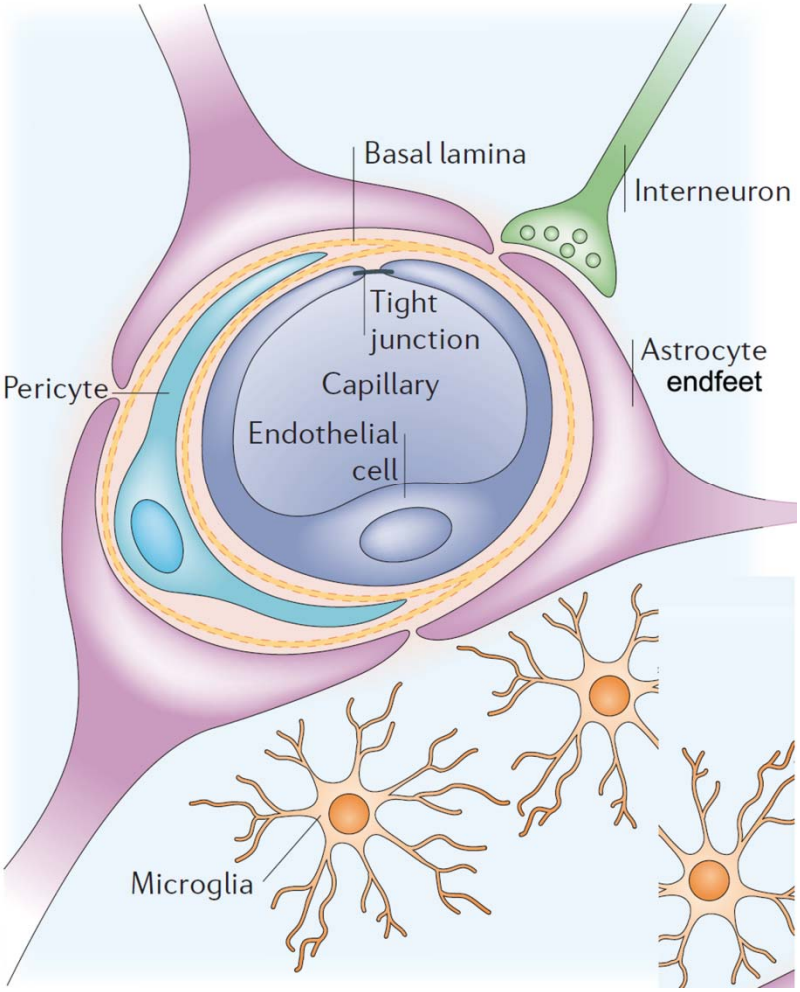

Fig. 1.5 Cellular constituents of the blood–brain barrier. The barrier is formed by capillary ECs, surrounded by basal lamina and astrocytic perivascular endfeet. Astrocytes provide the cellular link to the neurons. The figure also shows pericytes and microglial cells. Modified from (Abbott et al. 2006).

Endothelial cells

Endothelial cells (ECs) of the brain capillaries are the most important component of the BBB, as it is the ECs that form the physical, metabolic, and transporter barriers, which keep the brain in a more homeostatic environment than other parts of the body.

As a physical barrier, tight junctions of the ECs are formed by a number of membrane proteins, including claudin, occludin, and junction adhesion molecules. There are also other accessory proteins in the

cytoplasm, which help to anchor the membrane proteins onto cytoskeletons, such as actin. The tight junction at BBB is illustrated in Fig. 1.6.

The metabolic barrier concerns with enzymes expressed on the luminal and abluminal surfaces of the ECs. Especially in drug administration, such a barrier greatly protect brain from unwanted effects (Minn et al. 1991).

The transporter barrier is especially important in normal brain function. Brain needs high amount of nutrients for its metabolic demand. Transporters expressed in BBB ECs help to meet this demand and clear the toxic metabolites away. Examples are EAAT1-3 (excitatory amino acid transporters 1–3), GLUT1 (glucose transporter 1), LAT1 (L-system for large neutral amino acids), and Pgp (P-glycoprotein) (Abbott et al. 2006). As mentioned above, thyroxine transporter SLCO1C1 is important for T4 supply to the brain both during development and in adults.

During development, the first blood vessels could be detected in brain at E10 in the mouse and E11 in the rat (Bauer et al. 1993; Stewart and Hayakawa 1994; Qin and Sato 1995). This is a time at which astrocytes are not yet formed, but instead neurogenesis starts. Thus, the neural microenvironment is thought to assist the induction of BBB properties on these vessels. The maturation of BBB continues when gliogenesis starts later. Currently, there is no general agreement when BBB is fully developed. But at least during the early stage of neonatal life, BBB is still developing.

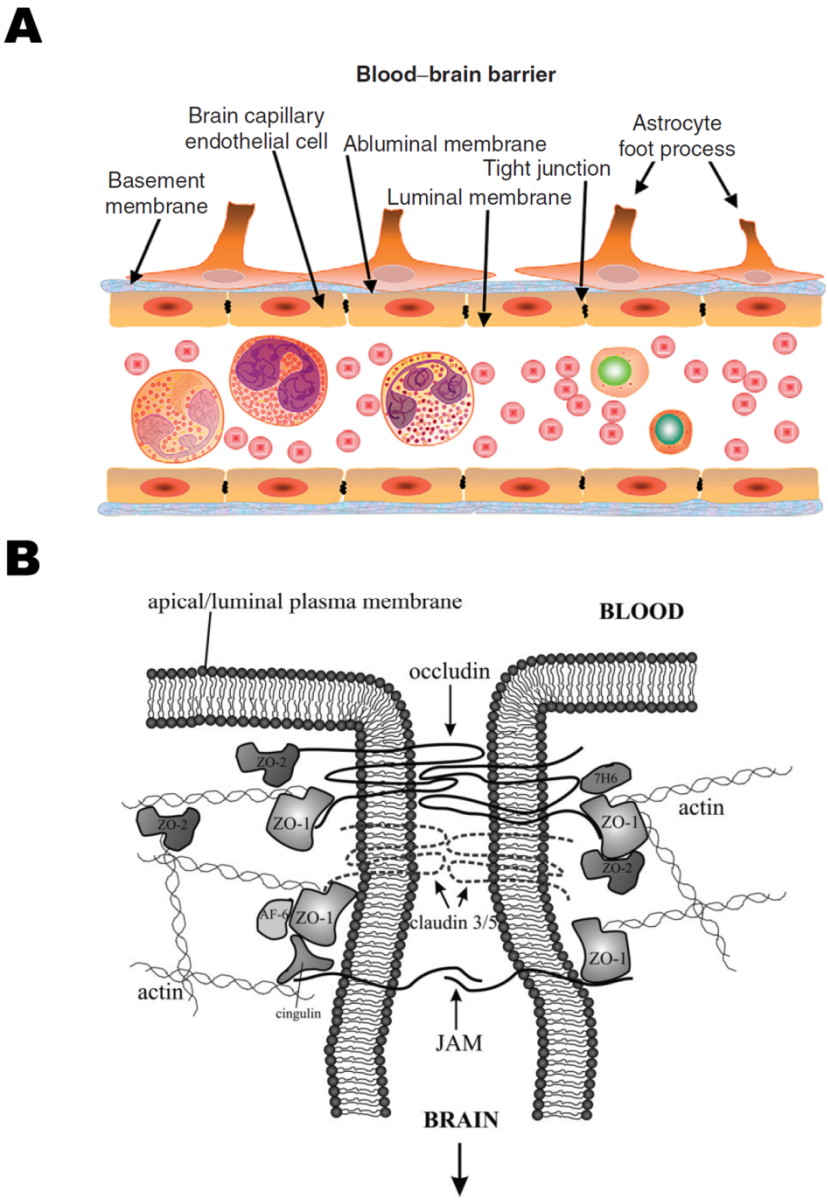

Fig. 1.6 BBB and tight junction. A. Diagram of BBB anatomy, including capillary endothelial cells sealed with tight junctions, a continuous basement membrane, and astrocyte foot processes. Tight junctions limit the paracellular diffusion of solutes from blood into brain parenchyma. Therefore, in order to cross the BBB, solutes must sequentially diffuse or be transported across both endothelial membranes. Adopted from (Westholm et al. 2008). B. Tight junction (TJ) at BBB. Transmembrane junctional adhesion molecule (JAM), occludin and claudins are components of the tight junction. Intracellularly ZO-1 and ZO-2 link the junctional proteins to cytoskeleton protein actin, which is important to the stability and function of TJs. Adopted from (Hawkins and Davis 2005)

Astrocytes

Astrocytes ensheathe brain capillaries by forming endfeet at the end of their processes. Endfeet are highly polarized structures. A characteristic feature is the localization of the water channel, aquaporin 4 (AQP4) (Nielsen et al. 1997). The expression of AQP4 is enriched in the endfeet membrane lining brain capillaries. A similar distribution was found for a potassium channel Kir4.1 (Nagelhus et al. 1999). The close contact and the characteristic polarization have already suggested that astrocytes be involved in the regulation of ECs. In *in vitro* models of BBB, astrocytes were shown to induce BBB properties in non-neural ECs (Janzer and Raff 1987; Hayashi et al. 1997). An *in vivo* study showed astrocyte loss leads to damaged barrier properties (Willis et al. 2004). Two mechanisms contribute to the induction of BBB perperties. One is contact-mediated, the other is soluble-factor mediated (Abbott 2002; Nedergaard et al. 2003). Identified soluble factors secreted by astrocytes include angiotensin II (Wosik et al. 2007), IL-6 (Takemoto et al. 1994), GDNF (Igarashi et al. 1999), and bFGF (Sobue et al. 1999).

Pericytes

Pericytes were discovered in the late 19th century by Eberth and Rouget (Sims 1986). They are found in many tissues and line the microvascular ECs. They contain contractile proteins, which are involved in local blood flow regulation (Peppiatt et al. 2006). Pericytes were suggested to be a component of the BBB first in 1981 (Broadwell and Salcman 1981). Their close physical contact with BBB ECs is reminiscent of the location of astrocyte endfeet. In cell culture, pericytes help to maintain BBB properties of ECs (Parkinson and Hacking 2005; Nakagawa et al. 2007). This effect is partially due to secretory factors from pericytes, such as angiopoietin-1 (Hori et al. 2004) and TGF-β1 (Dohgu et al. 2005; Takata et al. 2007).

Neurons

Neurons are involved in BBB development from the beginning when blood vessels invade into brain. Later in adults, neurons innervate the three above-mentioned BBB components, ECs, astrocytes, and pericytes to adapt blood flow to their high energy demand and dynamic activity (Hartman et al. 1972; Cohen et al. 1997; Vaucher et al. 2000; Peppiatt et al. 2006). This puts neurons in a central role in integrating/regulating BBB functions.

Extracellular matrix

The lamina membrane contains many molecules that are involved in anchoring and/or function of endothelial cells. Integrin receptors on ECs interact with laminin and other proteins in the lamina to

anchor ECs (Hynes 1992). When the lamina composition is disrupted, BBB permeability increases (Jian Liu and Rosenberg 2005).

Microglia

The function of microglia in the neurovascular unit is mainly discussed in the context of their interaction with other components and their contribution to BBB damage. For example, microglia amplify neurotoxicity caused by glutamate released from astrocytes (Bezzi et al. 2001). A rise in brain lactate could increase the production of cytokines in microglia, which could act on other components of the BBB (Andersson et al. 2005).

1.4 Choroid plexus

Choroid plexus is another barrier found in the brain. It is located along the wall of the ventricles (Fig. 1.7A). The cerebrospinal fluid (CSF) filling up the ventricles is secreted from choroid plexus. Similar to BBB, choroid plexus has also tight junctions, albeit formed by epithelial cells (Fig. 1.7B). Thus, choroid plexus serves as an active barrier to maintain normal brain function (Brown et al. 2004).

1.5 Cre-transgenic mice

Cre recombinase together with its recognition sites loxP form the base of knocking out genes in a tissue-specific manner (Yu and Bradley 2001; Branda and Dymecki 2004). If the expression of Cre is controlled (e.g. by fusion to a mutated estrogen receptor), a transient Cre activity can be achieved, thus avoiding: 1, ectopic embryonic expression other than in adults; 2, confounding influences from other tissues in a classical systemic knockout; 3, lethality due to the essential function of the gene of interest in development or compensation by other genes of similar functions.

The advantage of using BAC (bacterial artificial chromosome)-transgenic techniques for generation of Cre mice is the high fidelity of expression of the transgene to the endogenous expression pattern. Before the development of BAC-transgenic techniques, plasmids had been used. However, this approach usually results in aberrant expression of the transgene, genomic positional effects on transgene expression, or loss of expression after several generations. Later YAC (yeast artificial chromosome) was employed for transgenesis. Though also faithfully reflecting the endogenous expression pattern, the

tremendous work involved is prohibitive. BAC-transgenic technology revolutionized the way how transgenic mice can be generated. This technique features bacterial culture (easier to handle than yeast culture), long gene regulatory sequence (closer to the endogenous situation), high successful recombination rate (less screening), and copy-number dependent expression (tight transgene dose control).

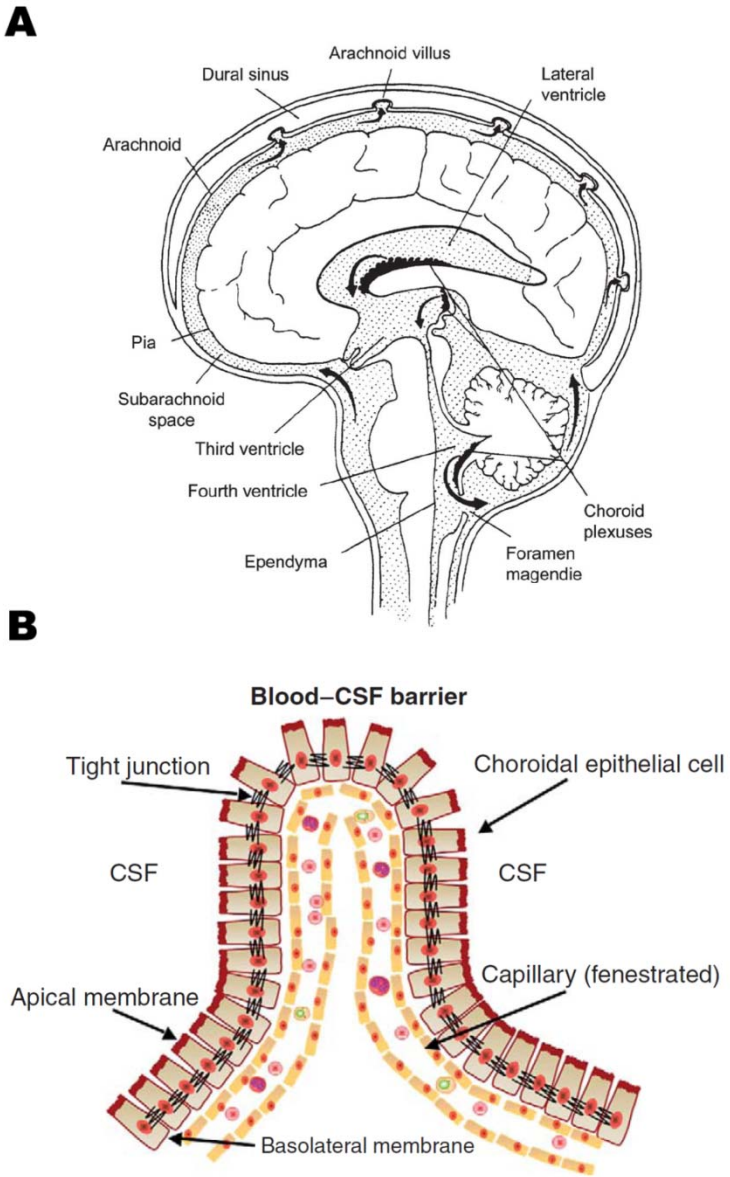

Fig. 1.7 Choroid plexus and its tight junction. A. The locations of the choroid plexuses and the distribution of CSF in the human brain. The CSF is shown as the stippled area and the choroid plexuses are shown as the solid black structures. Adopted from (Brown et al. 2004). B. Diagram of BCSFB anatomy consisting of polarized choroidal epithelial cells connected by tight junctions vascularized with a

fenestrated capillary bed. The presence of tight junctions between choroidal epithelial cells forces solutes to sequentially cross both the apical (CSF-facing) and basolateral (blood-facing) membranes in order to pass from CSF to blood or vice versa. Adopted from (Westholm et al. 2008).

While the essential role of T4 in fetal brain development is becoming clearer, exact mechanisms of T4 action in individual brain cell types remain vague. One approach to study the mechanisms is to know which cells are sensitive to T4 deficiency during development. According to recent studies, cellular uptake of T4 is facilitated by transporters. Therefore, brain-specific T4 transporters could be used as a marker to monitor cellular requirement of T4. In this study, a transgenic mouse line expressing Cre recombinase under the control of a T4 transporter gene locus was generated. Cell lineages expressing this transporter during brain development were identified. Especially the layer 2/3 cortical neurons were studied in details. Their development in the fetal brain was linked to the maternal T4 status during pregnancy. Among the factors causing hypothyroxinemia, iodine deficiency is most common. The World Health Organization (WHO) has stated that iodine deficiency is the second, after starvation, preventable cause of cerebral lesions (Perez-Lopez 2007). Today there are up to 0.5% pregnant women having overt hypothyroidism and up to 2.5% having subclinical hypothyroidism, even in industrialized countries (Morreale de Escobar et al. 2004; ACOG Committee 2007).

1.6 NF-κB in cerebral ischemia

The NF-κB family of transcription factors is composed of five members, p50, p52, p65 (RelA), RelB and c-Rel. They form hetero- or homo-dimers that can translocate from cytoplasm into nucleus to regulate the transcription of their target genes (Perkins 2007). One important function of NF-κB has been shown to contribute to cell survival by activating the transcription of pro-survival genes and/or suppressing that of the anti-survival genes. For example, a candidate tumor suppressor ING4 (inhibitor of growth family, member 4) interacts with p65. In glioma, downregulation of ING4 was found to be responsible for increased tumor blood vessel density by upregulating NF-κB targeted angiogenic genes (Garkavtsev et al. 2004).

In cerebral ischemia, NF-κB activation leads to neuronal cell death. Mice lacking p50 showed lower NF-κB activity and reduced infarct size (Schneider et al. 1999). Genes that could mediate the deleterious effect of NF-κB in cerebral ischemia include those involved in eicosanoid biosynthesis (Pla2g4a, encoding

cytosolic phospholipase A_2; Ptgs2, encoding cyclooxygenase 2; Ptges, encoding mitochondrial prostaglandin E_2 synthase) (Herrmann et al. 2005) and proapoptotic BH3-only genes (Bim and Noxa) (Inta et al. 2006).

However, since NF-κB regulates the expression of a wide range of genes (for details see the Target Genes at www.nf-kb.org), the outcome of the NF-κB regulation is complicated. Furthermore, the development of ischemic damage and its recovery adds another layer of complexity to the roles of NF-κB in cerebral ischemia. Nijboer and coworkers observed different effects depending on the time of NF-κB inhibition. An early inhibition (0 and 3 hrs after ischemic damage) of NF-κB activity could protect neurons from apoptosis induced by hypoxia-ischemia (Nijboer et al. 2008b). In contrast, a later (18 and 21 hrs) or prolonged (0, 6, and 12 hrs) inhibition of NF-κB activity resulted in aggravated neuronal damage (Nijboer et al. 2008a). Taking a closer look, they could demonstrate a difference in the transcription of cytokines and apoptotic factors, which might contribute to the effect of NF-κB inhibition in respective cases.

Recently, different stages of pathobiology in cerebral ischemia have been differentiated (Dirnagl et al. 1999; Endres et al. 2008; Lo 2008). When putting NF-κB in this context, it is not surprising that NF-κB plays different roles when the ischemic pathobiology progresses.

The neuronal activation of NF-κB was found to be the cause of ischemia-induced neuronal cell death (Herrmann et al. 2005; Zhang et al. 2005). NF-κB in other cell types in the brain may also play a role in other aspects in cerebral ischemia. Yepes and coworkers showed that NF-κB-mediated Tweak/Fn14 signaling pathway increased neurovascular unit permeability after cerebral ischemia (Polavarapu et al. 2005). Inhibition of this pathway could reduce NF-κB activity and the neurovascular unit permeability (Zhang et al. 2007b). Astrocytic NF-κB activity was suggested to mediate the effects seen in these two studies. Endothelial cells are a key component of the neurovascular unit. However, whether endothelial NF-κB plays a role in cerebral ischemia is unknown. This is partly due to lack of a tool to target NF-κB in BBB endothelial cells.

1.7 Aim of the study

Thyroxine plays an important role in brain development. During brain development, different brain structures may have different requirements for thyroxine. This may be reflected by the expression pattern of the thyroxine transporter SLCO1C1. In adults, its expression is found almost exclusively in BBB endothelial cells. However, during development when BBB is not fully established, the expression of SLCO1C1 could be different. In this study, to gain knowledge on how SLCO1C1 is expressed during brain development, we aim to,

1. Generate a transgenic mouse line that expresses Cre under the control of the Slco1c1 locus
2. Examine the expression pattern of Cre in the transgenic mice
3. Investigate the effect of hypothyroxinemia on Slco1c1-expressing cell lineages

2. Materials and Methods

2.1 Materials

2.1.1 Cells

COS-1 and PC12 cells were bought from DSMZ (Braunschweig, Germany).

2.1.2 Constructs

RcCMV-p65	Dr. Ioana Inta, University of Heidelberg, Heidelberg
pConst	Lab of Prof. Günther Schütz, DKFZ, Heidelberg
pERT2	Lab of Prof. Günther Schütz, DKFZ, Heidelberg
phRL-TK	Promega, Mannheim
pNF-κB-Luc	Stratagene, Amsterdam, The Netherlands

2.1.3 Enzymes

BamHI	Promega, Mannheim
EcoRI	New England Biolabs, Frankfurt
EcoRV	Roche, Penzberg
HiFi Phusion DNA polymerase	Biocat, Heidelberg
HindIII	Promega, Mannheim
MMLV reverse transcriptase	Promega, Mannheim
NheI	Promega, Mannheim
NotI	New England Biolabs, Frankfurt
Pfu DNA polymerase	Promega, Penzberg
Polynucleotide Kinase	Promega, Mannheim
SacI	Promega, Mannheim
Shrimp alkaline phosphatase	Roche, Penzberg
SnaBI	New England Biolabs, Frankfurt
T4 DNA ligase	Roche, Penzberg
XbaI	Promega, Mannheim

2.1.4 Kits

Cytotoxicity detection Kit	Roche, Penzberg
Gel extraction	Qiagen, Hilden
Mini, Midi plasmid purification	Promega, Mannheim
Mini, Midi, Maxi plasmid purification	Qiagen, Hilden
PCR purification	Qiagen, Hilden
SYBR-Green PCR core reagents	Applied Biosystems, Darmstadt
TaqMan Gene Expression Assay	Applied Biosystems, Darmstadt

2.1.5 Chemicals

2,2,2-Tribromoethanol (Avertin)	Sigma-Aldrich, Steinheim
5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal)	Peqlab, Erlangen
Acetic acid 100% p.A.	AppliChem, Darmstadt
Adenosine triphosphate (ATP)	AppliChem, Darmstadt
Agarose	Serva, Heidelberg
Ampicillin	AppliChem, Darmstadt
Boric acid	Grüssing, Filsum
Bovine serum albumin (BSA)	AppliChem, Darmstadt
Bromophenol blue	Sigma-Aldrich, Steinheim
Calcium chloride	Riedel-de Haen, Seelze
Chloramphenicol	Carl-Roth, Karlsruhe
Chloroform	Sigma-Aldrich, Steinheim
Cresyl violet	Sigma-Aldrich, Steinheim
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Steinheim
Dimethylformamide (DMF)	Merck, Darmstadt
Disodium hydrogen phosphate	Riedel-de Haen, Seelze
Embedding medium	Leica, Wetzlar
Eosin	Waldeck, Münster
Ethanol absolute p.A.	Merck, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Steinheim
Ethyleneglycoltetraacetic acid (EGTA)	AppliChem, Darmstadt
Eukitt	O. Kindler, Freiburg
Gelatin	Merck, Darmstadt
Glutardialdehyde 25%	Merck, Darmstadt
Glycerol ≥98%	Carl-Roth, Karlsruhe
Glycine	AppliChem, Darmstadt
Igepal (NP40 substitute)	Sigma-Aldrich, Steinheim
Isopropanol absolute p.A.	AppliChem, Darmstadt
Magnesium chloride	Riedel-de Haen, Seelze
Paraformaldehyde (PFA)	Riedel-de Haen, Seelze
Penicillin/Streptomycin	PAA Laboratories, Cölbe
peqGOLD RNAPure	Peqlab, Erlangen
Phenol	Carl-Roth, Karlsruhe
Potassium chloride	AppliChem, Darmstadt
Potassium dihydrogen phosphate	Riedel-de Haen, Seelze
Potassium ferricyanide	Riedel-de Haen, Seelze
Potassium ferrocyanide	Merck, Darmstadt
Rapid-hyb buffer	GE Healthcare, Munich
Redivue [^{32}P] dCTP	GE Healthcare, Munich
RPMI 1640 medium	PAA Laboratories, Cölbe
Select-Agar	Invitrogen, Karlsruhe
Sodium chloride	Merck, Darmstadt
Sodium deoxycholate	AppliChem, Darmstadt
Sucrose	ICN Biochemicals, Aurora, OH, USA
TransFast® reagent	Promega, Mannheim
Tris	Carl-Roth, Karlsruhe

Triton X-100	Sigma-Aldrich, Steinheim
Tryptone	Sigma-Aldrich, Steinheim
Tween 20	Carl-Roth, Karlsruhe
Yeast extract	Carl-Roth, Karlsruhe

2.1.6 Equipment

Autoclave	Systec, Wettenberg
Bio-Spin 6 and 30 columns	Bio-Rad, Munich
BlueMarine 200 gel electrophoresis	Serva, Heidelberg
Coverslip	Menzel-Glaser, Braunschweig
Cryotome	Leica, Wetzlar
Electroporator	Bio-Rad, Munich
Gene Amp 5700 sequence detector	Applied Biosystems, Darmstadt
Homogenizer PRO200	PRO Scientific, Oxford, CT, USA
Polysin® slide	Menzel-Glaser, Braunschweig
Spectrophotometer	Eppendorf, Hamburg
Whatman paper	GE Healthcare, Munich
Zeta Probe GT Nylon membrane	Bio-Rad, Munich

Note: If not specified, the location of a company is in Germany.

2.1.7 Primers

Name	Sequence (5'-3')
armA-5'	TAC GTA GGG TCT CGA TGG CAG GAT T
armA-3'	ATC CAT GAG AAT GTT TCT TAT CCC AGC
armB-5'	GCT CTA GAA TGG ACA CCT CAT CCA AAG AAA AT
armB-3'	GAT GCT AGC TAC GTA GCC TGC ACA GGA GGC AAA A
Rec1_UA5	GCT ATT CAT GTC TTG GAA GCC
Rec1_DA3	CAG GTT CTT CCT GAC TTC ATC
Rec1_UB5	CTC GAG GAA GTT CCT ATT CTC
Rec1_DB3	TCT CTG TCT CCT CTG CTT ATC
Cre_up	GAA CAA CAG GAA ATG GTT CCC
Cre_down	GGT GGA GAG ATG GAT CTC TGT
Lac1	TTA CCC GTA GGT AGT CAG GCA
Lac2	TTA CGA TGC GCC CAT CTA CAC
Slc_rt1	CAT GGA GAA GTA CAG CTA TGA G
Slc_rt2	TCT GCA CAC ACC CGA TGT AG
GAPDH-F	ATC CTG CAC CAC CAA CTG CTT A
GAPDH-R	TTC AAG AGA GTA GGG AGG GCT
pgrp_p1	AGC GCG GAT CCC CCT GGA GAA TGT GAG AGG C
pgrp_p2	AGC GCG GAT CCC TTT TGT TAG CCC CAC CTG C
pgrp_p3	AGC GCC TCG AGG GAC TGC AGG GCT GTG TAT G
pgrp _κB1_1	CTG AGA TCT AAG CCT TGG TAC CCC CAG GAA ACA ACT GAA TG
pgrp _κB1_2	CAT TCA GTT GTT TCC TGG GGG TAC CAA GGC TTA GAT CTC AG
pgrp _κB2_1	GGT CTG GGT CGG AAA GTC GAC CCC AGGTGTGAAGCAAGC
pgrp _κB2_2	GCTTGCTTCA CAC CTG GGG TCG ACT TTC CGA CCC AGA CC
Pglyrp_LC_m_1s	CAG TTC GCT ACG TGG TGA TCT
Pglyrp_LC_m_1as	AGG TGA TGC CAA TAG ACA TGG
cyc5	ACC CCA CCG TGT TCT TCG AC
acyc300	CAT TTG CCA TGG ACA AGA TG

2.1.8 Antibodies

Primary

Antibody	*Dilution*	*Cat #*	*Company*
Rabbit anti-Cre	1:3,000	Self-made	Prof. Günther Schütz
Mouse anti-Cre	1:200	MMS-106R	Covance
Mouse anti-NeuN	1:500	MAB377	Chemicon
Mouse anti-GFAP	1:50	Sc-33673	Santa Cruz
Rabbit anti-GFAP	1:500	Z0334	Dako
Mouse anti-Nestin	1:200	MAB353	Chemicon
Rat anti-mouse CD31	1:300	557355	BD Pharmingen
Rabbit anti-β-gal	1:5,000	AB986	Chemicon
Goat anti-doublecortin (Dcx)	1:200	Sc-8066	Santa Cruz

Secondary

Antibody	*Dilution*	*Cat #*	*Company*
Cy3 goat anti-rabbit	1:200	111-165-003	Jackson
Cy3 goat anti-mouse	1:200	115-165-003	Jackson
Cy3 donkey anti-goat	1:200	705-165-003	Jackson
FITC rabbit anti-goat	1:200	FI-5000	Vector Labs
AlexaFluor 488 donkey anti-mouse	1:200	A21202	Invitrogen
AlexaFluor 488 goat anti-rabbit	1:200	A11008	Invitrogen

2.1.9 Buffers

1X TE buffer

10 mM Tris-Cl, pH 7.5

1 mM EDTA

20X SSC – for Southern blotting

3 M NaCl

0.3 M sodium citrate

The chemicals are dissolved in dH_2O. Adjust pH to 7.0 with HCl and then autoclave.

NID buffer – for genotyping

50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2 mM $MgCl_2$; 0.1 mg/ml gelatin; 0.45% NP40

Fixing Solution (can be stored in dark at 4°C) – for LacZ staining

PBS 0.1M, pH7.3

+ 5 mM EGTA

+ 2 mM $MgCl_2$

+ 0.2% Glutaraldehyde

Wash Solution – for LacZ staining

PBS 0.1M, pH7.3

+ 5 mM EGTA

+ 2 mM $MgCl_2$

+ 0.01% Na-Deoxycholate

+ 0.02% NP-40

Staining Solution (freshly prepared, protected from light) – for LacZ staining

PBS 0.1M, pH7.3

+ 5 mM EGTA

+ 2 mM $MgCl_2$

+ 0.01% Na-Deoxycholate

+ 0.02% NP-40

+ 10 mM $K_3[Fe(CN)_6]$[e] (329.25 g/mol) III

+ 10 mM $K_4[Fe(CN)_6]$[f] (422.4 g/mol) II

+ 0.5 mg/ml X-Gal in DMF

X-Gal (5-Brom-4-Chloro-3-Indoxyl-β-D-Galacoside) – for LacZ staining

The stock X-gal solution was dissolved in DMF (Dimenthylformamide) at a concentration of 50mg/ml, stored at -20°C and protected from light.

10X MOPS running buffer – for RNA gel electrophoresis

0.4 M MOPS, pH 7.0

0.1 M sodium acetate

0.01 M EDTA

2X Formaldehyde Loading Dye – for RNA gel electrophoresis

40 µl Formaldehyde

100 µl Formamide

39 µl dH_2O

20 µl 10XMOPS

1 µl Ethidium Bromide

2.2 Methods

2.2.1 Modification of BACs by homologous recombination in bacteria

2.2.1.1 Competent cells

2.2.1.1a Competent cells for re-transformation of the BAC

E coli strain EL250 was used for competent cell preparation (Lee et al. 2001). The bacteria were grown at 32°C O/N. No antibiotics were added into the culture at this stage. The next day, the O/N culture was diluted 100-500-fold in LB medium and grown at 32°C till the OD600 reached 0.4 – 0.5. Cells were centrifuged for 10 min at 4,000 rpm at 4°C, followed by washing with ice-cold 10% glycerol for three times. Aliquots were snap-frozen in liquid N_2 and stored at -80°C. Competent cells are sensitive to temperature changes. During the preparation, the cells were kept around 4°C.

2.2.1.1b Competent cells for homologous recombination

EL250 cells carrying the BAC RP24-85B20 were grown in LB medium supplemented with Chloramphenicol (25 µg/ml) at 32°C O/N. The next day, the O/N culture was grown at 32°C till the OD600 reached 0.4 – 0.5. RED recombinase activity was induced by shaking the culture in a waterbath at 42°C for 15 min, followed by chilling on ice for 30 min. The washing step was the same as described in '2.2.1.1a'. These competent cells were prepared freshly each time for transformation.

2.2.1.1c Competent cells for plasmid transformation

Competent cells used for plasmid transformation were prepared by the $CaCl_2$ method, which is commonly used for transformation by heat-shock. Briefly, O/N culture were grown to OD600 = 0.4 – 0.5. Then cells were washed with ice-cold 0.1M $CaCl_2$ for three times. Aliquots of cells were stored at -80°C.

2.2.1.2 Construct preparation

Homology arms (armA and armB) were amplified from the BAC RP24-85B20 by PCR. For armA, primers armA5' and armA3' were used. For armB, primers armB5' and armB3' were used. The PCR protocol was 95°C 2min; 95°C30 sec-63°C1min -72°C 1min (30x); 72°C10min ; 4°C.

Both ends of armA were phosphorylated by PNK kinase. Then the armA was ligated to EcoRV-digested and dephosphorylated pConst vector (kindly provided from the lab of Prof. Günther Schütz, DKFZ, Heidelberg). The correct clone was confirmed by digestion with SnaBI and sequencing. This construct was named pConst+armA. The pConst+armA was digested by NheI and dephosphorylated, then was ligated with NheI and XbaI digested armB. The correct clone was confirmed by PCR (primers, armA3' and armB5') and sequencing. This construct was named pConst+armA+armB. Moue lines generated from this construct are called constitutive. At the same time, the homology arms were also cloned into a pERT2 vector (kindly provided from the lab of Prof. Günther Schütz, DKFZ, Heidelberg). The difference between pERT2 and pConst is the presence of the sequence of a mutated estrogen receptor ligand binding domain that is fused to Cre (Feil et al. 1997). Cre activity then can be induced by administration of tamoxifen. Mouse lines generated from this construct are called inducible.

2.2.1.3 Homologous recombination

BAC Mini-prep

BAC DNA was extracted from the DH10 host cells bearing RP24-85B20 by a BAC mini-prep protocol. Briefly, DH10 cells were grown from a single colony in LB medium supplemented with Chloramphenicol (25 µg/ml) O/N at 32°C. The O/N culture was centrifuged and resuspended in P1 buffer, lysed in P2 buffer and neutralized in P3 buffer (P1, P2 and P3 buffers are products of the company Qiagen). After centrifugation, the supernatant was precipitated by isopropanol (2/3 volume of supernatant). The mixture was then centrifuged at full speed for 10 min at 4°C. The pellet was washed with EtOH (70%) and dried and dissolved in appropriate volume of TE pH 8.0.

Re-transformation

An electroporation protocol was used for retransforming BAC RP24-85B20 into competent EL250 cells. Briefly, competent EL250 bacteria were thawed on ice in pre-cooled cuvettes (0.2 cm) for at least 5 min. The mini-prep BAC of 1, 2 or 5 µl was mixed with EL250 and electroporated at 2.3 kV (25 µF with Pulse controller set to 200 Ω). Then the mixture was grown in LB medium at 32°C for 1hr with

shaking (shaker, 200 rpm). Afterwards, the bacteria were spread on LB plates containing Chloramphenicol (25 µg/ml) and grown at 32 °C O/N.

Homologous recombination

Competent cells prepared according to '2.2.1.1b' were mixed with linearized pConst+armA+armB (digested by SnaBI). Electroporation was performed at 2.3 kV (25 µF with Pulse controller set to 200 Ω). Then cells were grown for 1hr and incubated O/N at 32°C on LB plate containing chloramphenicol (25 µg/ml) and ampicillin (50 µg/ml).

Removal of the Amp cassette

The ampicillin cassette, if not removed, may affect the expression of the transgene. Its removal was induced by 0.1% L-arabinose. Arabinose can induce the expression of the recombinase Flp in EL250 cells and thus mediate the Flp-FRT recombination. The above homologously recombined EL250 was incubated with 0.1% L-arabinose at 32°C for 1hr and then grown O/N in LB containing chloramphenicol (25 µg/ml).

2.2.1.4 Characterization of recombined BAC

Patterning

This is to compare the original, the homologously recombined, and the Amp-removed BACs after XbaI digestion. The digested DNA was run on 0.8% agarose gel (0.5XTBE) with ethidium bromide (1:20,000) in the gel and the running buffer.

Southern blotting

The original, the homologously recombined, and the Amp-removed BACs were digested with BamHI or SacI. The digests were run at the same conditions as in 'patterning'. Standard Southern blotting protocol was used to blot the DNAs from the gel onto nylon membrane. Briefly, the DNA gel was depurinated by 0.25 N HCl and denatured by 0.4 N NaOH. The DNA on the gel was transferred onto a nylon membrane with a blotting sandwich (from the bottom to the top: Whatman paper-gel-membrane-Whatman paper-dry towels-heavy balance, with the bottom Whatman paper soaked in 10X SSC buffer). The nylon membrane was then hybridized to a probe amplified by PCR. Before hybridization, the probe was radioactively labeled with Redivue [^{32}P] dCTP according to the

Rediprime protocol (GE Healthcare). The labeled probe was purified with Bio-Spin 6 and 30 columns (Bio-Rad) according to the manufacture's protocol. Hybridization was performed at 68°C for 1 hr. The hybridized membrane was washed with 2X SSC/0.1% SDS (RT) and 1X SSC/0.1% SDS (65°C) sequentially. Hybridization image was obtained by exposing the membrane to a radiosensitive film (Amersham Hyperfilm MP, GE Healthcare).

Sequencing

Sequencing was done by SeqLab (Göttingen) according to the company's suggestions.

Pulse field gel electrophoresis (PFGE)

The purpose of PFGE is to examine if the final BAC construct could be digested by NotI, which results in the release of the backbone.

First, BAC was midi-prepared. Briefly, the BAC was grown O/N in LB medium containing chloramphenicol (25 μg/ml) at 32°C. The O/N culture was pelleted by centrifugation. The pellet was resuspended in P1 buffer, lysed in P2 buffer and neutralized in P3 buffer (P1, P2 and P3 buffers are products of the company Qiagen). The lysate was centrifuged and the supernatant was filtered through 'cheesecloth'. Isopropanol (70% volume of the supernatant) was mixed with the filtered supernatant for precipitation. After centrifugation, the pellet was dissolved in QBT buffer and the resulted clear solution was run through QBT-equilibrated Q-500 column. After washing with QC buffer twice, BAC DNA was eluted with QF buffer (pre-heated to 65°C). Isopropanol (70% volume of the supernatant) was mixed with the flow-through for precipitation. After centrifuge, the pellet was washed with 70% EtOH. Finally, the pellet was dissolved in 1xTE with a volume according to the size of the pellet. (QBT buffer, QC buffer and Q-500 column are products of the company Qiagen.)

The NotI digested BAC DNA from mini-prep was run in 1% agarose in 0.5XTAE. The electrophoresis conditions were, initial t_{switch}=0.5s, final t_{switch}=20s, included angle α=120°, |E|=6V/cm, I≈140mA, total time T=14hrs. Lambda Ladder PFG Marker (N0340S, NEB) was also run as a size reference.

2.2.1.5 Purification of the recombined BAC for pronucleus injection

BAC DNA was digested by NotI. The digestion mixture was loaded onto the Sepharose CL4b Column (Pharmacia). Fractions were collected into 1.5 ml Eppendorf tube (200 μl/tube). About 30 – 40 fractions

were collected. The OD was measured to determine which fractions contained the most BAC DNA. Either one (backbone + BAC) or two peaks (backbone or BAC) could be seen. PFGE was run for the fractions containing the BAC DNA to check the separation of the backbone and the BAC. The fraction with the best separation (no backbone) and the best quality (good OD260/280 and concentration) was selected for injection by Frank Zimmerman (IBF, University of Heidelberg).

2.2.2 Characterization of transgenic mice

2.2.2.1 Ethics

All the animal experiments were conducted in accordance to the regulations of the Regierungspräsidium Karlsruhe, Germany.

2.2.2.2 Genotyping

Mouse tails were immersed in 200 µl NID buffer in Eppendorf tubes, containing 10 mg/ml proteinase K (added freshly). After Incubation at 56°C O/N, proteinase K activity was inactivated at 96°C for 10min. Primers armB_UB5'/armB_DB3' were used for Slco1c1-Cre and Lac1/Lac2 for Rosa26.

2.2.2.3 RT-PCR

RNA was extracted from freshly perfused organs by peqGOLD RNAPure (PeqLab) according to the manufacturer's instructions. The quality of the extracted RNAs was controlled by OD measurement and denaturing agarose gel electrophoresis. Briefly, the gel was prepared by dissolving 1 g of agarose in 100 ml 1XMOPS. About 3 µg of heat-denatured RNAs were loaded into the gel together with 2X Formaldehyde Loading Dye. Electrophoresis was run at 5-6 V/cm until the bromophenol blue had migrated to the middle of the gel. High quality RNAs (10 µg from each tissue) were reversely transcribed with Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega, Mannheim, Germany). The following primers were used for PCR amplification: GAPDH-F, GAPDH-R; Slc-r1, Slc-r2; Cre_up, Cre_down.

2.2.2.4 Tissue processing

Adult mice were anesthetized by i.p. injection of 150µl of 2.5% Avertin (2-2-2 tribromoethanol) per 10 g of body weight. Sequential transcardiac perfusion with Ringer's solution and ice-cold 4% PFA was done before organ collection. Brains were fixed in 4% PFA at 4°C for 4-12 hrs and then transferred into 30% sucrose solution for 24hrs. The brains were either directly sectioned or stored at 4°C in the sucrose solution. For long-term storage, brains were embedded in cryosection embedding medium and stored at -20°C. Neonates were anesthetized by ether and perfused and further processed as in adults. Embryos were taken out from anesthetised pregnant dam and decapitated. The further process was the same as in adults.

2.2.2.5 LacZ staining

Brains were embedded in embedding medium. Cryosections of 60 µm in thickness were attached onto Polysin® glass slides. Sections were dried for 30min (RT), fixed for 5min in 0.2% glutaraldehyde (RT), rinsed in Wash Solution for 5min, three times (4°C), and incubated in Staining Solution for 1-24hr in dark (37°C). The stained sections were then counter stained with Eosin for 1-4min, dehydrated and mounted with Eukitt.

2.2.2.6 Nissl staining

Cryosections of 60 µm thickness were stained in 0.1% cresyl violet solution for 3-5 minutes. After shortly rinsing with H_2O, sections were differentiated in 95% ethyl alcohol till a satisfactory purple colour was obtained.

2.2.2.7 Immunofluorescence

Cryosections of 60 µm thickness were stored in Cryoprotection solution at -20°C. For immuno-fluorescence, sections were washed three times in PBS for 5 min, permeabilized with 0.3% Triton X-100 in PBS for 30min, blocked with 5% BSA in 0.3% Triton X-100 in PBS for 30min, and incubated with primary antibodies in PBS (containing 5% BSA and 0.3% Triton X-100) at 4°C on a shaker. After 24 – 48hr, primary antibodies were washed away with PBS. Secondary antibodies in PBS (containing 5% BSA and 0.3% Triton X-100) were applied for detection of the primary antibodies (in the dark) for 4 hr at room temperature. Nuclei were stained with DAPI.

2.2.2.8 Middle cerebral artery occlusion (MCAO)

Mice were anesthetized by intraperitoneal injection of 150µl of 2.5% Avertin (tribromoethanol) per 10 g of body weight. A skin incision was made between the ear and the orbit on the left side. The parotid gland and the temporal muscle were removed by electrical coagulation. The stem of the MCA was exposed through a burr hole and occluded by microbipolar coagulation (Erbe, Tübingen, Germany). Surgery was performed under a microscope (Hund, Wetzlar, Germany). Mice were kept at a body temperature of 37 °C on a heating pad. For infarct measurement, mice were deeply anesthetized with Avertin and perfused intracardially with Ringer's solution at indicated time points. The procedure for

measuring the infarct on cryosections and correcting for cerebral edema has been described previously (Herrmann et al. 2003). Surgery was performed and the infarct was measured without knowledge of the genotype. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2.2.9 Hypothyroidism

Young adult female mice (C57BL/6) were mated. The day of mating (E0) was confirmed by vaginal plug. These mice were then divided into a control group and a propyl-thio-uracil (PTU) group. On E2, 0.1% propyl-thio-uracil (PTU) was added to the drinking water of the PTU group till 2 days after birth (P2; P0 being the day of birth). The control group received normal tap water. At P2, all the pups were decapitated. Brains were examined by LacZ staining in the pups that were genotyped as Cre/+; LacZ/+.

2.2.2.10 Serum free T3 and free T4 measurement

Blood was taken from the heart in deeply anesthetized adult mice. In the case of P2 pups, blood was collected after decapitation. The obtained blood was stored on ice for 4hr to allow coagulation. After centrifugation at 10,000g for 5min at 4°C, sera were carefully collected and stored at -20°C. The serum free T3 and free T4 measurement was performed in the Central Laboratory of the University hospital as routinely done with blood from patients.

2.2.2.11 Quantification

The number of LacZ-expressing cells in cortical layer 2/3 was quantified according to a previous published protocol (Lavado-Autric et al. 2003; Auso et al. 2004). Briefly, pups (P2) were taken from one to two litters per subgroup. LacZ-labeled cells were counted in a total of 11–23 fields per experimental subgroup (five to seven fields per pup). In primary somatosensory cortex (S1), fields represented by templates generated by the computer system (125 μm wide) were placed over the primary somatosensory cortex and spanned from layer 1 to the subcortical white matter. Values from each probe were later used for statistics. Data are presented as mean±S.E.M. Two groups were compared by student *t*-test.

2.2.3 PGRP-S in cerebral ischemia

2.2.3.1 Animals

PGRP-S−/− mice have been described previously (Dziarski et al. 2003). Because they had been backcrossed on the Balb/c background for nine generations, we used wild-type Balb/c mice (Harlan, Indianapolis) as controls. Germline deletion of RelA causes embryonic lethality (Beg et al. 1995). Therefore, we applied a conditional approach, introducing the nestin-Cre balancer gene into $RelA^{flox/flox}$ mice (Inta et al. 2006). For controls we used littermate $RelA^{flox/flox}$ mice that were negative for the nestin-Cre balancer gene.

2.2.3.2 Constructs

To construct PGRP-S reporter plasmids, we amplified a short (−330/+29) and a long part (−1093/+29) of the mouse PGRP-S promoter from mouse genomic DNA with the following primers: pgrp_p1, pgrp_p2, and pgrp_p3. The promoter sequences were subcloned into the BamHI/XhoI site of the firefly luciferase reporter vector pXP2 (Nordeen 1988). To mutate the NF-κB binding sites κB1 and κB2 of the PGRP-S promoter, we used a commercial mutagenesis kit (QuickChange II Site-Directed Mutagenesis Kit, Stratagene, Amsterdam, The Netherlands) and the following primers: pgrp _κB1_1, pgrp _κB1_2, pgrp _κB2_1, and pgrp _κB2_2.

2.2.3.3 Cell culture and transfection

PC12 cells were cultured in RPMI 1640 (PAA, Cölbe, Germany), supplemented with 10% horse serum, 5% fetal bovine serum, and 1× penicillin/streptomycin (PAA) at 37 °C in an atmosphere of 95% humidified air and 5% CO2. For transient transfection, PC12 cells were plated on 24-well plates precoated with rat tail collagen (Roche, Penzberg, Germany) at a density of 200,000 cells per well. After 24 h, cells were transfected with 0.5 µg per well of the PGRP-S reporter constructs and 0.1 µg of phRL-TK (Promega) using TransFast reagent according to the manufacturer's instructions (Promega). To express RelA, 0.1 µg of RcCMV-p65 (Schmitz and Baeuerle 1991; Inta et al. 2006) per well was cotransfected. For cotransfections a constant DNA concentration was used, which was maintained by adding pBluescript. Luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega).

2.2.3.3 Real-time RT-PCR

RNA from the cortex of mice was isolated using the acidic phenol extraction protocol followed by Qiagen RNeasy (Qiagen, Hilden, Germany) mini kit purification following the manufacturer's recommendations. cDNAwas synthesized from 5 µg total RNA using oligodT primers, superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) using standard conditions. For detection of pgrp-s expression in the brain, quantitative PCR was performed using the Lightcycler system (Roche Diagnostics, Mannheim, Germany) with SYBR-green staining of DNA doublestrands. Cycling conditions were as follows: 5 min 95 °C, 5 s 95 °C, 10 s 65 °C, 30 s 72 °C, 10 s 84 °C for 50 cycles. Melting curves were determined using the following parameters: 95 °C cooling to 50 °C; ramping to 99 °C at 0.2 °C/s. The following primer pairs were used: pglyrp_LC_m_1s and pglyrp_LC_m_1as. The Lightcycler PCR was performed using the SYBR green master mix, following the manufacturer's recommendations (Roche Diagnostics). Specificity of product was ensured by melting point analysis and agarose gel electrophoresis. cDNA content of samples was normalized to the expression level of cyclophilin (primers: cyc5 and acyc300). Relative regulation levels were derived after normalization to cyclophilin. Data are presented as mean±S.E.M. Three or more groups were compared by ANOVA, followed *post hoc* by the Tukey-HSD test. Two groups were compared by two-sided *t*-test.

For detection of pgrp-s expression in cell cultures, RNA was extracted from freshly perfused organs by peqGOLD RNAPure (PeqLab) according to the manufacturer's instructions. RNAs (10 µg from each sample) were reversely transcribed with Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega, Mannheim, Germany). PCR was performed according to the following protocol: 10 min at 95°C, 15 s at 95°C, and 1min at 60°C (40 cycles). Amplification was quantified with the Gene Amp 5700 sequence detector and the TaqMan Gene Expression Assay (Applied Biosystems, Darmstadt, Germany). The Assay ID for pgrp-s was Mm00437150_m1.

3. Results

3.1 PGRP-S in cerebral ischemia

The transcription factor NF-κB has been shown to contribute to ischemia-induced neuronal apoptosis (Herrmann et al. 2005; Inta et al. 2006). In searching for the downstream target genes, a microarray assay discovered that peptidoglycan recognition protein-S (PGRP-S) is regulated in a NF-κB dependent manner in cerebral ischemia. The real-time RT-PCR confirmed this by showing elevated levels of PGRP-S mRNA 24hrs after MCAO in the cortex of control mice (Fig. 3.1). The upregulation was significantly reduced in $RelA^{CNSKO}$ mice, which lack an NF-κB subunit RelA in the central nervous system (Inta et al. 2006). This suggests that RelA mediates the induction of PGRP-S in cerebral ischemia.

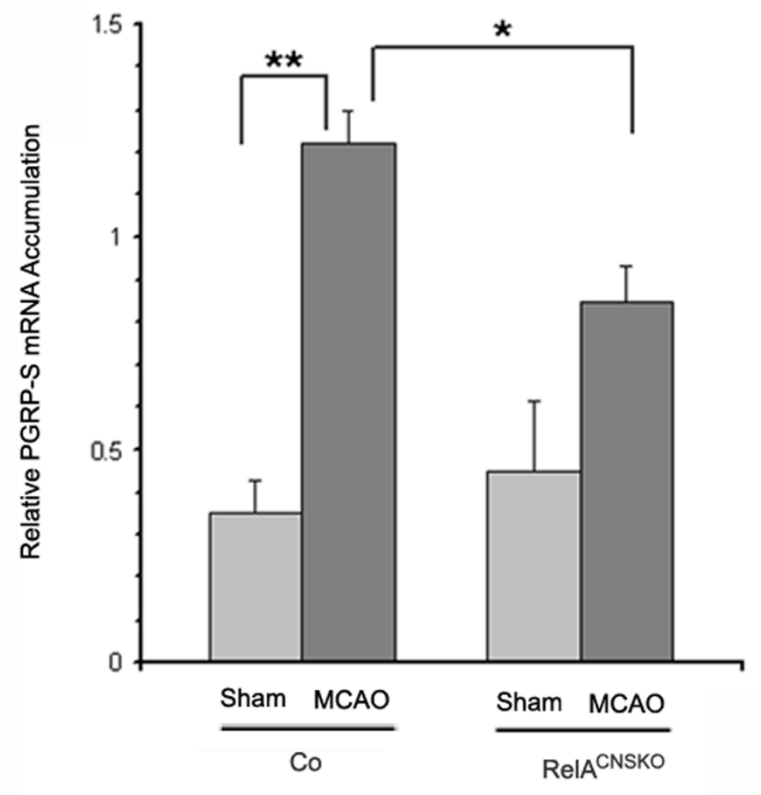

Fig. 3.1 PGRP-S mRNA accumulation 24 h after sham surgery or MCAO in littermate controls (Co) and RelACNSKO mice. Values are means±S.E.M. (n = 4). *p < 0.05; **p < 0.001 (ANOVA, Tukey-HSD *post hoc* test).

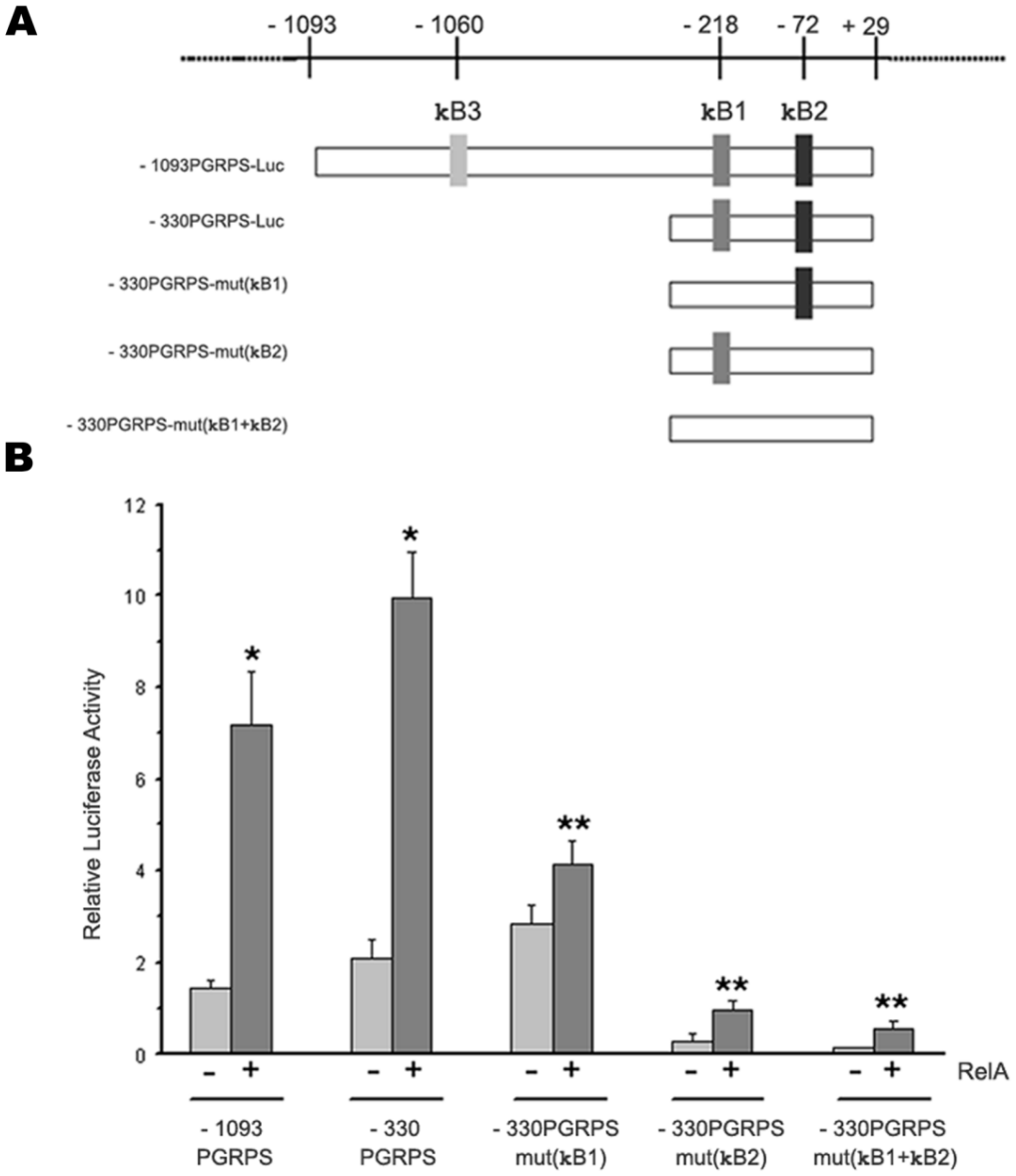

Fig. 3.2 Effect of the expression of RelA on the promoter activity of various PGRP-S promoter mutants. (A) Schematic diagram of the PGRP-S promoter and the mutants that were used for the reporter assay. The mouse PGRP-S promoter contains three putative NF-κB binding sites, κB1, κB2, and κB3. (B) PC12 cells were transiently cotransfected with the indicated PGRP-S promoter mutants and an expression plasmid of RelA. Luciferase activity is expressed relative to a control group in the same experiment. Values are means±S.E.M. (n = 5–15). *p < 0.001, compared to the group without RelA transfection; **p < 0.001, compared to −330PGRPS-Luc cotransfected with RelA (ANOVA, Tukey-HSD *post hoc* test).

The 5'-flanking sequence of the PGRP-S gene contains three putative NF-κB binding sites (Fig. 3.2A). To investigate whether the PGRP-S promoter is transactivated by RelA, a PGRP-S luciferase fusion gene (−1093-PGRPS-Luc) was constructed, in which luciferase expression is controlled by the 5'-flanking sequence of the PGRP-S gene (−1093/+29). Cotransfection of an expression plasmid for RelA stimulated luciferase expression, indicating that the PGRP-S promoter is transactivated by RelA (Fig. 3.2B). Deletion of the 5'-part of the promoter-containing κB3 (−330-PGRPS-Luc) did not affect the responsiveness of the PGRP-S promoter to RelA. However, the internal mutation of κB1 or κB2 or both significantly reduced stimulation by RelA. Mutation of κB2 severely impaired basal luciferase expression. In summary, these data show that RelA stimulates PGRP-S transcription through κB1 and κB2.

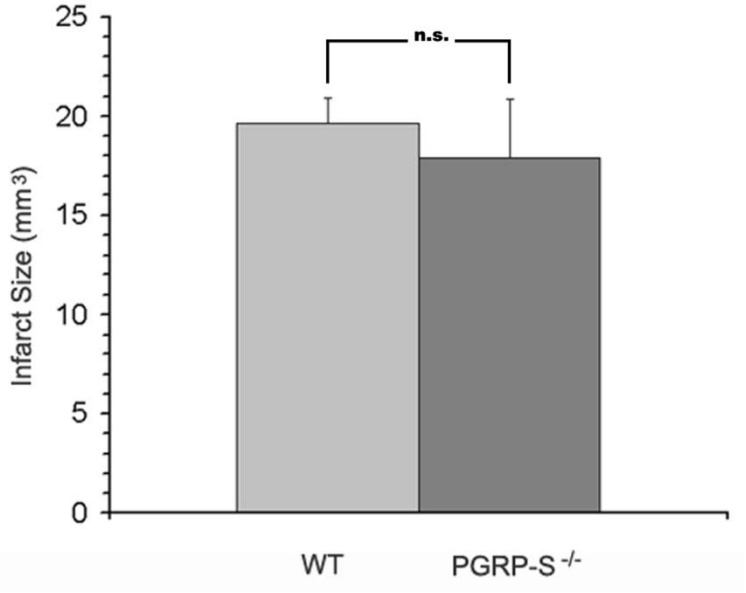

Fig. 3.3 Infarct size in wild-type (WT, *n* = 6) and PGRP-S−/− mice (*n*=9) 48h after MCAO. Values are means±S.E.M. n.s.=not significant, student *t*-test.

As PGRP-S has been reported to induce apoptosis in mammalian cells (Sashchenko et al. 2004; Sashchenko et al. 2007), we sought evidence showing that PGRP-S is involved in neuronal cell death and the development of the ischemic infarct. PGRP-S protein was expressed in COS-1 cells and was added into primary neuronal culture medium. However, PGRP-S did not induce neuronal cell death as judged by LDH release or TUNEL staining (data not shown). Moreover, the infarct size in PGRP-S-deficient mice 2 days after MCAO was the same as in Balb/c controls (Fig. 3.3). Thus, we were unable to detect a role for PGRP-S in ischemic brain damage.

Then, a question arises where the elevated PGRP-S comes from. By real-time RT-PCR, surprisingly, PGRP-S transcript was found in a blood-brain barrier cell line (TM-BBB4) (Hosoya et al. 2000; Terasaki and Hosoya 2001), but not in neurons and astrocytes (Fig. 3.4). This could mean that the elevated PGRP-S expression in cerebral ischemia was indirectly regulated by some unknown factors released from neurons or astrocytes and acting on BBB, since the BBB ECs in $RelA^{CNSKO}$ mice have functional RelA. BBB is a barrier that separates brain from the periphery. It is important for the brain homeostasis. BBB has a distinct gene expression profile from other brain cells. For example, the thyroxine transporter SLCO1C1 is specifically expressed in BBB endothelial cells, which mediates the transport of thyroxine into the brain parenchyma (Sugiyama et al. 2003; Tohyama et al. 2004).

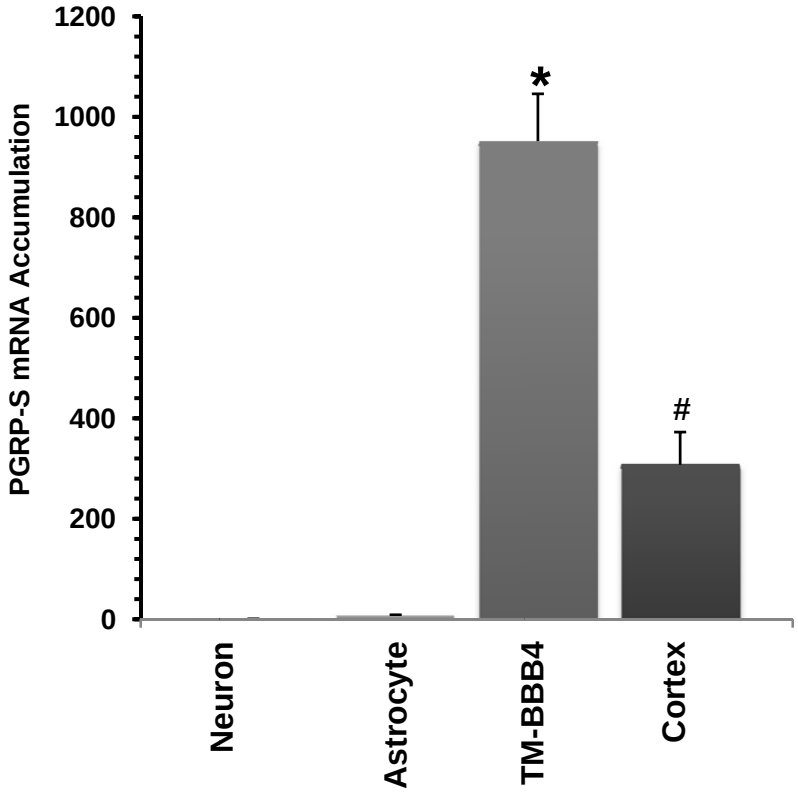

Fig. 3.4 PGRP-S mRNA accumulation. mRNAs from cultured primary neuron, cultured primary astrocyte, TM-BBB4 cells (a cell line for BBB endothelial cells), and cortex were reversely transcribed for cDNAs. The expression of PGRP-S at the transcription level was detected with the cDNAs by real-time PCR with TaqMan Gene Expression Assay for pgrp-s (Assay ID: Mm00437150_m1). *, $p<0.001$ (compared to PGRP-S expression in any of the other three groups); #, $p<0.05$ (compared to PGRP-S expression in neuron or astrocyte). Values are Mean±S.E.M., One Way ANOVA. Tukey test).

3.2 Generation of the Slco1c1-Cre transgenic mouse

In this study, a bacterial artificial chromosome (BAC)-transgenic approach was applied for the generation of the Slco1c1-Cre transgenic mice. This approach has been successfully used in many studies. As shown in Fig. 3.5, a codon-improved Cre (iCre) (Shimshek et al. 2002) was placed in the Slco1c1 gene. The expression of iCre is thus under the control of the regulatory sequence of the Slco1c1 gene. Fig. 3.6 shows the restriction sites on the BAC construct that were used for diagnosis in the cloning steps.

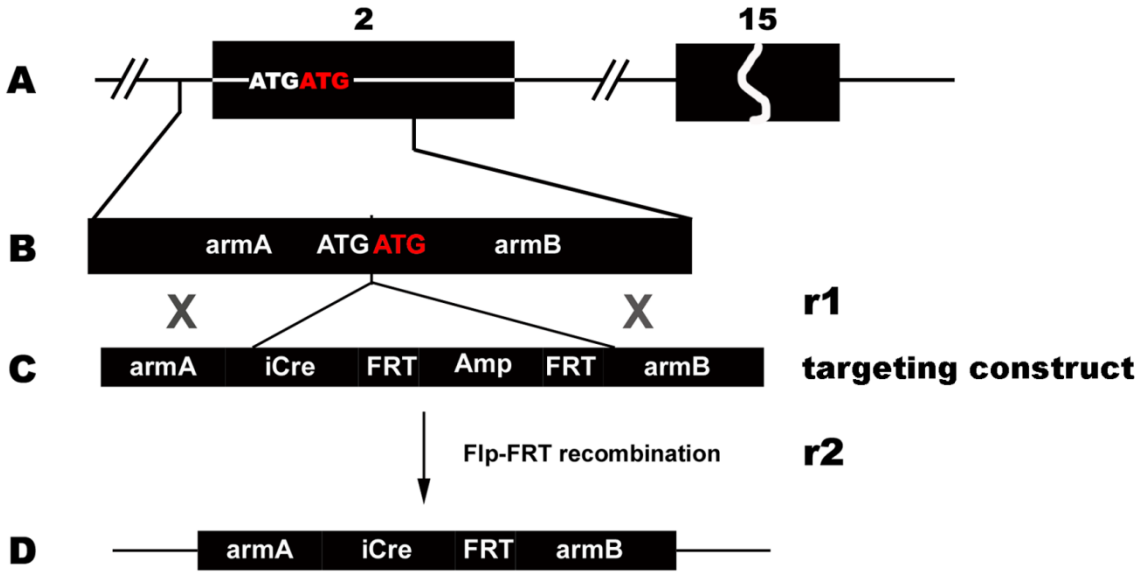

Fig. 3.5 Schematic representation of the Slco1c1 locus and the cloning strategy. A. The Slco1c1 gene has 15 exons. Exon 2 contains the translation start site (ATG in red). B. Before the translation start site, another ATG is found. The two homology arms were designed in such a way that armA ended with the first ATG, while armB started with the second ATG. The two homology arms were first cloned into the plasmid containing iCre and the selectable marker Amp (targeting construct). By homologous recombination (r1), the targeting construct shown in C was cloned into the region between armA and armB on the BAC RP24-85B20. The Amp was later removed by Flp-FRT mediated excision (r2). D shows the final organization on the BAC. The grey Xs indicate recombination events between the homology arms. The ATG at the end of armA is used as a start codon for the iCre translation. In the case of inducible lines, a mutated sequence of the estrogen receptor ligand binding domain (not shown) was fused after the iCre sequence. Thus, the Cre activity can be induced by administration of tamoxifen.

To ensure the correct recombination, a batch of experiments was performed. Restriction enzyme (XbaI) digested BAC constructs were loaded onto a gel (Fig. 3.7.1A). Compared to the wild type original BAC construct, after the first recombination (r1 in Fig. 3.5), there is an extra band (1.1kb), while an 11kb band is missing. After the second recombination (r2 in Fig. 3.5), the 1.1kb band is also missing. In addition,

southern blots were performed to characterize the BAC (Fig. 3.7.1B). By BamHI or SacI digestion, probes specific to either armB or Cre sequences detected different bands in the original (Ori), the iCre knock-in (r1), and the Amp-removal (r2) BAC constructs, confirming successful BAC cloning. The restriction sites used for Southern blotting are shown in Fig. 3.6. The targeted BAC construct (after the second recombination, r2) was then digested by NotI (illustrated in Fig. 3.6) to release the BAC from the vector (pTARBAC1). The digestion generated a band of 164kb and another 10kb band, which are shown in the pulse field gel electrophoresis (PFGE) in Fig. 3.7.1C. The PFGE proved that the BAC was intact for injection. The injected BAC was purified by Sepharose CL4b Column. Different fractions of the flow through were examined on PFGE, the fraction 15 in Fig. 3.7.1C was chosen for pronuclear injection, because in this fraction a good separation of insert and backbone was achieved (no vector band seen in the same lane) and the quality is high (OD A260/A280=1.779). Fig. 3.7.1D shows sequencing result of the targeted BAC. Usually the conjunctions of the two homology arms are most labile to mutation. The sequencing results proved that the sequence on the target BAC is not mutated.

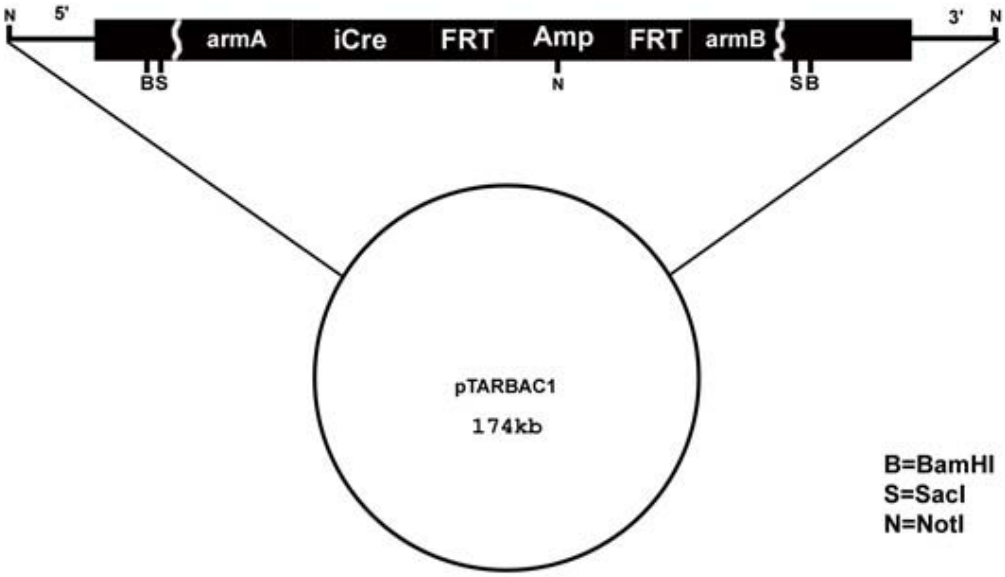

Fig. 3.6 Restriction sites on the targeted BAC RP24-85B20 before Amp removal. BamHI and SacI sites were used in Southern blotting. NotI sites were used to release the BAC from the backbone for injection.

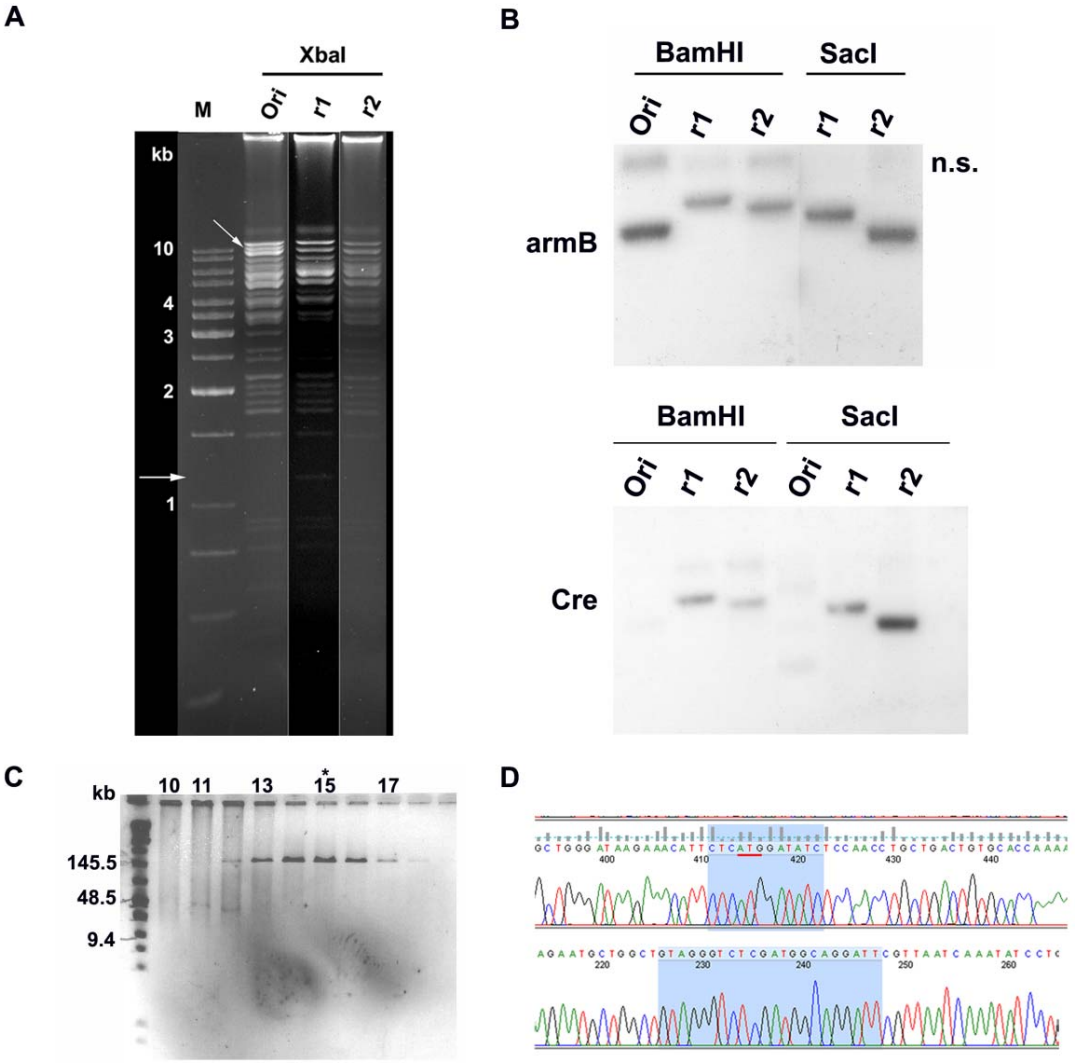

Fig. 3.7.1 Characterization of the BAC recombineering for the constitutive line. A. Gel electrophoresis of the original (Ori), the iCre knock-in (r1), and the Amp-removal (r2) BACs digested by XbaI. The most left lane is the Fermentas 1kb marker. The oblique arrow indicates an 11kb band, which is missing in r1 and r2. The horizontal arrow indicates a 1.1kb band, which is only found in r1. **B**. Sothern blotting of Ori, r1 and r2. In the upper panel, the BACs were digested with either BamHI or SacI. The hybridization probe was the sequence amplified by the primers armB5' and armB3'. The specific bands from left to right are 5.8kb, 8.5kb, 7.3kb, 6.2kb and 5.1kb in size, respectively (n.s . = non-specific). In the lower panel, the hybridization probe was the sequence amplified by Cre_up and Cre_down. The specific bands from left to right are 8.5kb, 7.3kb, 6.2kb and 5.1kb in size, respectively. There is no Cre in the Ori BAC. **C**. Pulse field gel electrophoresis (PFGE) of NotI digested r2. NotI digested r2 was purified through Sepharose

CL4b Column. Collected fractions were loaded for PFGE according to their ODs. The size marker (Low Range PFG Marker, NEB) is on the left. Fraction 15 (marked by an asterisk) was injected. **D**. Sequencing. In the upper panel, the highlighted region is the sequence flanking the first ATG (red underline). In the lower panel, the highlighted region is the 5' end of armA. Both regions were correctly recombined.

In Fig. 3.7.2, the characterization of the BAC recombineering for the inducible line was shown. The BAC recombineering for the inducible line differs with that for the constitutive line only in that Cre is fused to a mutated estrogen receptor ligand binding domain. Therefore, the principles of characterization for the inducible line are the same as those for the constitutive line. Successful recombination was shown by XbaI digestion (Fig. 3.7.2A), Southern blotting (Fig. 3.7.2B), PFGE (Fig. 3.7.2C), and sequencing (Fig. 3.7.2D).

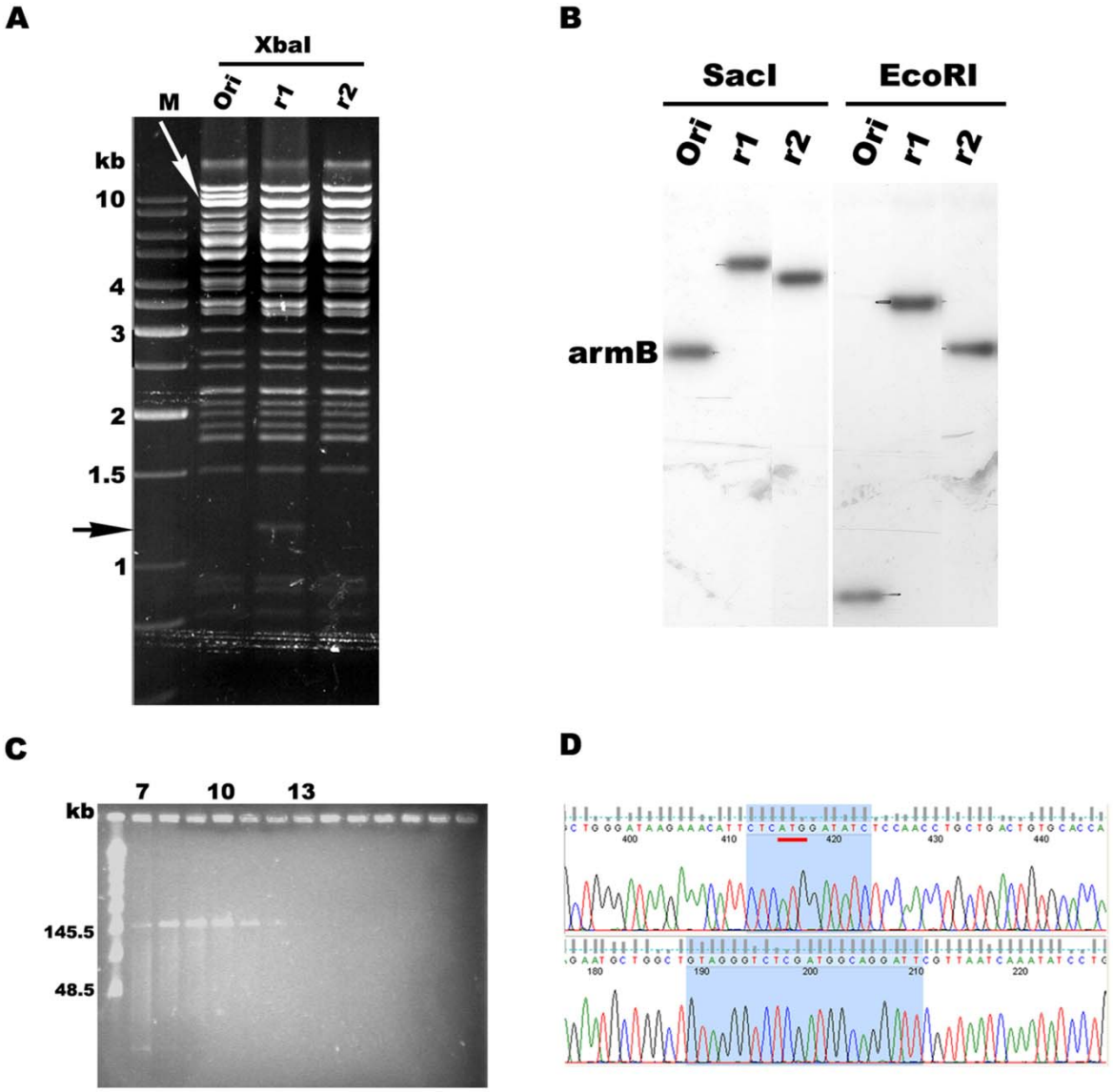

Fig. 3.7.2 Characterization of the BAC recombineering for the inducible line. A. Gel electrophoresis of the original (Ori), the iCreERT2 knock-in (r1), and the Amp-removal (r2) BACs digested by XbaI. The most left lane is the Fermentas 1kb marker. The oblique arrow indicates an 11kb band, which is missing in r1 and r2. The horizontal arrow indicates a 1.1kb band, which is only found in r1. **B**. Sothern blotting of Ori, r1 and r2. The BACs were digested with either SacI or EcoRI. The hybridization probe was the sequence amplified by the primers armB5' and armB3'. The specific bands from left to right are 3.6 kb, 7.1 kb, 5.9 kb, 1.2 kb, 4.7 kb and 3.5 kb in size, respectively. **C**. Pulse field gel electrophoresis (PFGE) of NotI digested r2. NotI digested r2 was purified through Sepharose CL4b Column. Collected fractions were loaded for PFGE according to their ODs. The size marker (Lambda Ladder PFG Marker, NEB) is on the left. Fraction 10 was injected. **D**. Sequencing. In the upper panel, the highlighted region is the sequence flanking the first ATG (red underline). In the lower panel, the highlighted region is the 5' end of armA. Both regions were correctly recombined.

3.3 Characterization of the Slco1c1-Cre transgenic mice

3.3.1 Founder mice

After pronuclear injection, 40 mice were born (Table 3.1). In these 40 mice, seven were genotyped as Cre+. Of the seven mice, three were male and four were female. Two female founders died at the age of about 20 weeks and did not produce any living offspring. The other two female were infertile. Therefore, all the following characterization was performed with offspring of the three male founders.

Founder/Born	Male : Female	Died/Founder	Infertile/Founder	Fertile/Founder
7/40 (17.5%)	3:4	2/7 (28.6%)	2/7 (28.6%)	3/7 (42.8%)

Table 3.1 Summary of founders. In total seven founders were selected by genotyping from 40 born after pronuclear injection. Two founders (female) died at the age of about 20 weeks old and were infertile. Another two (female) were infertile, too. All the mice further analyzed in this study were from the three male founders.

3.3.2 Expression pattern of Slco1c1-Cre

3.3.2.1 Detection of Cre and Slco1c1 transcripts in the brain

To know where the transgene Cre is expressed, mRNAs from various organs of Cre+ mice were extracted. The quality of extracted mRNA was checked by RNA gel electrophoresis (data not shown). The

cDNAs were reversely transcribed from these mRNAs. Primers specific for Cre (Cre_up/Cre_down) and Slco1c1 (Slc-rt1/Slc-rt2) were used to amplify the cDNAs of Cre and Slco1c1, respectively. Cre and Slco1c1 were detected in brain (cerebra and cerebella), but not from other organs (Fig. 3.8). This result is in line with the tissue distribution of SLCO1C1 (Sugiyama et al. 2003; Tohyama et al. 2004; Cheng et al. 2005). The concomitant expression of Cre and Slco1c1 suggests that Cre expression may reflect the endogenous expression of Slco1c1. Previous studies have shown that many transgenes introduced by BAC engineering faithfully mimic the endogenous situation (Copeland et al. 2001; Casanova et al. 2002; Mukherjee et al. 2006; Kwon and Hadjantonakis 2007).

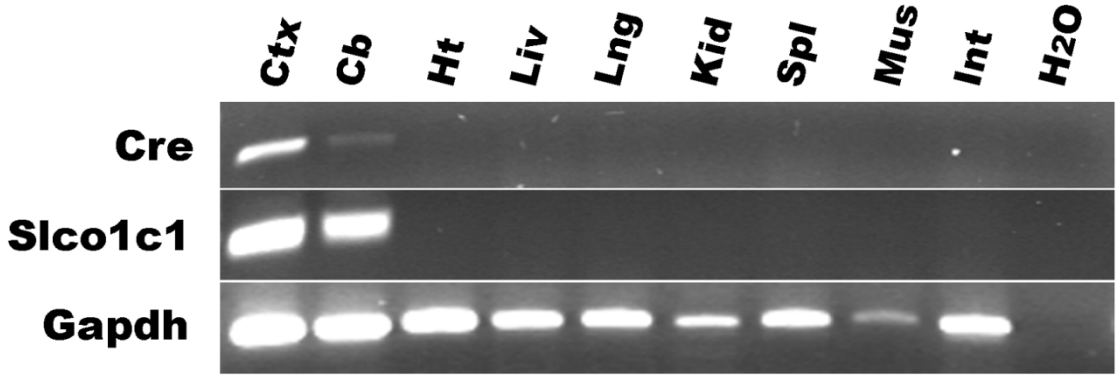

Fig. 3.8 In adult transgenic mice, at the transcription level, Cre and Slco1c1 expressions were found in the brain. Reversely-transcribed cDNAs from mRNAs of cerebra (Ctx), cerebella (Cb), heart (Ht), liver (Liv), lung (Lng), kidney (Kid), spleen (Spl), intestine (Int), and muscle (Mus) were used as templates to amplify Cre with primers Cre_up/Cre_down and Slco1c1 with primers Slc-rt1/Slc-rt2. Cre and Slco1c1 transcripts were found only in the brain. Gapdh was used as an input control and H_2O as a negative control.

3.3.2.2 Mice used for LacZ staining

In the founder mice, the targeted BAC may not integrate into the genome of the germ line cells or the integration could be mosaic, i.e. in different germ line cells there are different copy numbers of the BAC or different integration sites in the chromosome. Further analysis has to be done in their offspring. As listed in Table 3.2, in total ten mice (Cre+; LacZ+) from the three male founders were included in LacZ staining. At the time of staining (> 5 weeks of age), most of the brain development had been completed.

Founder	6	19					31			
F1	89	51	57	98		123	69		70	
LacZ Staining	89	145	174	263	264	315	240	245	327	328
Age (weeks)	29	22	19	13	11	11	12	12	5	5

Table 3.2 Summary of mice used for LacZ expression pattern. Offspring from the three fertile male founders were examined for their Cre expression pattern by LacZ staining. The first row shows the three founders. The second shows the F1s from the founders. The third row shows the mice that were used for LacZ staining. Mouse 89 is a F1. The rest mice are offspring from corresponding F1s.

3.3.2.3 LacZ staining in adults

In adult mice that were genotyped as Cre+;LacZ+, brain sections were stained for LacZ expression. The bacterial LacZ gene is inserted into the mouse Rosa26 locus (Soriano 1999). The expression of Cre can remove the floxed neomycin cassette containing a triple PolyA sequence in front of the LacZ gene, thus leading to its expression. LacZ encodes beta-galactosidase (β-gal), which can cleave its substrate X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside). The cleaved products then are oxidized to 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. By matching the blue color on the sections to known brain structures, one can find the anatomical expression pattern of LacZ, i.e. in this study Slco1c1-Cre.

Table 3.3 summarizes the brain structures that were found stained blue in LacZ staining. As expected, previous reported sites of Slco1c1 expression were found stained for LacZ, e.g. blood vessels and choroid plexus. However, other structures were also stained. The expression in Table 3.3 is graded for the density of staining, with the highest 5 and lowest 1, and 0 meaning no staining. Since blood vessels (capillaries) are everywhere in the brain (Abbott et al. 2006), incubation of the sections with X-gal needs to be controlled, otherwise the sections would be totally blue in color. The expression in this table

mainly came from non-vessel structures, since difference in blood vessel staining was not obvious at first sight.

The expression densities in non-vessel structures from different mice (offspring of different founders and F1s) are quite uniform, with variations no more than 1 grade (Table 3.3). This result again supports the concept that BAC-transgenic mice have the advantage in mimicking the endogenous gene expression. The most densely stained structures are the neuronal cell layers in the olfactory bulb (Gl and Ml), a superficial cortical layer of cells (later defined as layer 2/3), piriform cortex (Pir), hippocampus (CA1-CA3, dentate gyrus and the surrounding areas), choroid plexus (chp), anterodorsal thalamic nucleus (AD), and white matters that connecting the two hemispheres (anterior commissure and corpus callosum). Other structures were stained at various densities ranging from 1 to 4. In all the animals analyzed, at least some grade of staining was detected in all brain regions investigated.

Location	Expression
Olfactory bulb	
glomerular layer (Gl)	4-5
external plexifrom layer(EPl)	1-2
mitral cell layer (Mi)	4-5
granule layer, olf bulb (GrO)	4-5
anterior olfactory nu (AO)	3-4
accessory olfactory bulb (AOB)	3-4
Cerebral cortex	
layer 1	4
layer 2/3	5
layer 4	4
layer 5	3
layer 6	3
piriform cortex (Pir)	5
dorsal endopiriform nu (DEn)	2-3
Basal ganglia	
accumbens nu, core (AcbC)	2-3
accumbens nu, shell (AcbSh)	2-3
caudate putamen (CPu)	2-3
lateral globus pallidus (LGP)	3
medial globus pallidus (MGP)	3
ventral pallidum (VP)	2-4
claustrum (Cl)	2-4
olfactory tubercle (Tu)	5
islands of Calleja (iCj)	4-5
substantia nigra, compact (SNC)	2-3
substantia nigra, lat (SNL)	2-3
substantia nigra, reticular (SNR)	2-3
substantia innominata (SI)	2
subthalamic nu (STh)	3-4
Amygdala	
basolateral amygdaloid nu (BLA/V)	3-4
central amygdaloid nu (Ce…)	3
basolateral amygdaloid nu, post (BLP)	3-4
basomedial amygdaloid nu, post (BMP)	3-4
medial amygdaloid nu (Me…)	2-3
lateral amygdaloid nu (La…)	3-4
posterolat. cortical amygdaloid nu (PLCo)	3-4
posteromed. cortical amygdaloid nu (PMCo)	3
amygdalostriatal transition area (AStr)	2-3
amygdalohippocampal area (AHi…)	2-4
Hippocampus	
CA1 field	5
CA2 field	5
CA3 field	5
dentate gyrus (DG)	5
subiculum (S)	3-5
fimbria hippocampus (fi)	4-5
cingulum (cg)	4-5
Hypothalamus	
periventricular hypothalamic nu (Pe)	3-4
paraventricular hypothalamic nu (Pa…)	2-3
dorsomedial hypothalamic nu (DM)	2-4

Location	Expression
Hypothalamus	
anterior hypothalamic area (AH)	2-3
lateral hypothalamic area (LH)	3
arcuate hypothalamic nu (Arc)	3-4
supraoptic hypothalamic nu (SO)	2-4
ventralmedial hypothalamic nu (VMH)	3-4
med preoptic area (MPA)	2-3
lateroanterior hypothal nu (LA)	2-3
Circumventricular Organs	
choroid plexus (chp)	5
median eminence (ME)	2-3
subfornical organ (SFO)	4-5
Thalamus	
anterodorsal thalamic nu (AD)	4-5
anteroventral thalamic nu, dor med (AVDM)	2-3
anteroventral thalamic nu, ven lat (AVVL)	2-3
paraventricular thalamic nu (PV)	1-2
central medial thalamic nu (CM)	1-2
stria medullaris, thalamus (sm)	1-2
paracentral thalamic nu (PC)	1-2
medial habenular nu (MHb)	4-5
lateral habenular nu (LHb)	3-4
mediodorsal thalamic nu (MD)	1-2
parataenial thalamic nu (PT)	1-2
intermediodorsal thalamic nu (IMD)	1-2
Cerebellum	
Purkinje cell layer	3
med cerebellar nu (MED)	2-3
prepositus hypoglossal nu (Pr)	2-3
pontine ret. Nu (PnC)	2-3
nu trapezoid body (Tz)	2-3
spinal trigeminal nu (Sp5)	3
spinal vestibular nu (SpVe)	3
Miscellaneous	
laterodorsal tegmental nu (LDTg)	3-4
dorsal tegmental nu (DTg)	3-4
gigantocellular reticular nu (Gi)	3
anterior commissure, intrabulbar (aci)	1-2
anterior commissure, ant (aca)	4-5
forceps minor of the corpus callosum (fmi)	4-5
forceps major of the corpus callosum (fmJ)	4-5
external capsule (ec)	4
internal capsule (ic)	3-4
medial septal nu (MS)	1-2
nu of the vertical limb of the diagonal band (VDB)	2-3
lateral septal nu, intermediate (LSI)	3-4
lateral septal nu, ventral (LSV)	3
septofimbrial nu (SFi)	1-2
median preoptic nu (MnPO)	3-4
sublentic extended amygdale (SLEA)	2
lateral septal nu, dorsal (LSD)	3-4
triangular septal nu (TS)	3
med mammillary nu, med (MM)	3-4
med mammillary nu, lat (ML)	3-4
lateral mammillary nu (LM)	3-4

Table 3.3 Expression of the Slco1c1-Cre detected by LacZ staining. Continuous coronal sections (60 μm in thickness) were cut from 10 Cre+;LacZ+ mice (as shown in Table 3.2). The entire olfactory bulbs and forebrain and part of cerebellum were analysed. Adjacent sections were stained by either LacZ or Nissl staining. For LacZ staining, the sections were incubated in the staining solution for 4 hours at 37°C. Relative densities of the LacZ staining were evaluated and ranked as follows: 5, maximum density; 4, very dense; 3, dense; + 2, moderate; 1, low; 0, below detection. The listed brain structures were identified from sections with Nissl staining. The anatomical nomenclatures are adopted from 'The Mouse Brain in Stereotaxic Coordinates' (Franklin and Paxinos 1997).

The most noticeable stained structures other than blood vessel and choroid plexus are a layer of cells in the cerebral cortex and hippocampus. On the sagittal sections in Fig. 3.9 and the coronal sections in Fig. 3.10, these two structures can be easily observed. Choroid plexus in the lateral ventricle and dorsal 3rd ventricle are stained densely.

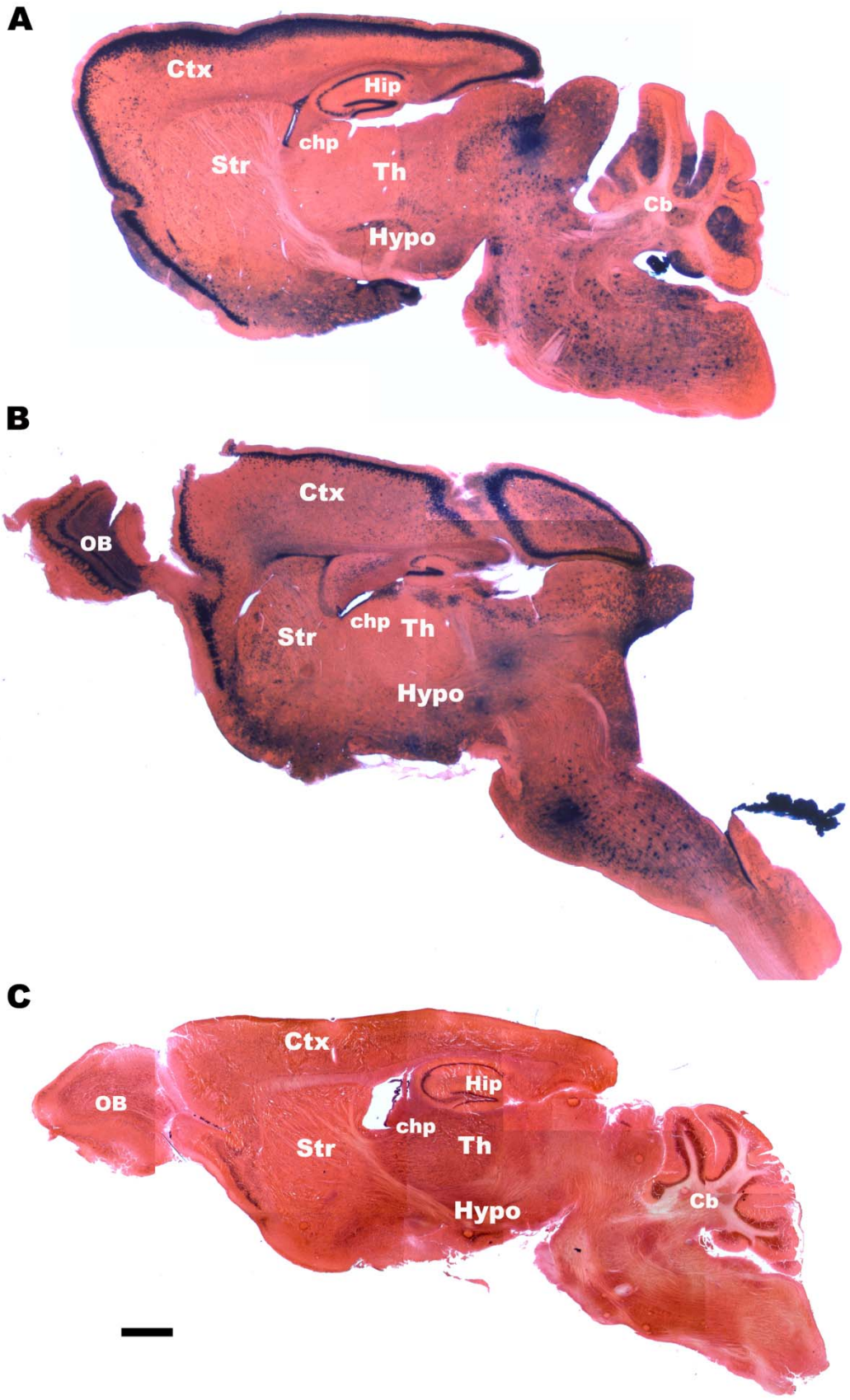
A
Ctx
Hip
chp
Str
Th
Cb
Hypo
B
Ctx
OB
Str
chp
Th
Hypo
C
Ctx
Hip
OB
chp
Str
Th
Hypo
Cb

Fig. 3.9 Expression of Slco1c1-Cre detected by LacZ staining (sagittal). Mice harboring both the Cre and the LacZ alleles were examined for their Cre expression. On the sagittal sections shown in A and B, blue color is detected in cortex (Ctx), hippocampus (Hip), choroid plexus (chp), striatum (Str), thalamus (Th), hypothalamus (Hypo), cerebellum (Cb), olfactory bulb (OB), and other brain regions. Mice that do not express LacZ (C) were also examined as a negative control. There was no blue color detected in the negative controls. Scale bar = 500µm.

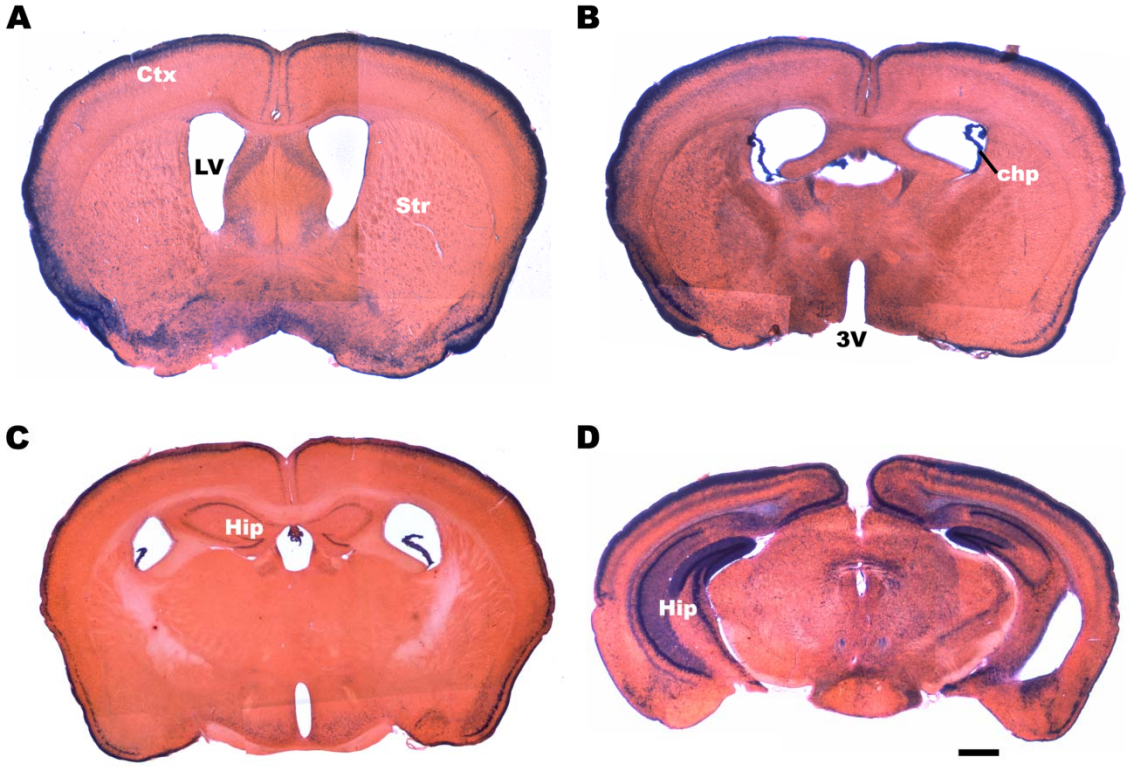

Fig. 3.10 Expression of Slco1c1-Cre detected by LacZ staining (coronal). Mice harboring both the Cre and the LacZ alleles were examined for their Cre expression. On the coronal sections shown in A-D, blue color is detected in cortex (Ctx), hippocampus (Hip), choroid plexus (chp), striatum (Str), and other brain regions. LV, lateral ventricle; 3V, 3rd ventricle. Scale bar = 500µm.

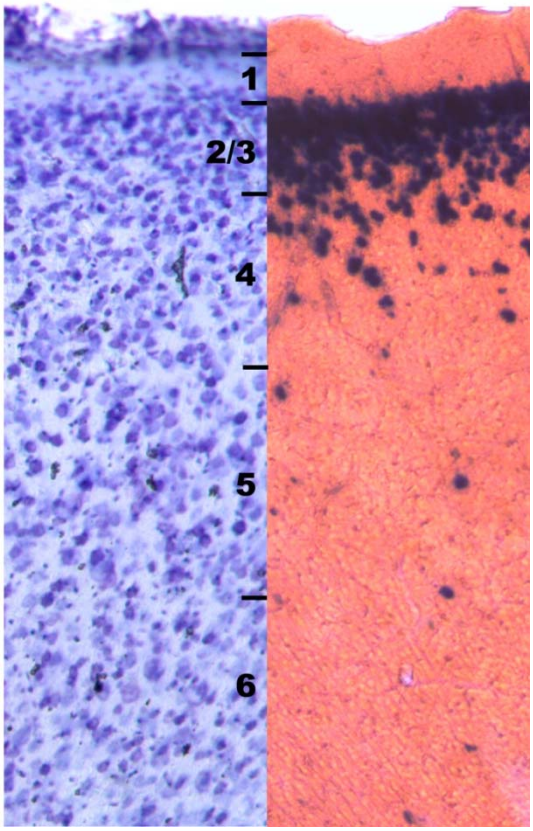

Fig. 3.11 Cortical layer 2/3 cells are LacZ positive. The cortical staining found in LacZ staining is located at layer 2/3 of the cortex. On the left side, Nissl staining shows the cortical lamination. The LacZ-positive region shown on the right side corresponds to layer 2/3.

The superficial cortical layer that is stained by LacZ staining corresponds to layer 2/3 in the cortex. By aligning to Nissl stained sections (Fig. 3.11), it is clearly seen that the cells in this layer showed the same closely packed arrangement in both Nissl and LacZ staining.

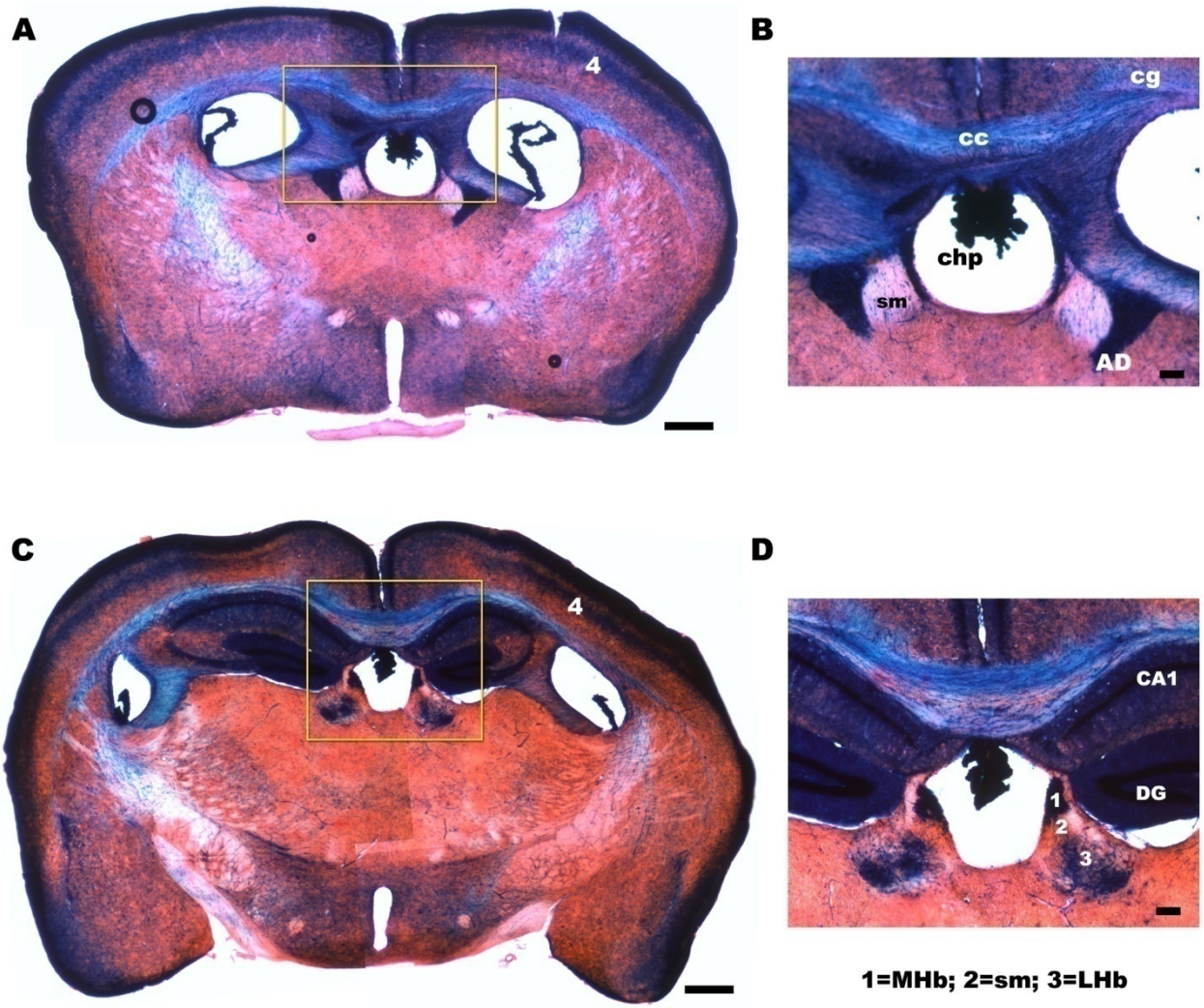

Fig. 3.12 Examples of LacZ expression in different brain regions. The region in the yellow frames in A and C are magnified in B and D, respectively. cc, corpus callosum; cg, cingulum; chp, choroid plexus; sm, stria medullaris, thalamus; AD, anterodorsal thalamic nu; MHb, medial habenular nu; LHb, lateral habenular nu; CA1, CA1 field, hippocampus; DG, dentate gyrus. The number 4 in A and C indicate a region that also shows blue color. But a detailed examination only found diffusive blue color without any apparent cell-like structure. Scale bar, = 500µm (A and C); =125µm (B and D).

In Fig. 3.12, two examples are shown to illustrate how the staining intensity was graded. Anterodorsal thalamic nucleus (AD) and choroid plexus (chp) were graded as '5' in Fig. 3.12B, CA1, dentate gyrus (DG) and medial habenular nucleus (MHb) in Fig. 3.12D were also graded as '5'. Corpus callosum (cc) and cingulum (cg) were '4-5' in Fig 3.12B, since in different sections they were stained a little bit differently,

which is the case in many brain structure evaluated. Lateral habenular nucleus (LHb) is defined as '3-4' and stria medullaris (sm) as 1-2.

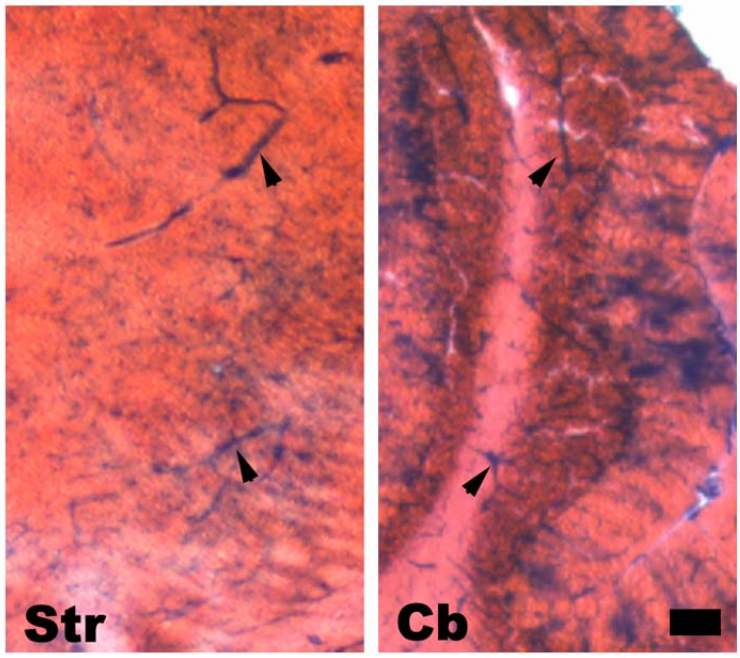

Fig. 3.13 Blood vessels detected by LacZ staining in striatum (Str) and cerebellum (Cb). Arrow heads indicate blood vessels with blue staining in striatum and cerebellum. Scale bar=100µm.

In Fig 3.13, blood microvessels in striatum (Str) and cerebellum (Cb) are displayed. The arrowheads point to bigger microvessels. The smaller ones cannot be easily recognized, since they are so fine (5-10µm in diameter) that they appear as diffusive staining. This diffusive staining is seen everywhere in the brain, including the cortical region in Fig. 3.12 (number 4). If long incubation with substrate was allowed, most areas of the brain sections would stained blue, making recognition of stained brain structures impossible.

3.3.2.4 LacZ staining in embryos

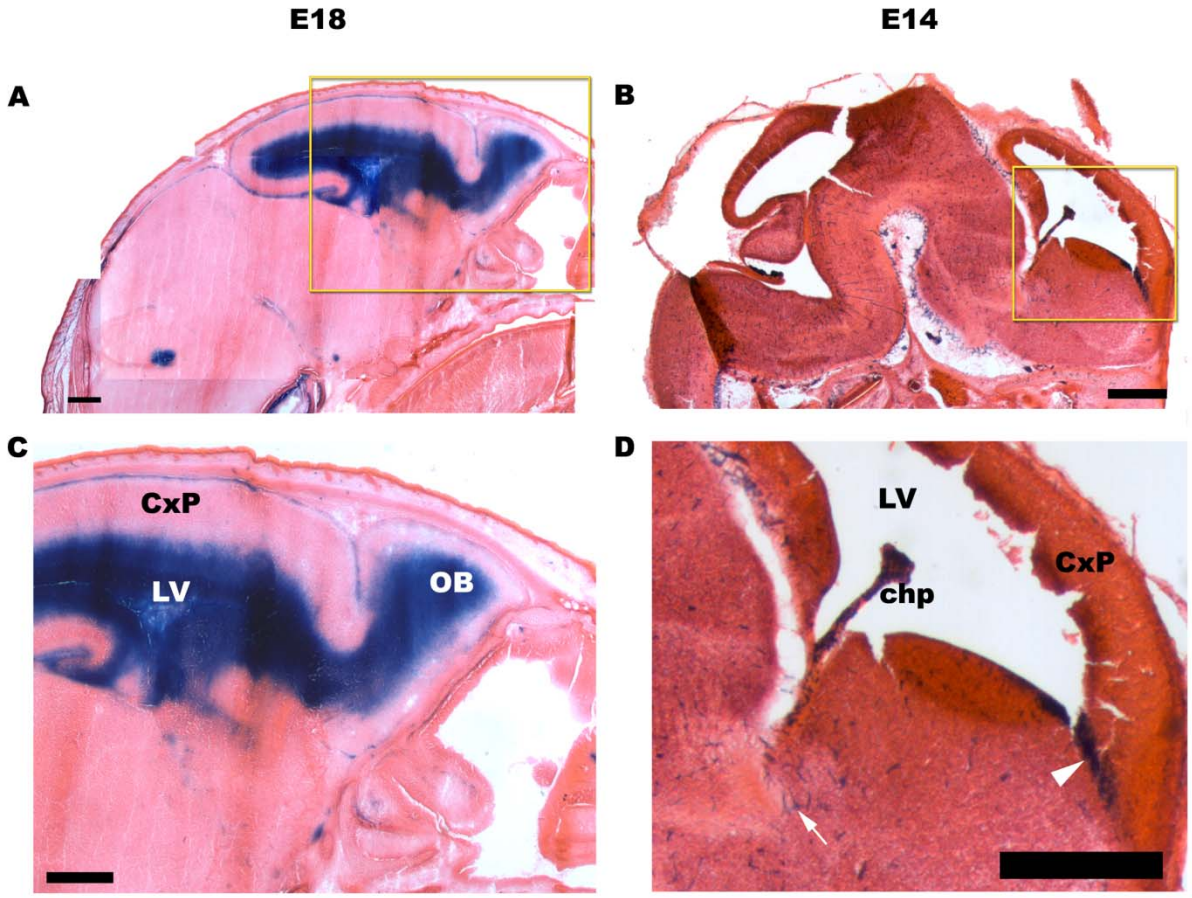

Fig. 3.14 LacZ staining of E18 and E14 embryos. E18 (A and C) and E14 (B and D) embryos were stained for LacZ expression. The yellow frames in A and B were magnified in C and D, respectively. LV, lateral ventricle; OB, olfactory bulb; CxP, cortical plate; chp, choroid plexus. The arrow in D indicates blood vessels. The arrowhead in D indicates LacZ-expressing cells near the ventricular zones. Scale bars=500µm.

The LacZ staining seen in the neuronal structures (cortical layer 2/3, hippocampus and others) raises the question when these cells express Slco1c1-Cre. As early as at E14, in addition to the wide spread staining of blood vessels, LacZ staining can be observed around the lateral ventricle (LV), especially the rostral part (Fig. 3.14 B and D). The area near the lateral ventricle contains neurogenic zones during development, ventricular zone (VZ) and subventricular zone (SVZ). This finding suggests that the stained

neuronal structures seen in adults may originate in these zones. The more obvious staining in the rostral part of the lateral ventricle also supports this hypothesis. From the rostral neurogenic zones, new born neurons migrate to the olfactory bulb, where neurons are replaced throughout life in rodents. At E18, this migration is even more clearly detected by LacZ staining (Fig. 3.14A and C). A strong staining can be seen extending from lateral ventricle to olfactory bulb. The glomerular cell layer (Gl) and mitral cell layer (Ml) of the olfactory bulb are strongly stained (Table 3.3).

In Fig. 3.15, a detailed nomenclature of an E14 brain section is shown. In this figure, blood vessel and choroid plexus staining is already present.

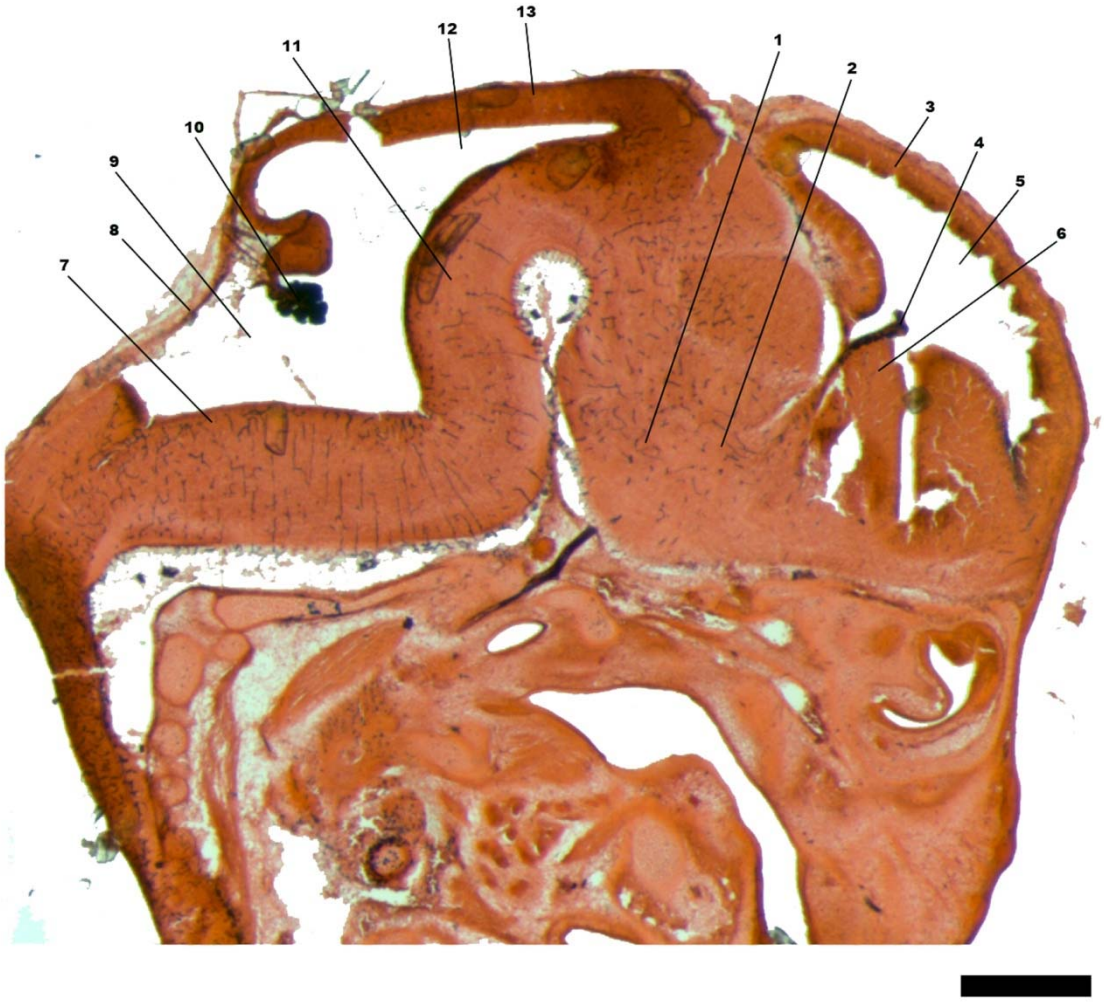

Fig. 3.15 LacZ staining of a sagittal brain section (E14). Blue staining on blood vessels are found in the whole brain. The listed structures are, 1. diencephalon (hypothalamus), 2. diencephalon (thalamus), 3. roof of neopallial cortex (future cerebral cortex), 4. choroid plexus extending into lateral ventricle, 5. lateral ventricle, 6. copus striatum mediale (medial aspect of ganglionic eminence), 7. ventral part of medulla oblongata, 8. roof of fourth ventricle, 9. fourth ventricle, 10. choroid plexus within central part of lumen of fourth ventricle, 11. pons, 12. mesencephalic vesicle, 13. roof of midbrain. Scale bar=500 µm.

3.3.2.5 Adult neurons have no Slco1c1-Cre activity

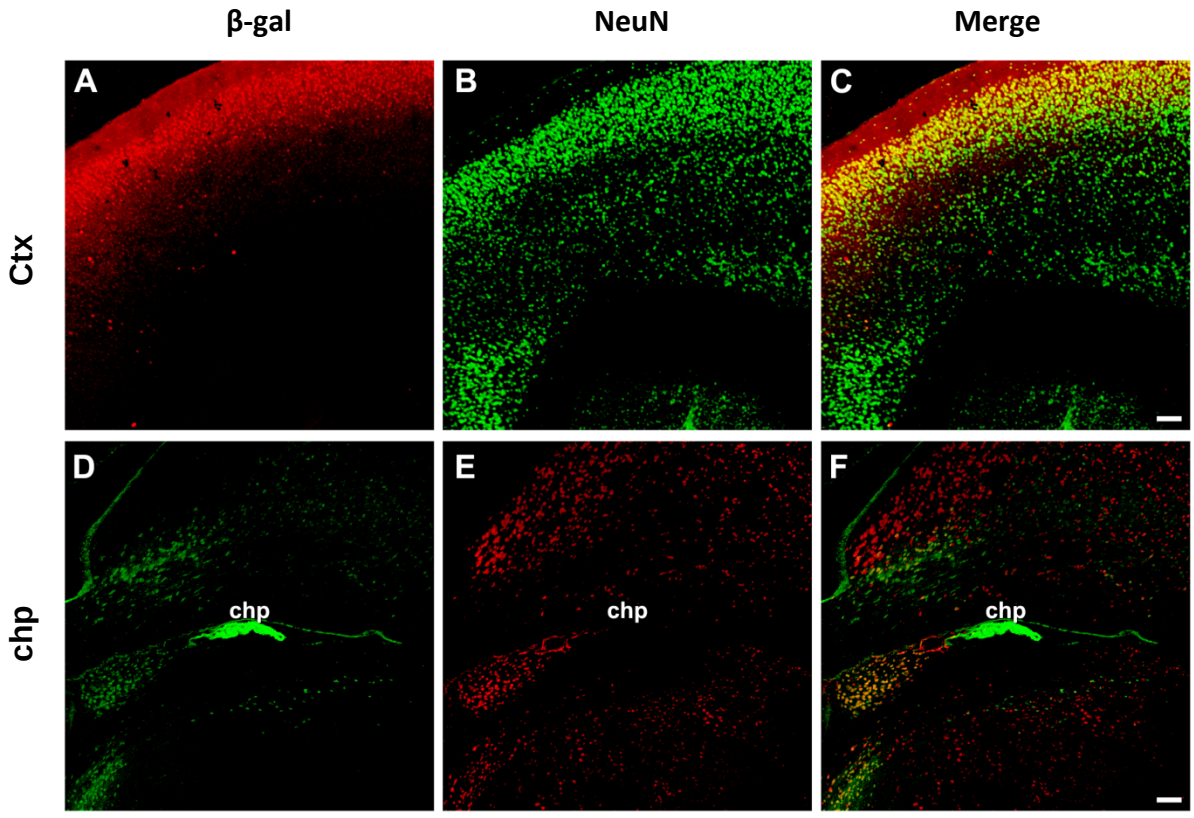

Fig. 3.16 Colocalization of β-gal-expressing cells with neurons. Coronal cryosections of brain were stained by antibodies against β-gal and NeuN. The secondary antibody for β-gal was labeled with Cy3 in cortex (Ctx) and Alexa488 in choroid plexus (chp), for NeuN was with Alexa 488 in Ctx and Cy3 in chp. The stained sections were then examined by confocal microscopy. In Ctx (A-C), the expression of β-gal colocalizes with a neuronal marker (NeuN). In non-neuronal structure chp (D-F), β-gal expression was found. Scale bars = 100µm.

To identify the cell type of the LacZ-labeled cells in cortical layer 2/3, brain sections were stained immunofluorescently with β-gal and NeuN (neuronal marker). In the upper panel of Fig. 3.16, in the cortex, β-gal colocalizes with NeuN, indicating that the β-gal-expressing cells in this layer are neurons. In the lower panel, choroid plexus, a non-neuronal structure, is stained by β-gal, but has no NeuN staining.

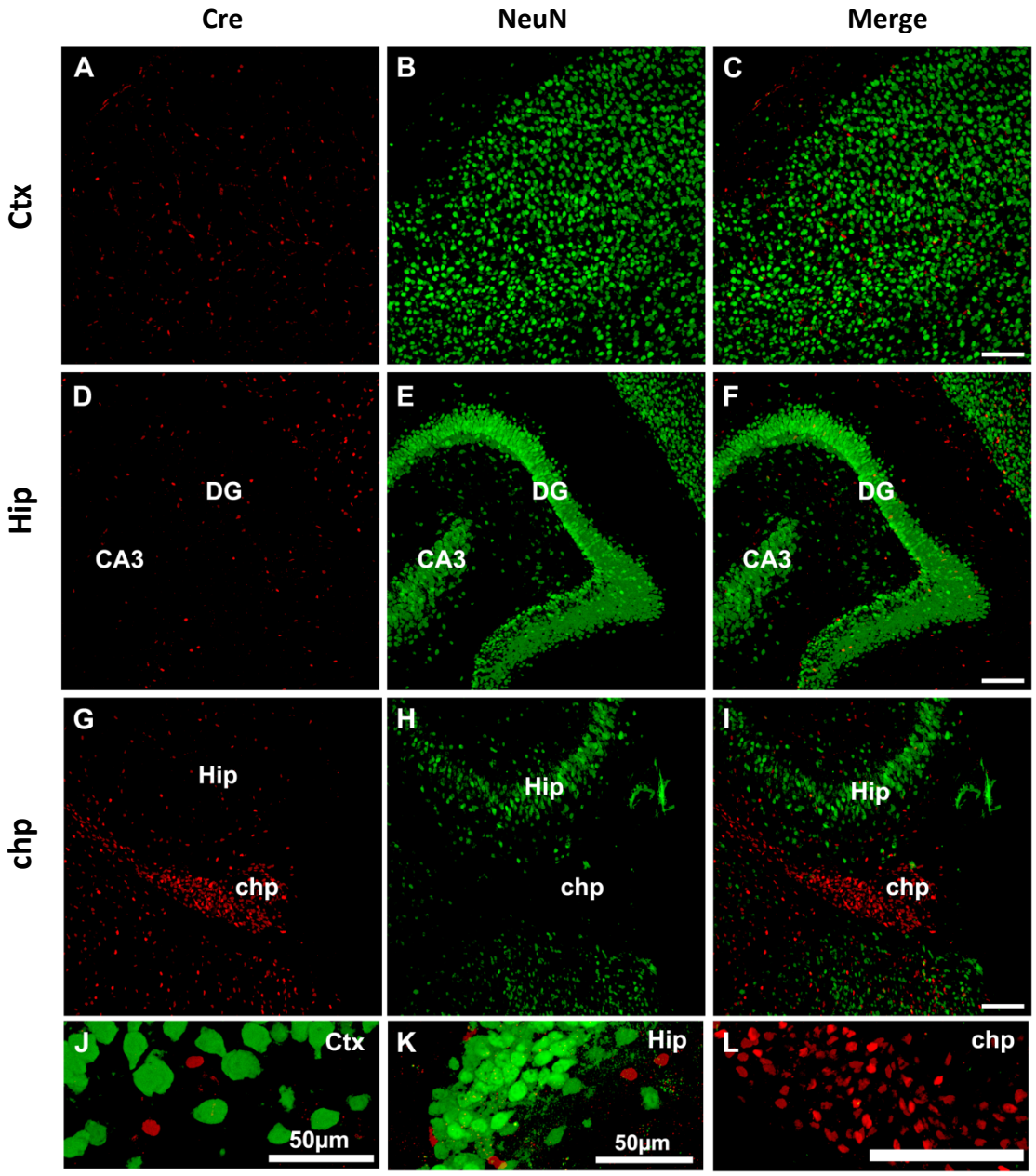

Fig. 3.17 Cre-expressing cells do not colocalize with neurons. Coronal cryosections of brain were stained by antibodies against Cre and NeuN. The secondary antibody for Cre was labeled with Cy3, for NeuN was with Alexa 488. The stained sections were then examined by confocal microscopy. In cortex (Ctx, A-C), hippocampus (Hip, D-E), and choroid plexus (chp, G-H), the expression of Cre does not colocalize with a neuronal marker (NeuN). At higher magnification in cortex (J), hippocampus (K), and choroid plexus (L), Cre expression does not colocalize with NeuN. DG, dentate gyrus. If not indicated, scale bars = 100 µm.

To investigate if Cre is expressed in adult neurons, brain sections were stained by Cre and NeuN antibodies. Cre-labeled cells have no colocalization with NeuN-labeled cells in cerebral cortex, hippocampus and choroid plexus (Fig. 3.17). Together with the results of the LacZ staining and β-gal/NeuN staining, we conclude that Slco1c1-Cre is expressed in neurons during early development, but not in adult neurons. Its expression in blood vessels and choroid plexus is sustained from embryonic development till adulthood.

Furthermore, in an inducible line, in which Slco1c1-Cre activity is controlled by a mutated estrogen receptor ligand binding domain (ERT2), induction of Cre activity in adults by tamoxifen (2x1mg/day, for 5 days) does not reveal LacZ-expressing cells in cortical layer 2/3 and hippocampus (Fig 3.18), while the blood vessel and choroid plexus staining was preserved. This result further supports that Slco1c1-Cre is not expressed in adult neurons.

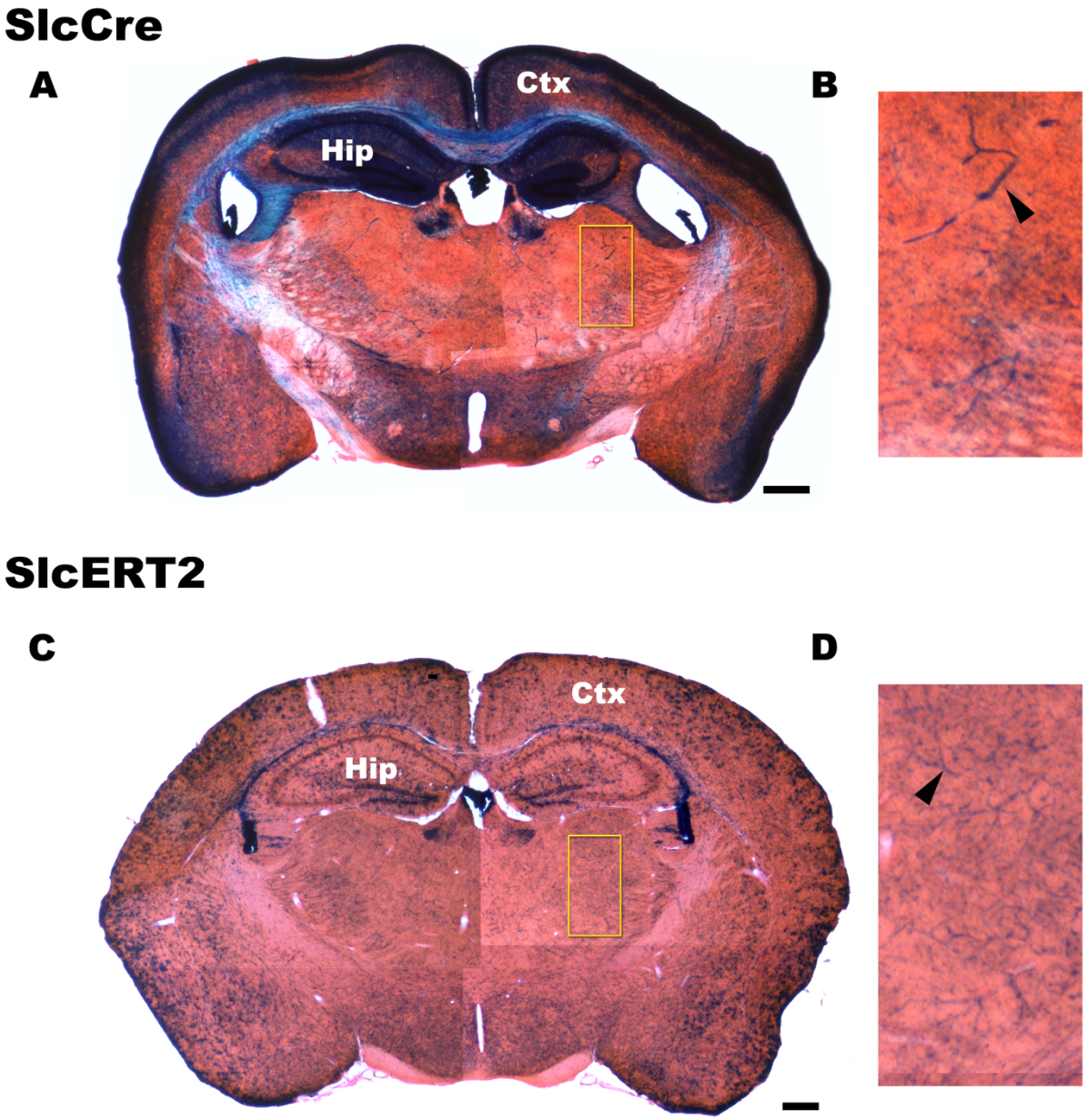

Fig. 3.18 LacZ staining of the constitutive line (SlcCre) and the inducible line (SlcERT2). A. In the constitutive line, a notable feature is the staining in layer 2/3 of the cortex (Ctx) and hippocampus (Hip). B. Higher magnification of the region in the yellow frame in A. C. In the inducible line, compared to the constitutive line, the staining in layer 2/3 of the cortex (Ctx) and hippocampus is not detected. D. Higher magnification of the region in the yellow frame in C. Arrow heads in B and D indicate staining in blood vessels. Scale bars = 500 µm (in A and C).

3.3.2.6 Slco1c1-Cre is expressed in brain blood vessels in adult mice

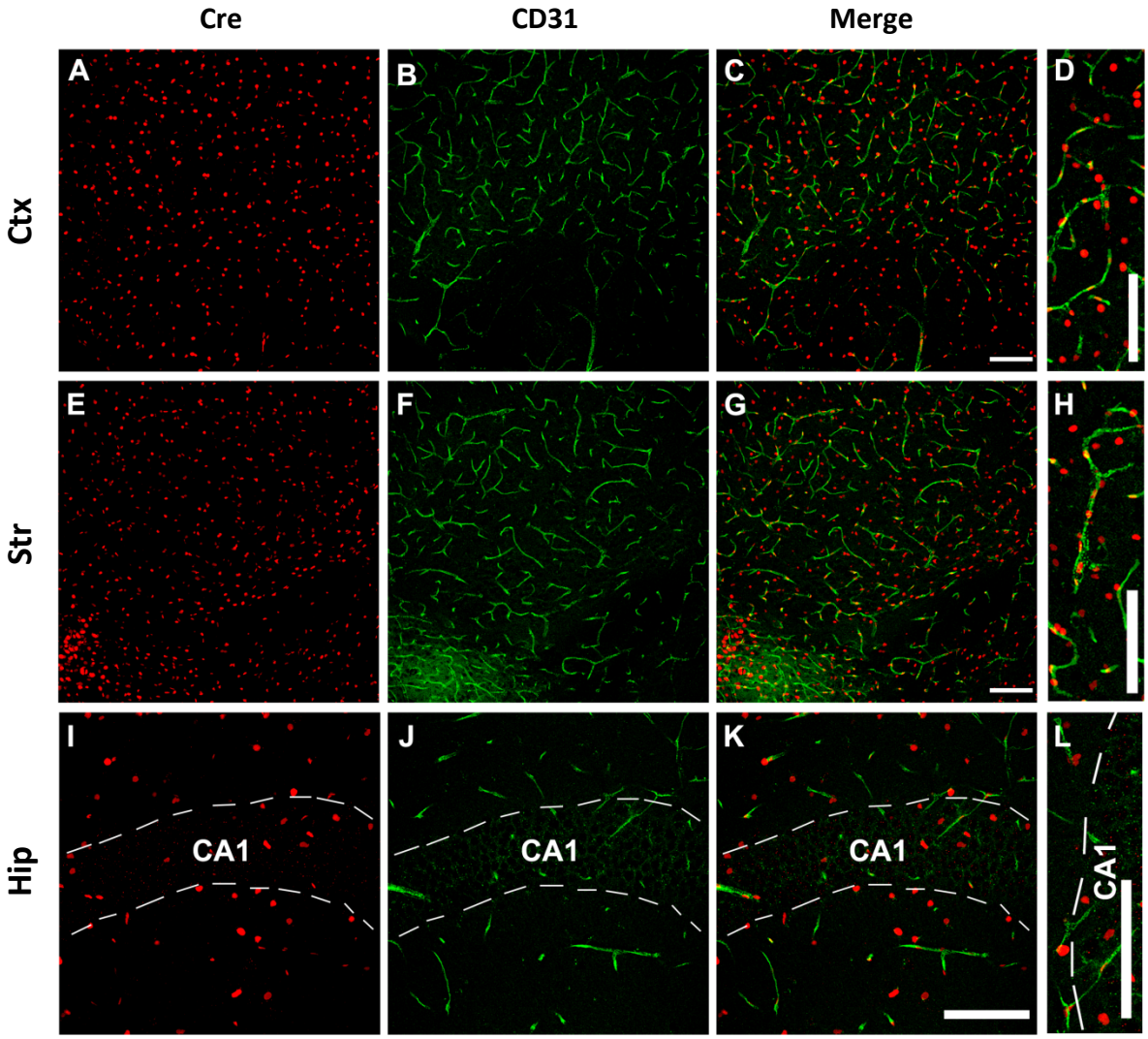

Fig. 3.19 Colocalization of Cre-expressing cells with BBB endothelia. Coronal cryosections (60 µm thick) of the brain were stained by antibodies against Cre and CD31. The secondary antibody was labeled with Cy3 for Cre and with Alexa 488 for CD31. The stained sections were then examined by confocal microscopy. In different cerebral regions, cortex (Ctx, A-D), striatum (Str, E-H), and hippocampus (Hip, I-L), the expression of Cre colocalizes with an endothelial cell marker (CD31). The dashed lines in I-L indicate the CA1 region of hippocampus. D, H and L show higher magnification in cortex (D), striatum (H), and hippocampus (L). Scale bars = 100 µm.

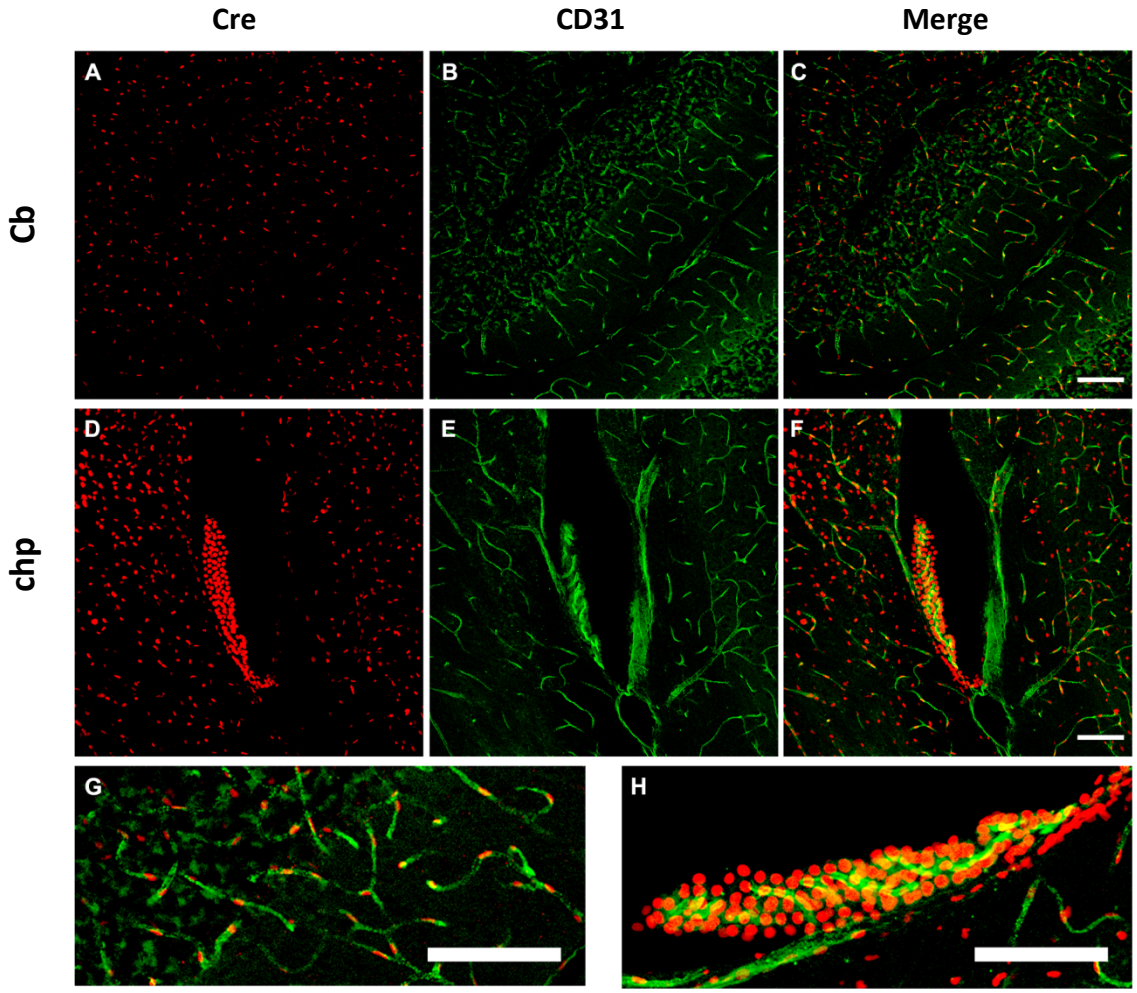

Fig. 3.20 Colocalization of Cre-expressing cells with BBB endothelia in cerebellum and choroid plexus. Coronal cryosections (60µm thick) of brain were stained by antibodies against Cre and CD31. The secondary antibody was labeled with Cy3 for Cre and with Alexa 488 for CD31. The stained sections were then examined by confocal microscopy. In cerebellum (Cb, A-C), the expression of Cre colocalizes with an endothelial cell marker (CD31). In choroid plexus (chp, D-F), Cre expression is found in close relation to CD31. G and H are higher magnification in cerebellum (G) and choroid plexus (H). Scale bars = 100µm.

Since in adult neurons Slco1c1-Cre is not expressed, it is interesting to know if Cre activity is present in blood vessels, where Slco1c1 is expressed. An endothelial cell marker CD31 (PECAM) was co-stained with Cre. As in Fig. 3.19 and Fig. 3.20, in cortex (Ctx), striatum (Str), hippocampus (Hip), and cerebellum (Cb), most (if not all) Cre-labeled cells colocalize with or are located in the immediate vicinity of CD31-labelled structures, which are blood vessels. In choroid plexus, blood vessels and Cre are also seen next to each other. This is because endothelial cells and epithelial cells in choroid plexus are in close contact (illustrated in Fig. 1.7).

3.3.2.7 A role of SLCO1C1 in embryonic neurogenesis

The above results demonstrated that Slco1c1-Cre expression closely resembles the endogenous expression pattern in adults, while neuronal expression is only found in embryonic development. Thyroid hormones are important for brain development. The Slco1c1-Cre line may provide a tool to study particularly the influence of thyroxine on those neurons that express Slco1c1 in their development. To test this possibility, mice were rendered hypothyroxinemic by administrating 0.1% PTU to their mothers in the drinking water from E2 to P2. This protocol is sufficient to reduce the serum level of thyroxine (T4), while keeping T3 unaltered (Fig. 3.21). In the PTU group, the fT4 level in serum of mothers is 8.57±1.40 ng/l, significantly decreased compared to the control level at 19.01±1.66 ng/l ($p<0.005$). The fT3 levels, however, show no difference in the control (2.37±0.12 ng/l) and in the PTU group (2.71±0.32 ng/l). The thyroid hormone levels in P2 pups also showed difference in fT4 (12.12±0.24 ng/l in the control vs. 7.35 ng/l in the PTU group). Since the limited blood volume from these pups, blood from several pups was pooled for one measurement in the PTU group. Thus, no statistical test to compare the data was performed.

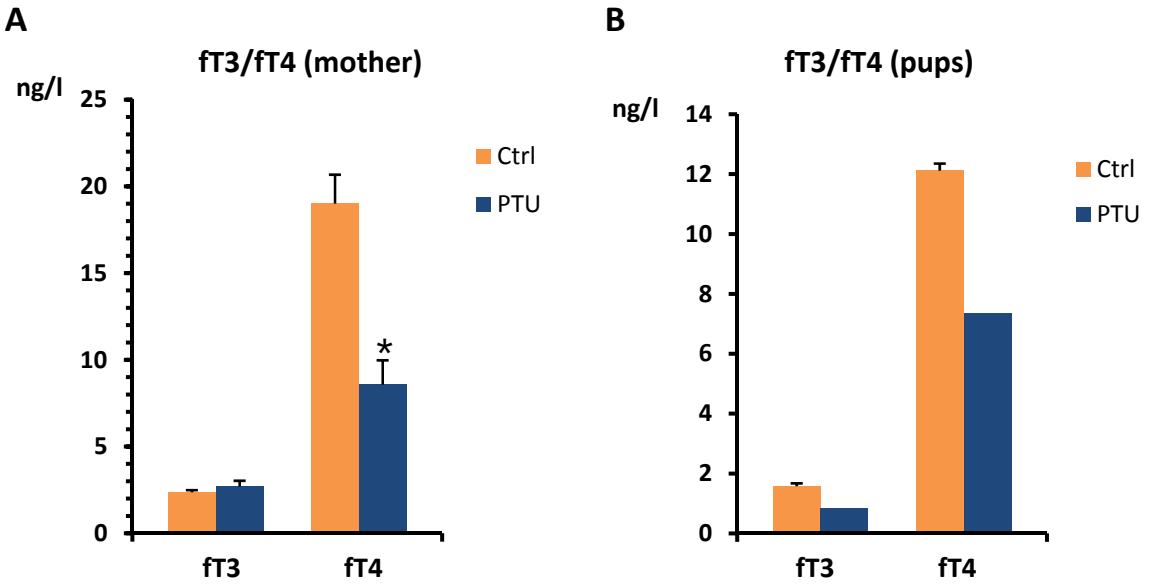

Fig. 3.21 Serum fT3 and fT4 levels in mother and pups two days after labor. Mice were rendered hypothyroxinemic by 0.1% PTU (E2-P2). Blood was collected from heart in the mothers (A) and the pups (B) two days after labor. Sera levels of fT3 and fT4 were measured in the Central Laboratory of the University hospital. In mothers, fT4 is significantly decreased by PTU treatment, while fT3 is not different. In pups, the same fT4 and fT3 pattern is observed. But due to the limited amount of blood, no

statistical test was performed. n = 4 -6 in A. Values are Mean±S.E.M. (*, p=0.0014, p<0.05 as significant, student *t*-test)

The body weight from both groups was also compared. Pups (P0) in the control group had significantly higher body weight (1.39±0.03 g) than those in the PTU group (1.18±0.02 g).

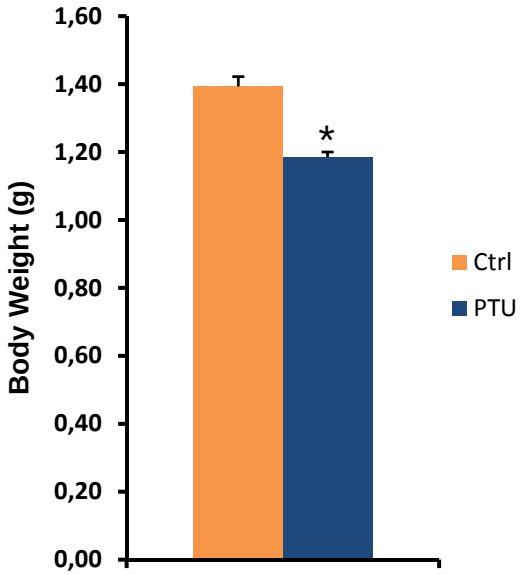

Fig. 3.22 Body weight of pups on the day of birth (P0). PTU treated pups have significantly lower body weight than the control. (*, p<0.0001, student *t*-test, n = 13 - 15)

The influence of thyroid hormones on neurogenesis has been shown by others (Lavado-Autric et al. 2003; Auso et al. 2004; Cuevas et al. 2005). Particularly migration of newborn neurons to their final destination is affected. In this study, coronal brain sections of P2 pups were stained for LacZ and the LacZ-expressing cells in cortical layer 2/3 were counted and compared between the control and the PTU group. The number of LacZ-expressing cells in cortical layer 2/3 per visual field (47.00±4.17) in the PTU group decreased significantly (p<0.05) compared to that in the control (60.65±2.86) (Fig. 3.23A). The percentage of LacZ-expressing cells in layer 2/3 was also significantly decreased in the PTU group (33% in the control vs. 29% in the PTU group, p<0.05) (Fig. 3.23B). This result shows that neurons that expressed Slco1c1 during brain development are sensitive to changes in thyroxine.

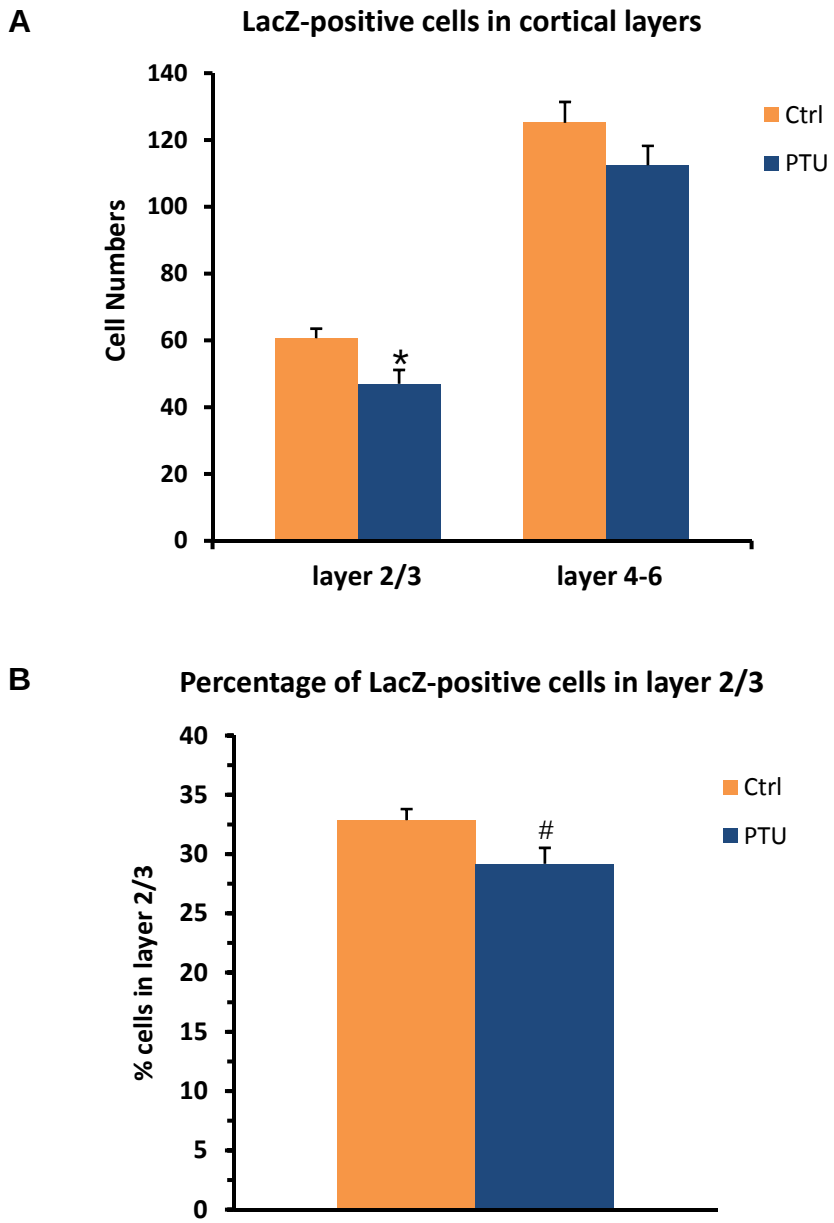

Fig. 3.23 Hypothyroxinemia reduces the number of LacZ-positive cells in cortical layer 2/3. Pregnant mice were rendered hypothyroxinemic by adding 0.1% PTU into their drinking water from the second day after conception (E2) till the second day after labor (P2). A. The number of LacZ-positive cells in cortical layer 2/3 decreases significantly (*, p=0.0102, student *t*-test) in the PTU group, compared to the control. By contrast, in layer 4-6, there is no statistically significant difference. B. Values shown as a percentage of lay 2/3 LacZ-positive cells in the total number of cells in layer 2-6. As in A, the percentage is significantly decreased in the PTU group (#, p=0.0312, student *t*-test), compared to the control. Values are mean±S.E.M., p<0.05 as significant. (n=2-4)

3.3.2.8 A role of SLCO1C1 in adult neurogenesis?

LacZ staining not only revealed expression of Slco1c1-Cre in the developmental VZ and SVZ, but also in the adulthood SVZ. Recently, adult neurogenesis has become widely accepted (Ming and Song 2005; Zhao et al. 2008). To test if Slco1c1 is expressed in neural stem cells, the Slco1c1 transcript was first examined in cultured neural stem cells. Neurospheres derived from adult mice were cultured and mRNA was extracted. Using RT-PCR, Slco1c1 expression can be detected in neural stem cells (Fig. 3.24).

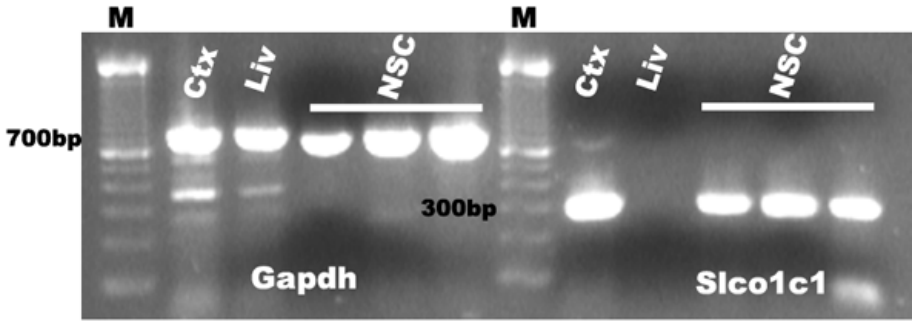

Fig. 3.24 Cultured neural stem cells express Slco1c1. Reversely-transcribed cDNAs from mRNAs of cortex (Ctx), liver (Liv), and cultured neural stem cells (NSC) were used as templates to amplify Slco1c1 with primers Slc-rt1/Slc-rt2. As shown on the right side, neural stem cells express Slco1c1. Ctx serves as a positive control, while Liv as a negative control. On the left side, cDNA input was controlled by primers amplifying Gapdh. 700bp and 300bp indicate sizes of Gapdh and Slco1c1 amplicons, respectively. M, DNA size marker.

Adult SVZ is known to contain progenitor cells. These cells are divided into three types. Type B cells (GFAP-positive) are neural stem cells, type C cells are transient amplifying cells, and type A cells (Dcx-positive) are neuroblasts (Ming and Song 2005; Zhao et al. 2008). In transgenic mice, GFAP and Cre were found colocalized in the SVZ (Fig 3.25). GFAP-positive neural stem cells in adults can divide and differentiate into all the three major cell types in the brain (neuron, astrocyte and oligodendrocyte). A study has shown that thyroid hormones are essential in generating new neural stem cells (Lemkine et al. 2005). Colocalization of GFAP- and Cre-expressing cells in the SVZ suggests that Slco1c1 is expressed in neural stem cells.

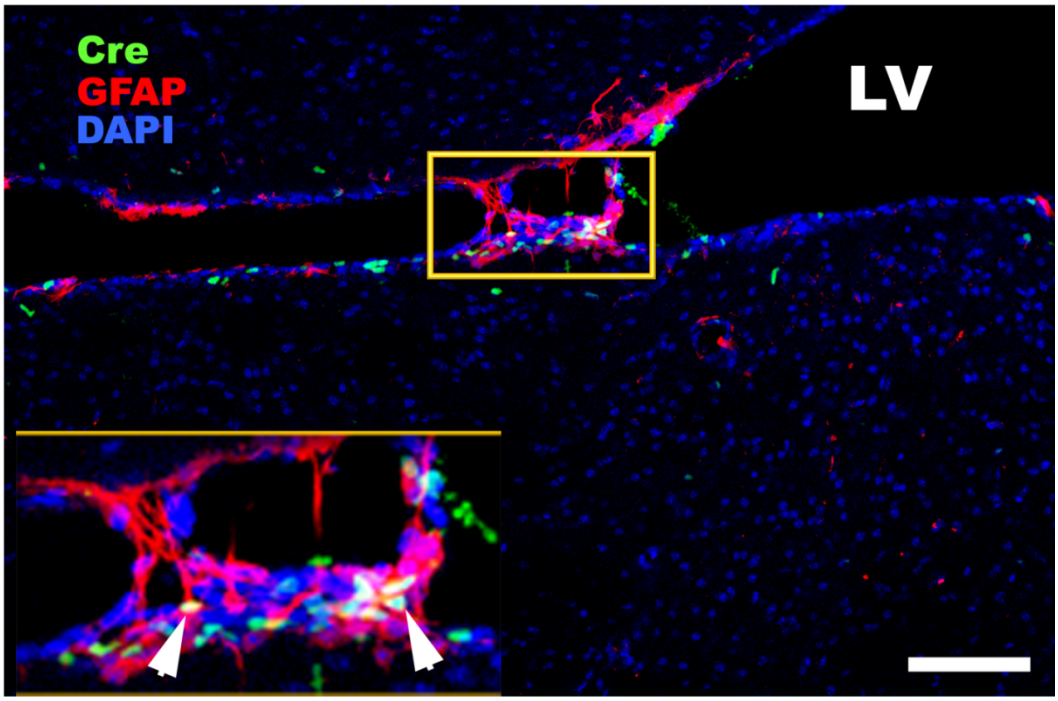

Fig. 3.25 Colocalization of Cre-expressing cells with GFAP-positive cells in the SVZ. Confocal images were obtained from adult brain sections stained for Cre and GFAP. The inset shows colocalization (arrowhead) of Cre- and GFAP-expressing cells, which are located in the SVZ of the lateral ventricle (LV). Nuclei were stained with DAPI. Scale bar=100µm.

Type A cells are immature neurons. They migrate out of the SVZ towards olfactory bulb under normal conditions. Under pathological conditions, such as cerebral ischemia, neurogenesis is potentiated and Dcx-positive cells migrate towards the site of infarct (Lichtenwalner and Parent 2006; Zhang et al. 2007a). Fig. 3.26 shows that under normal conditions, Dcx and Cre expression are colocalized, suggesting that A cells express Slco1c1.

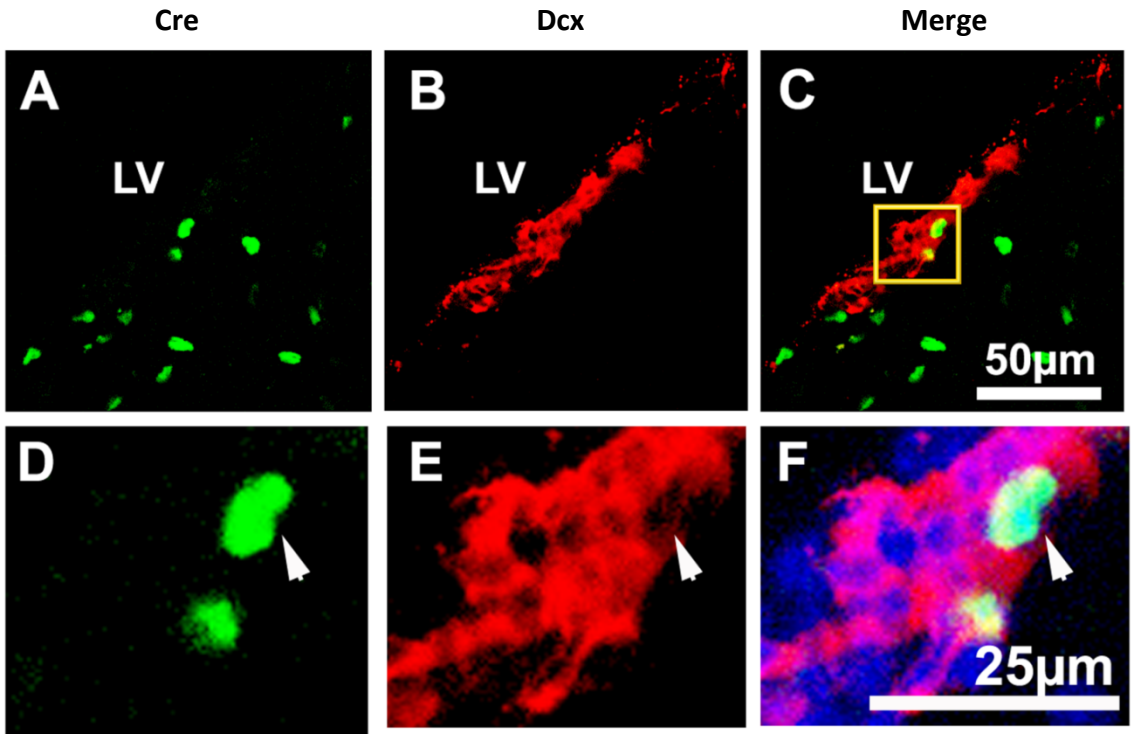

Fig. 3.26 Cre expression colocalizes with doublecortin (Dcx) in the subventricular zone (SVZ). Coronal sections were stained with Cre and Dcx antibodies. Near the lateral ventricle (LV), Cre expression colocalized with Dcx-expressing cells. Higher magnification of the region in the yellow frame in C is shown in D-F. The blue color in F is DAPI staining.

Fig. 3.27 shows the colocalization of Dcx and Cre in brains of Slco1c1-Cre mice after cerebral ischemia. Seven days after middle cerebral artery occlusion (MCAO), when neurogenesis peaks (Zhang et al. 2004), Dcx/Cre double positive cells can be more easily detected (Fig. 3.27A-F). These double positive cells clearly are among those that migrate out of the SVZ towards the site of the infarct (arrowheads in Fig. 3.27D-F; Fig, 3.27G-J).

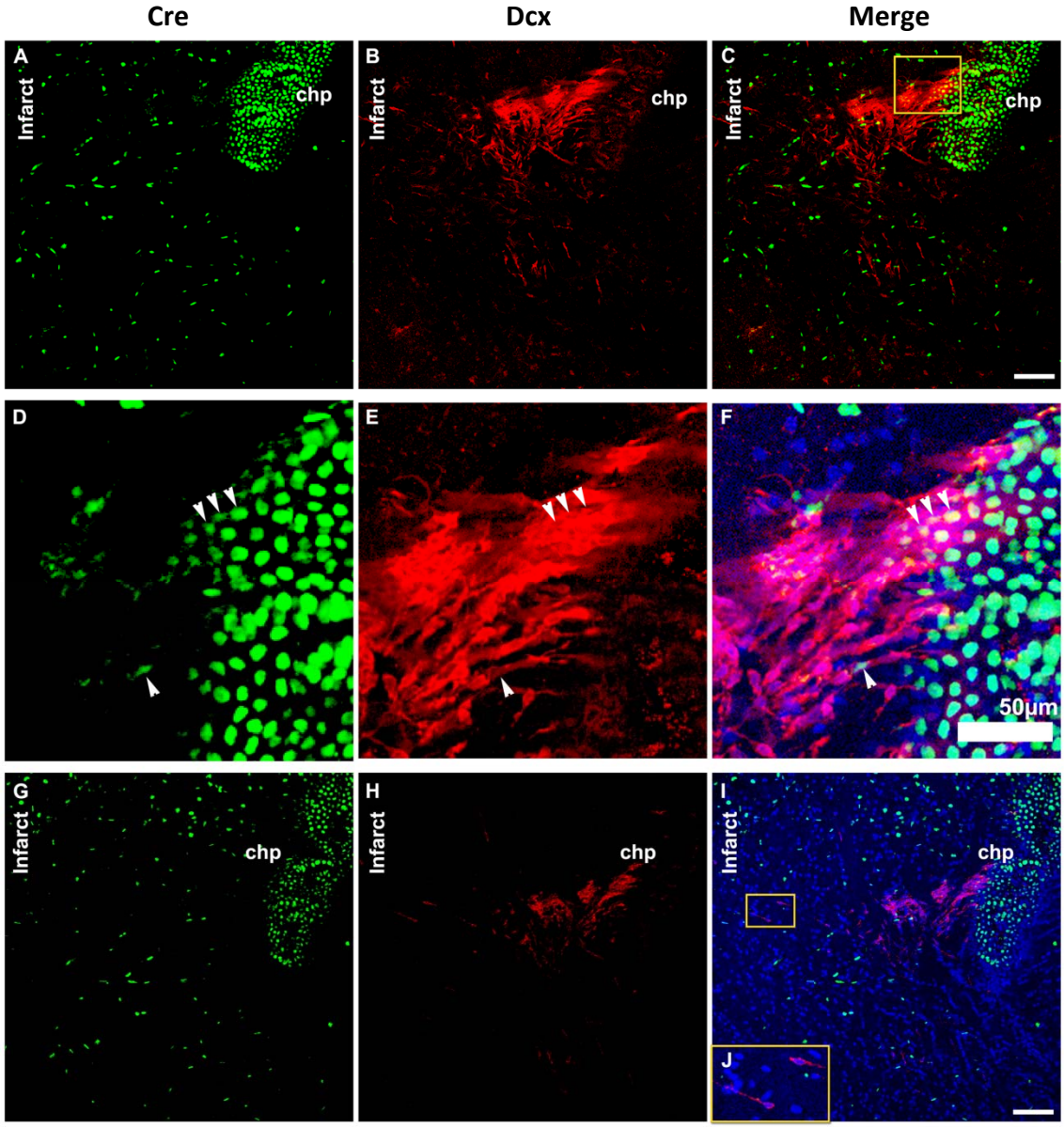

Fig. 3.27 Cre expression colocalizes with doublecortin (Dcx) in cells migrating out from the subventricular zone (SVZ) after MCAO. Seven days after MCAO, coronal brain sections were stained with Cre and Dcx antibodies. The location of infarct is shown in A-C and G-I. Near the lateral ventricle (LV), Cells expressing Cre and Dcx are migrating out from the SVZ. Higher magnification of the region in the yellow frame in C is shown in D-F. Arrow heads indicate colocalization of Cre and Dcx. The inset J is the higher magnification of the region in the yellow frame in I, showing two Dcx-positive cells migrating towards the site of infarct that are Cre-negative. Chp, choroid plexus; the blue color in F, I and J is DAPI staining. Scale bars=100µm, if not indicated.

4. Discussion

4.1 PGPR-S in cerebral ischemia

In the first part of the results, the regulation of PGRP-S (PGLYRP1) in cerebral ischemia is demonstrated. The detrimental role of NF-κB in cerebral ischemia has been shown in our lab and by others (Schneider et al. 1999; Herrmann et al. 2005; Schwaninger et al. 2006; Kunz et al. 2008; Nijboer et al. 2008b). Discovering its downstream target genes will allow the development of more specific therapeutic targets in cerebral ischemia, since NF-κB is also involved in other important functions. PGRP-S belongs to the recently discovered family of peptidoglycan recognition proteins. This protein family is evolutionally conserved and plays an important role in the innate immune system (Steiner 2004; Dziarski and Gupta 2006b; Dziarski and Gupta 2006a; Royet and Dziarski 2007). Besides its bactericidal effect (Lu et al. 2006), other functions have been described, such as induction of apoptosis of mammalian cells (Sashchenko et al. 2004; Sashchenko et al. 2007). Though PGRP-S has no effect on the infarct size, it may well be that it is involved in protecting the ischemic brain against infection at a later time point that was not investigated in this study.

The regulation of PGRP-S had not been shown before. The putative NF-κB binding sites on its promoter indeed control its expression as demonstrated in the luciferase assay with PRPG-S promoter mutants. Another family member PGRP-L (PGLYRP2) was shown to be regulated by NF-κB in keratinocyte, enabling its differential expression (Li et al. 2006). The induction of PGRPs by the same transcription factor underscores the importance of NF-κB in the regulation of this gene family. Interestingly, a PGRP homolog in Drosophila has been shown to activate NF-κB signaling (Choe et al. 2002). This mechanism may further potentiate the production of PGRPs, though it is also possible to counterbalance the beneficial effect of PGRP-S by NF-κB dependent damage in brain ischemia.

A surprising finding in this study is the PGRP-S expression in BBB endothelial cells. Previously, PGRP-S was shown to be expressed mainly in immune cells. In brain ischemia, with the disruption of BBB and inflammation in the brain, blood cells (e.g. neutrophils) infiltrate into the brain to take part in the inflammatory responses, which is harmful (Buck et al. 2008; Kunz et al. 2008). Neutrophils have been shown to be a major site of PGRP-S action. The high expression of PGRP-S in the BBB may extend the

source of PGRP-S production in the body. A burst of PGRP-S production in BBB endothelial cells and in the infiltrated neutrophils could contribute to protection from infection.

Compared with neurons and astrocytes, BBB endothelial cells are less well studied in cerebral ischemia. The slow progress in BBB endothelial cell study is partly due to the lack of a tool to target these cells. Generating a BBB endothelial Cre mouse line would make it feasible to study NF-κB or other molecules specifically in these cells. Therefore, it is necessary to search for a gene that is specifically expressed in BBB endothelial cells. The newly found gene encoding the thyroxine transporter, SLCO1C1, is a good candidate.

4.2 Brain thyroid hormone transporters

BBB endothelial cells are unique in that they form tight junctions. This structure builds a physical barrier. Substances exchange between the two sides of the barrier is thus limited and regulated. The regulation of such exchange is achieved by the expression of transporters and enzymes in the BBB endothelial cells. The enzymes that are expressed on the outer surface of a cell membrane are called ectoenzymes. They have their active sites facing the exterior of a cell. Together with the intracellularly expressed enzymes, they metabolizes and inactivate compounds that are harmful to brain functions (Abbott et al. 2006). The transporters that are expressed in the BBB endothelial cells are important to mediate the entry and exit of substances. These transporters can overcome the restricted movement of substances across the BBB due to tight junctions and less transcytosis/endocytosis. SLCO1C1 is such an interesting transporter.

SLCO1C1 is a thyroxine (T4) transporter that is almost exclusively expressed in the brain, BBB endothelial cell and choroid plexus epithelial cell (Sugiyama et al. 2003; Tohyama et al. 2004). According to the most prevalent model, SLCO1C1 is the only source of brain T4 (Heuer 2007; Visser et al. 2007). It has been shown that the expression of Slco1c1 is tightly controlled by thyroid status (Tohyama et al. 2004). A T4 activating enzyme, D2, has similar thyroid status dependent regulation (Bianco et al. 2002). Hence, SLCO1C1 is not only involved in the normal T4 transport, but can adjust the transportation according to the requirements.

Study of the thyroid hormone transporters is one way to better understand how thyroid hormones are utilized by cells. A successful example is to link mutations of MCT8 with an X-linked inherited mental

retardation (Allan–Hernon–Dudley syndrome) (Schwartz and Stevenson 2007; van der Deure et al. 2007). The patients have inactivating mutations of the T3 transporter MCT8, which is the only transporter for neuronal T3 uptake found till now. Treatment with thyroid hormones has failed, strengthening the concept that transporters are a key component in thyroid hormone metabolism (Herzovich et al. 2007).

Because of the presence of BBB, neurons cannot get access to blood T3 directly. SLCO1C1 is thus the first step that permits neurons to be exposed to enough T3. Currently, it is not clear if there is any disease that is caused by SLCO1C1 dysfunction (van der Deure et al. 2007). Since SLCO1C1 is more specific to T4 transport and its expression sites are closely related to brain functions, it is necessary to study its function in brain development.

4.3 Identifying thyroxine-sensitive structures by Slco1c1-Cre transgenic mice

The expression of Slco1c1 is well studied in adults. The major sites of expression are BBB endothelial cells and choroid plexus epithelial cells (Sugiyama et al. 2003; Tohyama et al. 2004). But its expression in development had not been studied. The development of BBB starts at about the same time as neurogenesis (Bauer et al. 1993; Stewart and Hayakawa 1994). At this early stage, the barrier function is not established. Therefore, it is interesting to know how brain cells take up thyroid hormones. To solve this question, the expression pattern of Slco1c1 during development needs to be examined.

Cre recombinase has been used to generate conditional gene knockout mice (Gaveriaux-Ruff and Kieffer 2007). Cre transgenic mice can be also used as a cell lineage tracer. By crossing Cre transgenic mice to reporter mice (e.g. Roas26 or EGFP mice), the progeny of Cre-expressing cells can be detected. This strategy has a clear advantage. Firstly, one has more options concerning which reporter to use. When Rosa26-LacZ reporter mice are used, LacZ (X-gal) staining can be used, which is easy and less costly. When EGFP reporter mice are used, confocal microscopy can aid to determine the cell types that express Cre. Secondly, if necessary, Cre mice can be crossed to mice containing floxed alleles, conditionally knocking out genes-of-interest in Cre-expressing cells.

The best choice of creating a Cre transgenic mouse line is to use the recently developed BAC-recombineering technique (Copeland et al. 2001; Lee et al. 2001). This method is widely used in

transgenic research. The advantages are mainly: 1, faithful recapitulation of the endogenous gene expression pattern due to the long regulatory sequences (100-200kb); 2, handy cloning and recombination protocols; 3, tight transgene dose control (copy-number dependent expression). To allow Cre to use the Slco1c1 regulatory sequence for its expression *in vivo*, the coding sequence of Cre was inserted to the Slco1c1 locus by homologous recombination. The Amp gene in the targeting construct was later removed by Flp mediated recombination, because the presence of the Amp sequence could influence the expression of the transgene.

In this study, the expression pattern of Slco1c1-Cre was analyzed in 10 mice from 3 founders, since expression patterns of the Sclo1c1-Cre could differ in different founders. The modified BAC was injected into pronucleus, when BAC integration into the genome is most easily achieved. The BAC, however, can integrate into different sites in the genome and/or with different copy numbers. This may potentially influence the transgene expression. However, analysis of the 10 mice showed no big differences. This again proves that BAC-transgenic technology is highly reproducible.

According to the Slco1c1-Cre expression pattern found by crossing with the Rosa26 reporter mice, a striking discovery is the staining in neuronal structures (olfactory bulb, cortical layer 2/3, hippocampus, anterodorsal thalamic nucleus, etc.). Due to the design of the Rosa26-LacZ reporter mice, the positive staining only tells that the cells once expressed the Cre gene before, but not at the time of, LacZ staining. To gain knowledge of the Slco1c1-Cre expression in adults, immunofluorescence was performed. The results were as expected. The Cre-expressing cells are endothelial cells of the brain microvessels and also found in choroid plexus. Adult neurons do not express Slco1c1-Cre. This expression is exactly as what has been published for Slco1c1 expression in adults (Sugiyama et al. 2003; Tohyama et al. 2004).

Since neurons do not express Slco1c1-Cre in adults, the expression of LacZ must be initiated early in development. As early as E14, brain microvessels and choroid plexus were stained for LacZ. Therefore, the expression of Slco1c1 starts at the early stage of BBB development.

At this stage, embryos were also stained positive for LacZ in the region near the lateral ventricle. The areas near the lateral ventricle during development are neurogenic zones, including ventricular zone (VZ) and subventricular zone (SVZ). Neural stem cells are located in the VZ. They divide and differentiate

into the three major cell types in the brain, neuron, astrocyte and oligodendrocyte. Neurogenesis starts at about E10 and lasts till E17 in the mouse. Gliogenesis starts later from E17 and last into adult period. Therefore, the staining in the neurogenic zones at E14 might be localized in neuronal precursor cells. Intermediate progenitor cells (IPCs) have been proposed to contribute to neuronal output from the neurogenic zones (Pontious et al. 2008). IPCs produce only neurons (Noctor et al. 2004). According to the current hypothesis of cerebral cortex development, IPCs give rise to neurons of the upper neocortical layers (layer 2/3 and layer 4) (Pontious et al. 2008). In this study, the cortical layer 2/3 was found strongly stained for LacZ. Results from immunofluorescence in adults and LacZ staining (X-gal) in embryos suggested that Slco1c1-Cre is expressed in neurons only early in brain development, but not in adults. Therefore, thyroxine may be important for the development of the IPCs. Indeed, maternal hypothyroxinemia during fetal brain development led to decreased LacZ-positive cells in this layer. This decrease should not come from downregulation of the expression of Slco1c1, since hypothyroidism upregulate its expression (Tohyama et al. 2004). Previous studies by BrdU labeling have shown that layer 2/3 cells are born predominantly between E15 and E17. Thyroid hormone deficiency can reduce BrdU-positive cells in this layer (Lavado-Autric et al. 2003; Auso et al. 2004). The finding in this study is in agreement with these studies. Furthermore, it is tempting to speculate that IPCs directly acquire T4, since they may express SLCO1C1. T4 could exert its effect in the IPCs either through a non-genomic mechanism or by conversion to T3.

Another LacZ-positive structure is the hippocampus. Peak neurogenesis in hippocampal CA1 and CA3 areas takes place between E15 and E17 (Bayer 1980). This period coincides with the birth of the cortical layer 2/3 neurons, which suggests that E15-E17 is a critical period for T4 utilization by these brain structures. New pyramidal neurons in CA1 and CA3 are generated in the VZ that runs along the contour of hippocampus. They then migrate radially to their corresponding final positions in hippocampus. Hypothyroidism can influence the cell numbers in CA1 and CA3 (Lavado-Autric et al. 2003; Auso et al. 2004).

Olfactory bulb is a site that undergoes continuous neurogenesis in development and in adults. In rodents, adult neurogenesis in olfactory bulb is especially prominent, which functionally relates to their sensitive odour discrimination. Neurons that are born in the SVZ migrate to the olfactory bulb in a so called rostral migration stream (RMS). In adults, Slco1c1-Cre expression is detected in the SVZ-located cells, some (though not all) of which are co-labeled by GFAP. GFAP-positive cells in the adult SVZ are

recognized as neural stem cells (B cell). Thus, these cells may be sensitive to thyroxine and possess a different metabolism mechanism than postmitotic neurons.

The anterodorsal thalamic nucleus (AD) is another structure that was found strongly expresses LacZ. This expression was not only found in the constitutive line, but also in the inducible line when Cre activity was induced in adults. AD is located lateral to the dorsal 3rd ventricle and ventral to hippocampus. AD neurons have been found to project to the retrosplenial cortex, which belongs to the limbic system (Van Groen and Wyss 1995). The limbic system consists of brain structures that are believed to have functions in emotion, learning and memory (The Hippocampus Book 2007). The involvement of AD in learning and memory was also suggested (Mitchell and Dalrymple-Alford 2006). It was also shown that AD neurons play a role in detecting environmental cues and participate in orienting the head (Zugaro et al. 2001). Concerning the expression of Slco1c1-Cre, AD differs from other neuronal structures, which express Slco1c1-Cre transiently during development. In AD, neurons continue to express Slco1c1-Cre in adults. This finding implies that the T4 transporter SLCO1C1 transports T4 directly into AD neurons. The functional meaning of this direct transport is unclear. However, it is possible that AD neurons could serve as a sensor to brain T4 levels in adults. A selective depletion of AD neurons might help to find out if this hypothesis is right.

Neurons born in adult SVZ are found to migrate to site of brain damage. In cerebral ischemia, it was clearly shown that new-born neurons make their way to striatum and retained their ability to proliferate during migration (Yamashita et al. 2006; Zhang et al. 2007a). Some growth factors have been found to promote this process, such as EGF (Ninomiya et al. 2006). The role of thyroid hormones in adult neurogenesis was also studied. THs act on neural stem cells (NSCs) in the SVZ through binding to thyroid hormone receptor α (Lemkine et al. 2005). Hypothyroidism can impair the proliferation and migration of NSCs, but reduce their apoptosis. In the hippocampal subgranular zone (SGZ), neurogenesis is also influenced by THs. Hypothyroidism greatly decreases NSCs survival and differentiation, resulting in less neurogenesis in the dentate gyrus (DG) (Ambrogini et al. 2005; Desouza et al. 2005). These studies have linked the action of THs on NSCs to the expression of thyroid hormone receptors (TRs). The expression of Slco1c1-Cre in NSCs found in this study provides a mechanism how NSCs can use thyroid hormones. Based on the general influence of THs on cell growth and differentiation, it is reasonable to think that the Slco1c1-expressing NSCs should respond to the thyroid status in the body under different conditions. Thus, the Slco1c1-Cre transgenic line can be used to trace the fate of these NSCs under

pathological conditions. DCX (doublecortin)- and Slco1c1-Cre-positive cells that migrate to the site of infarct were more easily found after MCAO, which implies that thyroxine plays an important role in migrating neuroblasts. Further studies on quantification and fate mapping will clarify how thyroxine influences these cells in the ischemia-induced neurogenesis.

4.4 Thyroid hormone metabolism in brain development

Now it is well accepted that maternal thyroid hormone status is a key regulator of fetal brain development. The presence of thyroid hormones in fetal brain tissues are found well before the start of fetal thyroid function (FTF) (de Escobar et al. 2004; Morreale de Escobar et al. 2004). Deficiency in maternal thyroid hormones leads to defects in fetal brain development. Though there is high D3 activity in the placenta, sufficient amount of thyroid hormones can enter the fetus (Koopdonk-Kool et al. 1996; de Escobar et al. 2004; Morreale de Escobar et al. 2004). In fetal development, higher concentration of T3 was found in brain than in circulating blood (Kester et al. 2004). This is due to the local generation of T3 in brain from T4. The level of maternal T4 was found to be more important to fetal brain development than that of maternal T3 (Morreale de Escobar et al. 2000). Normal maternal T4, but not T3, level could correct the brain development defects in fetus (Calvo et al. 1990).

Early in brain development, BBB is not well established (Engelhardt 2003; Ballabh et al. 2004). The blood vessels at this stage in the brain are thus as leaky as those in other tissues. At E14, brain blood vessels have already expressed Slco1c1-Cre, based on result of LacZ staining in the current study. This result suggests that even at early stages of BBB development, SLCO1C1 could play a role in transporting T4 into the brain parenchyma. However, it is unknown how much brain T4 is transported by SLCO1C1. The metabolism of thyroid hormones in brain endothelial cells is less well known. It is reported that brain endothelial cells express D2 (Kohrle 2007). Thus, T4 in endothelial cells can be converted to T3. Besides SLCO1C1, MCT8 (T3 transporter) was also suggested to be expressed in BBB endothelial cells, which could explain partially the mild brain developmental effect in D2 KO mice, though further studies are necessary to prove this hypothesis (Galton et al. 2007).

Astrocytes are generally accepted as a major source of brain T3. D2 expression in brain is found mainly in astrocytes, although in hypothyroidism cortical interneurons were also found to express D2 (Guadano-Ferraz et al. 1997; Guadano-Ferraz et al. 1999). It is still a mystery which transporter mediates

the entry of T4 into astrocytes. As to SLCO1C1, till now there has been no convincing data to show its expression in astrocytes.

MCT8 is a neuronal T3 transporter in the brain (Heuer et al. 2005). Inactivating mutations of MCT8 lead to psychomotor retardation as found in AHDS patients. In this study, the discovery of transient SLCO1C1 expression in neurons suggests another mechanism by which thyroid hormones can influence brain development. Since D2 activity is not found in neurons, it may well be that T4 exerts non-genomic (non-transcriptional) effects in these SLCO1C1-expressing neurons. Previous studies showed that T4 (and rT3), but not T3, promoted actin polymerization in cultured astrocytes (Siegrist-Kaiser et al. 1990). This actin polymerization-promoting effect was also seen in developing neurons *in vivo* (Farwell et al. 2006). Regulation of actin polymerization is very important in neuronal migration (Ayala et al. 2007; Leonard 2008). Developmental hypothyroxinemia reduced the number of LacZ-positive (SLCO1C1-exprssing) neurons in cortical layer 2/3 in the current study. Taken together, it is possible that T4 influences the migration of the SLCO1C1-expressing neurons by modulating actin polymerization.

Of note, though LacZ-positive neurons could be detected in all the brain regions examined, the cell number in some regions (e.g. cortical layer 4-6) were not as high as that found by Nissl staining. This difference could mean that not all neurons express SLCO1C1 in their development. Previous studies also found a migration defect of neurons in cortical layer 4-6 induced by hypothyroxinemia (Lavado-Autric et al. 2003; Auso et al. 2004). Since neurons in cortical layer 4-6 may not express SLCO1C1, there may be at least two populations of neurons, differing in the mechanisms of T4-mediated migration. Thus, the Slco1c1-Cre transgenic mice could provide a tool to find thyroxine-sensitive brain structures and to differentially study their behaviors during development.

Abbreviations

AHDS	Allan–Hernon–Dudley syndrome
BAC	bacterial artificial chromosome
BBB	blood-brain barrier
BW	body weight
CNS	central nervous system
Cre	Causes recombination of the bacteriophage P1 genome
D1	type 1 deiodinase
D2	type 2 deiodinase
EC	endothelial cell
Fig	figure
GFAP	glial fibrillary acidic protein
HAT	heterodimeric amino Acid transporter
hr	hour
i.p.	intraperitoneal
KO	knockout
loxP	locus of crossover (x) in P1
MCAO	middle cerebral artery occlusion
MCT	monocarboxylate transporter
nu	nucleus
O/N	over night
Oatp1c1	organic anion transporting polypeptide, member 1c1
PCR	polymerase chain reaction
PTU	propyl-thio-uracil
rT3	reverse T3
Slco1c1	solute carrier organic anion transporter family, member 1c1
SVZ	subventricular zone
T3	3,5,3’-triiodothyronine

T4	thyroxine
TH	thyroid hormone
TSH	thyroid-stimulating hormone

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Publication

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