Brain Phenotype of *Car9*$^{-/-}$ Knock-out Mouse

Master's Thesis
Institute of Medical Technology
University of Tampere
May 2008
Salla Autio

Acknowledgements

This study was carried out at the Institute of Medical Technology, University of Tampere in the Carbonic anhydrase and hemochromatosis research group. I want to sincerely thank Docent Anna-Kaisa Parkkila, M.D. Ph.D and Professor Seppo Parkkila for their excellent guidance, support and patience. I also want to thank Mr. Mika Hilvo and Mr. Teemu Kivioja their expertises. And most of all, I want to thank Mrs. Aulikki Lehmus for technical assistance and expertises.

I dedicate this thesis to my father Olli (25.04.1924-09.03.2007), who did not live to see my graduate but who always believed in me and supported me in every aspect of my life. I also want to thank my family, my mother Marjo, husband Heikki and our baby boy Jami for bearing with me during this process. Without their motivation, support and help this would not been possible.

May 2008, Tampere

Salla Autio

PRO GRADU –TUTKIELMA

Paikka:	TAMPEREEN YLIOPISTO Lääketieteellinen tiedekunta Lääketieteellisen teknologian instituutti
Tekijä:	AUTIO, SALLA VILHELMIINA
Otsikko:	*Car9*$^{-/-}$ poistogeenisen hiiren aivofenotyyppi
Sivumäärä:	61
Ohjaaja:	LT, DOS Anna-Kaisa Parkkila
Tarkastajat:	Professori Markku Kulomaa ja LT, DOS Anna-Kaisa Parkkila
Aika:	Toukokuu 2008

Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Hiilihappoanhydraasi IX (CA IX) on solukalvoon kiinnittyvä isoentsyymi, joka ilmentyy vain vähäisessä määrin tai ei lainkaan normaaleissa ihmisen aivoissa. Aikaisemmin *Car9*$^{-/-}$ poistogeenisten hiirien on todettu olevan agressiivisia, yliaktiivisia ja niiden aivokudoksessa on todettu olevan vakuolaarisia muutoksia. Meidän tavoitteenamme oli tutkia *Car9*$^{-/-}$ poistogeenisten hiirien aivofenotyyppiä käyttäytymistesteillä, morfologisilla analyyseillä sekä cDNA mikrosirumenetelmällä.

Tutkimusmenetelmät: *Car9*$^{-/-}$ poistogeenisten hiirien käyttäytymistestit tehtiin hieman muunnellulla SHIRPA protokollalla käyttäen villityypin hiiriä kontrolleina. Käyttäytymistestien kesto oli 12 kuukautta, jonka aikana hiiret testattiin kahden kuukauden välein. Aivokudosnäytteet otettiin käyttäytymistestien eri aikapisteissä lopetetuilta hiiriltä. Aivokudosnäytteet värjättiin Hematoksyliini-Eosiini –värjäyksellä ja elektronimikroskopia tehtiin solumuutosten havaitsemiseksi aivoissa. *Car9*$^{-/-}$ poistogeenisten hiirien geenien ilmentymistä aivoissa tutkittiin cDNA mikrosiruanalyysilla.

Tutkimustulokset: Käyttäytymistestit osoittivat *Car9*$^{-/-}$ poistogeenisten hiirien olevan aktiivisempia testattaessa ”locmotor activity-”, ”transfer arousal-” ja ”spontaneous activity-” testeillä. ”Morris water maze”-testi osoitti, että *Car9*$^{-/-}$ poistogeenisillä hiirillä oli vaikeuksia oppia ja että niillä oli huonompi näkömuisti. Hematoksyliini-Eosiini –värjäys ja elektronimikroskopia osoittivat vakuolaarisia muutoksia *Car9*$^{-/-}$ poistogeenisten hiirien aivokudosnäytteissä. Elektronimikroskopia osoitti myös myeliinin ja neuronien hajoamista, tyhjiä aksoneita/dentriittejä sekä solujen tuhoutumista aivokudoksessa. Mikrosirumenetelmä osoitti 12 alisäädeltyä ja 11 ylisäädeltyä geeniä, joiden fold-change –arvot olivat yli 1.40 ja alle -1.40. Useita geenejä oli alisäädeltynä kromosomissa 4, joka on myös *Car9* geenin sijainti.

Johtopäätökset: Olemme identifioineet hiirikannan, jolla on muutoksia käyttäytymisessä sekä aivokudoksessa. *Car9*$^{-/-}$ poistogeenisillä hiirillä oli degeneratiivisia muutoksia aivokudoksessa. Käyttäytymistestien mukaan *Car9*$^{-/-}$ poistogeeniset hiiret olivat aktiivisempia ja osoittivat epänormaaleja muistitoimintoja. Koska useita geenejä oli alisäädelty *Car9*$^{-/-}$ geenin välittömässä läheisyydessä lisätutkimukset ovat välttämättömiä, jotta pystytään selvittämään liittyvätkö meidän löydöksemme CA IX vajaavuuteen.

MASTER'S THESIS

Place:	UNIVERSITY OF TAMPERE Faculty of Medicine Institute of Medical Technology (IMT)
Author:	AUTIO, SALLA VILHELMIINA
Title:	Brain Phenotype of *Car9*$^{-/-}$ Knock-out Mouse
Pages:	61
Supervisor:	Docet Anna-Kaisa Parkkila, MD PhD
Reviewers:	Professor Markku Kulomaa and Docent Anna-Kaisa Parkkila, MD PhD
Date:	May 2008

Abstract

Background and aims: Carbonic anhydrase IX (CA IX) is a membrane-bound isozyme, which shows only low expression in the normal human brain. Previously *Car9-/-* knock-out mice have been stated to be aggressive and hyperactive and their brain tissues showed vacuolar changes. Our aim was to study *Car9-/-* knock-out mice brain phenotype using behavioural tests, morphological analysis and microarray.

Methods: The behavioural analysis for the *Car9-/-* knock-out mice was done with a slightly modified SHIRPA protocol using wild-type mice as controls. Duration of the behavioural analysis was 12 months and the mice were tested every two months. The brain tissue samples were obtained from the mice sacrificed at each time point after the behavioural tests. The brain tissue samples were stained with Haematoxylin-Eosin (HE) –staining and electron microscopy was performed to detect cellular changes in brain. Gene expression in the brain of *Car9-/-* knock-out mice was studied with a cDNA microarray analysis.

Results: The behavioural tests showed that *Car9-/-* knock-out mice were more active in their response tested for locomotor activity, transfer arousal and spontaneous activity. Morris water maze test showed that *Car9-/-* knock-out mice had difficulties to learn and they had also poorer visual memory. Haematoxylin-Eosin –staining and electron microscopy revealed vacuolar changes in the brain samples obtained from *Car9-/-* knock-out mice. Electron microscopy showed also myelin and neuron degeneration, empty axons/dentrites and cell decomposition in brain tissue. Microarray showed 12 down-regulated and 11 up-regulated genes with a fold –change over 1.40 and under -1.40. Several genes were down-regulated in chromosome 4, which is also the location of *Car9* gene.

Conclusions: We have identified a mouse strain, which has alterations in behaviour and brain tissue. The *Car9-/-* knock-out mice had degenerative changes in their brain tissue. According to the behavioural tests the *Car9-/-* knock-out mice were more active and showed abnormal memory functions. Because several genes were down-regulated in the close proximity of *Car9-/-* gene further investigations are warranted to find out if our findings are related to CA IX deficiency.

CONTENTS

Abbreviations

AP1	activator protein 1
CA	carbonic anhydrase
CA IX	carbonic anhydrase IX
CA9	carbonic anhydrase 9 (referes particularly to the human gene)
Car9	carbonic anhydrase 9 (referes particularly to the mouse gene)
$Car9^{-/-}$	a null mutation of the *Car9* gene
CA-RP	carbonic anhydrase related protein
CAI	carbonic anhydrase inhibitor
ccRCC	clear cell renal carcinoma
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSF	cerebrospinal fluid
DNase	deoxyribonuclease
GIP	glycosyl phosphaditylinositol
HNPCC	hereditary nonpolyposis colorectal cancer
HNSCC	head and neck squamous cell carcinoma
HRE	hypoxia response element
OD	optical density
PR	protected region
pVHL	von Hippel-Lindau protein
RCC	renal cell carcinoma
RNase	ribonuclease
RPTP	receptor-type protein tyrosine phosphatase
sCA IX	soluble form of CA IX
SHIRPA	a systematic, objective protocol for phenotype analyze
SP	specificity protein
VHL	von Hippel-Lindau
WT	wild-type

1. Introduction

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes, which catalyze the reversible hydration of carbon dioxide and therefore participate in the maintenance of pH homeostasis of the cell. Mammals have at least 13 active CA isozymes that belong to α-CA gene family. CA isozymes have different cell localisations, tissue distributions and functions.

Carbonic anhydrase IX is a highly active, membrane-bound glycoprotein. CA IX has a limited distribution in normal tissues and it is mainly expressed in gastrointestinal track (Pastoreková et al., 1997). Normal human brain tissue shows only low or no expression of CA IX. In the peripheral and central nervous systems CA IX expression is detected in the ventricular lining cells and in the Choroid plexus (Ivanov et al., 2001). CA IX is also expressed in some neuronal axons and Purkinje cells of mouse brain tissue (Hilvo et al., 2004). Human CA IX has a special position due to its tumor-related expression pattern as well as its proposed dual function as a highly efficient enzyme and an adhesion molecule (Závada et al., 2000). The adhesion properties of CA IX may be involved in maintenance of mucosal integrity contributing to proper intercellular contacts and communication (Ortova Gut et al., 2002).

Ortova Gut et al. (2002) obtained CA IX -deficient mice with a null mutation of the *Car9* gene (*Car9*$^{-/-}$) by targeted gene disruption. The CA IX -deficient mice were healthy and fertile and showed no visible deviations from their wild-type littermates. However, homozygous mice developed gastric hyperplasia of the glandular epithelium with numerous large pathological cysts. No morphological and histological abnormalities were observed in lung, spleen, liver, kidney, jejunum, ileum or colon. While maintaining *Car9*$^{-/-}$ knock-out mice we observed that they were more aggressive and hyperactive than their wild-type littermates. Unexpectedly, the *Car9*$^{-/-}$ knock-out mouse brain tissue samples also showed vacuolar changes. The brain phenotype of the *Car9*$^{-/-}$ knock-out mice was studied using behavioural tests, morphological analysis and cDNA microarray. In these analyses our aim was to explore in what time scale the alterations in brain become detectable and if the behavioural changes could be observed in the behavioural tests.

2. Review of the Literature

2.1 Carbonic anhydrases (CAs)

2.1.1 General aspects

There are three evolutionarily unrelated carbonic anhydrase gene families, which are called the α-CAs, β-CAs and γ-CAs. Evidence shows that only α-genes are present in vertebrates but that they are also present in many algae, cytoplasm of green plants and some eubacteria. The β-genes are present in eubacteria, archaebacteria, algae and chloroplasts of mono- and dicotyledons. The γ-genes are found mainly in archaebacteria and in some eubacteria (Hewett-Emmett, 2000; Supuran et al., 2003). This thesis will focus on mammals and therefore α-CAs.

The α-carbonic anhydrases are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide, $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Mammals have at least 13 enzymatically active α-CAs: CA I, II, III, VII and XIII are cytoplasmic (Lehtonen et al., 2004a; Sly & Hu, 1995), CA IV, IX, XII, XIV and XV are membrane-bound (Hilvo et al., 2005; Ivanov et al., 1998; Mori et al., 1999; Pastoreková et al., 1997; Sly & Hu, 1995; Türeci et al., 1998), CA VA and VB are mitochondrial (Fujikawa-Adachi et al., 1999a; Nagao et al., 1993), and CA VI is the only secretory isoform (Kivelä et al., 1999). There are also three inactive carbonic anhydrase-related proteins (CA-RPs): CARP VIII (Kato et al., 1990; Skaggs et al., 1993), CARP X (Hewett-Emmett & Tashian, 1996) and CARP XI (Lovejoy et al., 1998). CA-RPs also belong to the CA gene family (Tashian et al., 2000). CAs are involved in several important physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs. These physiological processes include pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs, biosynthetic reactions such as gluconeogenesis, lipogenesis and urea synthesis in animals, CO_2 fixation in plants and algae, bone resorption, calcification, tumorigenicity and many other physiological or physiopathological processes (Supuran et al., 2003).

The CAs efficiently catalyze the hydration of CO_2 to bicarbonate and proton, and the Zn(II) ion of CAs is essential for the catalysis. Evidence suggests that the catalytic reaction involves two steps. First step is a reaction between CO_2 and zinc-bound OH^- ion yielding a coordinated HCO_3^- ion, which is displaced from the metal ion by water molecule (Equation 1.). Second step is the regeneration of OH^-, which involves the transfer of H^+ from the zinc-bound water molecule to the solution (Equation 2.). This second reaction is the rate limiting step in this catalysis, i.e., the proton transfer that regenerates the zinchydroxide species of the enzyme. (Lindskog & Silverman, 2000; Supuran et al., 2003)

(1.) $EZn^{2+} - OH + CO_2 \leftrightarrow EZn^{2+} - HCO_3^- \overset{H_2O}{\leftrightarrow} EZn^{2+} - OH_2 + HCO_3^-$

(2.) $EZn^{2+} - OH_2 \leftrightarrow EZn^{2+} - OH^- + H^+$

There are two main classes of CA inhibitors (CAIs): inorganic metal-complexing anions and aromatic heterocyclic sulfonamides. These unsubstituted sulfonamides bind to the Zn(II) ion of the enzyme either by substituting the nonprotein zinc ligand (Equation 3.) or by adding to the metal coordination sphere (Equation 4.) generating trigonal-bipyramidal species. Sulfonamides are the most important CAIs binding in a tetrahedral geometry of the Zn(II) ion in deprotonated state with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II). Anions may bind either in tetrahedral geometry of the metal ion or as trigonal-bipyramidal adducts. (Supuran et al., 2003)

(3.) $E - Zn^{2+} - OH_2 + I \leftrightarrow E - Zn^{2+} - I + H_2O$ (substitution)

Tetrahedral adduct

(4.) $E - Zn^{2+}\ OH_2 + I \leftrightarrow E - Zn^{2+} - OH_2(I)$ (addition)

Trigonal-bipyramidal adduct

2.1.2 Carbonic anhydrase I

The physiological function of the major red cell isozyme carbonic anhydrase I (CA I) is still unknown (Supuran el al., 2003). CA I is the next most abundant protein after the haemoglobin in erythrocytes. In low levels CA I have also been found in intestinal epithelium, colon epithelium,

vascular endothelium, corneal epithelium and the lens of the eye (Brady et al., 1991; Fraser et al., 1989). Low-active cytosolic CA I is four to five times less active than CA II in CO_2 hydration (Lindskog & Silverman, 2000). The carbonic anhydrase 1 (*CA1*) gene is expressed in adult human and mouse erythroid cells and colon epithelia from two distinct promoters with different tissue specificities (Brady et al., 1991; Fraser et al., 1989). The distal *CA1* promoter is active in reticulocytes and erythroleukemic cells, whereas the proximal promoter determines expression in colon epithelial cells (Brady et al., 1991). The use of these promoters appears to be exclusive and so they are never active in the same cell type (Sowden et al., 1992).

2.1.3 Carbonic anhydrase II

Cytoplasmic carbonic anhydrase II (CA II) is the most widely distributed isozyme, which is expressed practically in every human tissue. It is also one of the fastest known enzymes. CA II was originally found in erythrocytes where it catalyzes the hydration of CO_2 to HCO_3^-. CA II plays a major role in contributing to acid base homeostasis and has very diverse physiological roles in different cell types. In renal tubules and collection ducts CA II acidifies urine by elimination of H^+ and in osteoclasts it provides H^+ to acidify the bone-resorbing compartment. It also contributes to H^+ secretion by gastric parietal cells and promotes HCO_3^- secretion to pancreatic juice by pancreatic duct cells. It also provides HCO_3^- for bile, catalyzes the production of HCO_3^- to saliva by salivary gland acinar cells and is involved in the production of aqueous humor by ciliary body epithelium cells. CA II regulates pH of the cerebrospinal fluid produced in Choroid plexus. In distal colonic epithelium H^+ and HCO_3^- secretion catalyzed by CA II are coupled to Cl^- and Na^+ reabsorption and contribute to electrolyte and water balance. It has also been suggested that CA II contributes to fatty acid and amino acid synthesis. (Sly & Hu, 1995; Chegwidden & Carter, 2000)

2.1.4 Carbonic anhydrase III

Carbonic anhydrase isozyme III (CA III) is a cytosolic protein and it is particularly rich in skeletal muscle, adipocytes and liver. CA III has several characteristics, which distinguish it from the other isozymes. It has especially very low specific activity, which is only 3% of that of CA II. Because of the low specific activity it has been suggested that its physiological function is not that of hydrating CO_2 (Kim et al., 2004). Although CA III has been studied quite a lot, the main physiological

function of the enzyme is yet unknown. It is suggested that CA III may not act simply as a carbonic anhydrase (Cabiscol & Levine, 1996; Kim et al., 2004). It has also been suggested that CA III functions as an oxyradical scavenger and thus protects cells from oxidative damage. In CA III overexpressing cells a lower level of free radicals may affect the growth signalling pathways (Räisänen et al., 1999). In a study CA III knock-out mice showed that the mice were viable and fertile, had normal life span and had no anatomic alterations. These mice had also the same response to a hyperoxic challenge as did their wild-type littermates, which suggests that more investigations is needed to understand the real function of this enzyme (Kim et al., 2004). CA III has also previously thought to have intrinsic phosphatase activity, but this assumption has shown to be false (Kim et al., 2000).

2.1.5 Carbonic anhydrase IV

Carbonic anhydrase isozyme IV (CA IV) is high affinity, membrane-associated carbonic anhydrase anchored to the cell membrane by a glycosylphosphatidylinositol (GIP) tail. CA IV works in tandem with CA II in respiration and acid-base regulation (Chegwidden & Carter, 2000). CA IV is expressed in several tissues. In kidney CA IV is localized in apical plasma membranes of the proximal renal tubule and the thick ascending limb of Henle where it is involved in bicarbonate reabsorption. Studies also suggest that CA IV plays a role in bicarbonate transport across the basolateral plasma membrane in these segments (Brown et al., 1990). In the lungs CA IV is highly expressed on the luminal surface of the alveolar capillary endothelium where it participates in carbon dioxide exchange and local pH regulation (Fleming et al., 1993). CA IV is also localized to the apical plasma membrane of the mucosal epithelium in distal small intestine and large intestine where it participates in the extensive ion and fluid transport (Fleming et al., 1995). CA IV has been shown to be present in capillary endothelium of rat and human skeletal muscles (Sender et al., 1994), and in sarcolemma and sarcoplasmic reticulum of rat muscle (Decker et al., 1996). CA IV is also expressed on the endothelial cell surface of other microcapillary beds including the choriocapillaris of the human eye (Hageman et al., 1991), cortical capillaries of mouse and rat brain (Ghandour et al., 1992), and in the human gallbladder epithelium (Parkkila et al., 1996).

2.1.6 Carbonic anhydrase V

Carbonic anhydrase V (CA V) is low-activity isozyme located in the mitochondrial matrix. CA V is important in gluconeogenesis and ureagenesis where it provides HCO_3^- as a substrate for pyruvate carboxylase and carbamyl phosphate synthetase. This mitochondrial isozyme is also required for lipogenesis, which occurs in cytoplasm (Chegwidden & Carter, 2000). The cDNA for human CA V was originally cloned from human liver and the *CA5* gene has been mapped to chromosome 16 (Nagao et al., 1993). Because another mitochondrial CA was later characterised these two isozymes have been termed CA VA and CA VB (Fujikawa-Adachi et al., 1999a; Shah et al., 2000). The cDNA for CA VB was isolated from human pancreas and salivary glands and the *CA5B* gene is located on chromosome X (Fujikawa-Adachi et al. 1999a). CA VB is expressed in pancreas, kidney, salivary glands, spinal cord, heart and skeletal muscle but not in the liver, whereas CA VA expression is observed mainly in liver but also in skeletal muscle and kidney. These findings indicate that these are two genetically distinct isoforms of human CA V. They have different patterns of tissue-specific distribution and physiological roles (Fujikawa-Adachi et al., 1999a; Shah et al., 2000). It is observed that CA VB is 3.3 times catalytically more efficient than CA VA for the physiological reaction of CO_2 hydration (Nishimori et al., 2007).

2.1.7 Carbonic anhydrase VI

Carbonic anhydrase isoenzyme VI (CA VI) is the only secretory isozyme of the mammalian CA gene family. It has several properties that distinguish it from the well characterized cytoplasmic isoenzymes. CA VI is one of the key enzymes maintaining homeostasis on the surfaces of the oral cavity and upper alimentary canal. It is exclusively expressed in the serous acinar cells of the parotid and submandibular glands from where it is secreted into the saliva (Kivelä et al., 1999). Although the physiological role of CA VI has remained undefined it has been suggested that it may participate in protecting the teeth from caries (Kivelä et al., 1999), in neutralizing excess acid in the mucous layer of the esophageal and gastric epithelium (Parkkila et al., 1997), and has growth supporting role in taste buds (Thatcher et al., 1998). CA VI is also expressed into the milk from the mammary gland and it is suggested that milk CA VI not only is linked to the functions of the upper gastrointestinal tract but also may participate in normal growth and development of the whole alimentary tract (Karhumaa et al., 2001b). A stress-inducible intracellular form of CA VI has also

been described in mouse fibroblasts and it has been suggested that it might participate in cellular adaptation to stress (Sok et al., 1999).

2.1.8 Carbonic anhydrase VII

The cytoplasmic carbonic anhydrase VII (CA VII) appears to be the most highly conserved of the active mammalian carbonic anhydrases (Earnhardt et al., 1998). The CO_2 hydration activity of CA VII is about 4% that of the high-activity CA II isozyme (Lakkis et al., 1996). The CA VII gene was isolated and characterized from a human genomic library and its gene was located to chromosome 16 (Montgomery et al. 1991). The salivary gland is major site of CA VII expression (Montgomery et al., 1991), but a wide expression pattern is also detected in the brain (Lakkis et al., 1997).

2.1.9 Carbonic anhydrase IX

CA IX is discussed detail in section 2.2.

2.1.10 Carbonic anhydrase XII

Carbonic anhydrase XII (CA XII) is a tumor-associated carbonic anhydrase identified from a human renal cell carcinoma (RCC). CA XII is in addition to CA IX the second catalytically active membrane-bound CA overexpressed in certain cancers (Ivanov et al., 1998; Türeci et al., 1998). CA XII expression has been detected in the normal human kidney, colon, prostate, pancreas, ovary, testis, lung, and brain (Ivanov et al., 1998, 2001). It is also expressed in the enterocytes of the normal large intestine (Kivelä et al., 2000a), the endometrium, uterus, efferent ducts and in sporadic cells of epididymal duct (Karhumaa et al., 2000, 2001a). CA XII has been localized to the basolateral plasma membranes of the epithelial cells. This basolateral location of CA XII in the endometrial epithelium suggests that the enzyme participates in the regulation of both the luminal and mural pH homeostasis in human uterus (Karhumaa et al., 2000, 2001a). The expression of CA XII is weak in the normal gastric mucosa and is slightly increased in gastric tumors (Leppilampi et al., 2003). CA XII is expressed at much higher levels in the RCC than in normal kidney tissue in 10% of the patients with RCC (Türeci et al., 1998). Most colorectal tumors also display abnormal

expression of CA XII (Kivelä et al., 2000a). The *CA9* and *CA12* genes are identified as von Hippel-Lindau (VHL) target genes and also seem to be regulated by similar mechanisms (Ivanov et al., 1998).

2.1.11 Carbonic anhydrase XIII

CA XIII is a recently characterized cytosolic enzyme, which is expressed in several human tissues including salivary gland, kidney, uterus, thymus, small intestine, spleen, prostate, ovary, colon, and testis. In mouse it is expressed in the spleen, lung, kidney, heart, brain, skeletal muscle, and testis (Lehtonen et al., 2004a, 2004b). CA XIII has a moderate CA catalytic activity similar to those of mitochondrial CA V and cytosolic CA I (Lehtonen et al., 2004a). CA XIII probably plays an important role in pH regulation in several tissues and its inhibition by sulfonamides may lead to detecting novel therapeutic applications (Lehtonen et al., 2004b). It is indicated that the expression of CA XIII is down-regulated in tumor cells compared to the normal tissue and its role as tumor suppressor should be investigated (Kummola et al., 2005).

2.1.12 Carbonic anhydrase XIV

Transmembrane carbonic anhydrase XIV (CA XIV) was first characterized from the mouse kidney (Mori et al., 1999). This high-active isozyme is highly homologous with extracellular CAs including CA XII, IX, VI, and IV. The highest similarity CA XIV has with CA XII (Fujikawa-Adachi et al., 1999b; Whittington et al., 2004). The expression of CA XIV is most abundant in the kidney and heart followed by the skeletal muscle, brain, lung and liver in mouse tissues (Mori et al., 1999). In humans its expression has been demonstrated in the heart, brain, liver and skeletal muscle, and at lower levels in the small intestine, colon, kidney and urinary bladder (Fujikawa-Adachi et al. 1999b). CA XIV is localised in the mouse and rat kidney to the proximal tubules and thin limbs of Henle. Its presence in the proximal tubules suggests a role in renal acidification (Kaunisto et al., 2002). CA XIV has also been detected in the plasma membrane of hepatocytes in the mouse liver (Parkkila et al., 2002) and in the neuronal membranes and axons of both the human and mouse brain (Parkkila et al., 2001). It is suggested that CA XIV may have an important role in modulating excitatory synaptic transmission in the brain (Parkkila et al., 2001). XIV also contributes to

extracellular buffering in the central nervous system and it is suggested that CA XIV contributes to buffering of the interstitial space in mouse hippocampus (Shah et al., 2005).

2.1.13 Carbonic anhydrase XV

Carbonic anhydrase XV (CA XV) is GIP –anchored membrane protein. The membrane-bound low-activity CA XV is an exceptional member of CA family because it is expressed in numerous species but is absent in humans and chimpanzees. CA XV has become a non-processed pseudogene in humans and chimpanzees. Phylogenetic analysis shows that mouse CA XV is related to CA IV and shares several properties with it. CA XV is expressed in kidney, brain and testis of mouse tissues (Hilvo et al., 2005). Mouse CA XV is a moderately active enzyme, which may play a physiological role at least in the kidney. It is possible that other isozymes have substituted this protein in humans (Hilvo et al., 2008).

2.1.14 Carbonic anhydrase related proteins

There are three inactive carbonic anhydrase-related proteins (CA-RPs): CA-RP VIII (Kato et al., 1990; Skaggs et al., 1993), CA-RP X (Hewett-Emmett & Tashian, 1996), and CA-RP XI (Lovejoy et al., 1998). In addition there are two receptor-type protein tyrosine phosphatases (PTPs): RPTPβ and γ and they contain N-terminal CA-RP sequences (Barnea et al., 1993; Krueger & Saito, 1992; Levy et al., 1993). All CA-RPs lack some of the three zinc-binding histidine residues required to bind the zinc ion, which is crucial for biological activity of the catalytic CA isozymes. This is why CA-RPs are believed to be enzymatically inactive isozymes (Taniuchi et al., 2002). The biological functions and the cellular distribution of these inactive CA isozymes are still undefined. It is suggested on the distribution and the developmental expression of CA-RPs in the human brain that they participates in various biological process of the CNS (Taniuchi et al., 2002).

2.2 Carbonic anhydrase IX

2.2.1 General aspects

Carbonic anhydrase IX (CA IX) was initially described as a tumor-associated membrane antigen MN and was originally detected in the cervical carcinoma cell line HeLa (Pastoreková et al., 1992). Later MN cDNA was cloned and the analysis revealed strong structural homology between the central region of the MN protein and carbonic anhydrases (Pastorek et al., 1994). After this the human genomic structure of *CA9* was subsequently characterized and located to chromosome 17 (Opavský et al., 1996, Ivanov et al., 1998).

Human CA IX contains 459 amino acids (aa) and it consists of an N-terminal signal peptide, a large extracellular part, a transmembrane region and a short intracellular C terminal tail. The extracellular part of CA IX is formed from two distinct domains: a proteoglygan-like (PG-like) region and a carbonic andhydrase (CA) domain. The coding sequence of *CA9* is divided into 11 exons and 10 introns. A signal peptide and a PG-like region are encoded by exon 1. CA domain has a highly conserved active site and it is coded by exons 2-8. Exons 10 and 11 encode a transmembrane region and an intracellular tail (Opavský et al., 1996). There is also other form of CA IX: a soluble protein sCA IX, which is released into the culture medium or into the body fluids. It is suggested that sCA IX is released by proteolytic cleavage of the extracellular part from transmembrane region and intracellular tail by membrane-associated proteases (Závada et al., 2003).

Human CA IX has a special position due to its tumor-related expression pattern as well as its proposed dual function as a highly efficient enzyme and an adhesion molecule. The PG-like region is a feature that distinguishes CA IX from the other CA isoenzymes and it seems to be responsible for its capacity to mediate cell adhesion (Závada et al., 2000). The adhesion properties of CA IX may be involved in maintenance of mucosal integrity contributing to proper intercellular contacts and communication. CA IX may also participate in acid-base balance on the basolateral surfaces of the gastrointestinal epithelial cells (Ortova Gut et al., 2002). The CA domain of CA IX has a high enzymatic activity in the catalysis of the hydration of CO_2 comparable to CA II and it is also strongly inhibited by three classic sulfonamides and cyanate (Wingo et al., 2001).

2.2.2 CA IX in normal tissues

The expression pattern of CA IX is quite unusual. Versatile studies provide a good opportunity to compare the CA IX distribution pattern among different species. The CA IX protein is normally present mainly in epithelial linings of the gastrointestinal tract with the highest level observed in the gastric mucosa of human and rat tissues. CA IX is expressed throughout the gastric mucosa from the gastric pits to the deep gastric glands and it is confined to the basolateral surface of the epithelial cells. All major cell types of the gastric epithelium express CA IX. The intestinal epithelium is another important source of CA IX expression. In the rat intestine CA IX is present in the duodenum and colon but is absent from jejunum and ileum. The basolateral membranes of the epithelial cells are positive for CA IX throughout the colon; however, the signal decreases considerably in the distal segments. In the human intestine CA IX is present in the ileum and proximal colon but the signal also decreases in the middle colon. In the rat colon the positive signal is most intense in the surface epithelium and in the human colon the signal intensity increases from the surface towards the base of the crypt of Lieberkühn. Submandibular and parotid glands show no signal for CA IX. A faint positive reaction is detected in the epithelial cells of the human pancreatic ducts but the rat pancreas is negative. Both human and rat bile ducts are positive for CA IX. In addition to the gastric and intestinal epithelium, the gallbladder epithelium is important site of CA IX expression (Pastoreková et al., 1997). CA IX is expressed in the peripheral and central nervous systems in the ventricular lining cells and in the Choroid plexus (Ivanov et al., 2001).

To summarise CA IX is present in the rat stomach, duodenum, colon, and biliary tract as well as the human stomach, duodenum, jejunum, ileum, colon, biliary tract, pancreas, efferent ducts, rete testis, rete ovarii, and the lining cells of the body cavities. It is also detected in the stomachs of guinea pig and chicken (Ivanov et al., 2001; Karhumaa et al., 2001a; Pastoreková et al. 1997; Saarnio et al. 1998b). It is shown that gastric mucosa has the highest expression of CA IX enzyme in human, rat and mouse. In mouse tissues the colon enterocytes and pancreatic acini clearly express CA IX. Low expression of CA IX is seen in kidney, liver, thymus, testis, epididymis, ileum, and spleen of mouse tissues. Interestingly the kidney and muscle shows strong positive signal for CA IX mRNA but IHC and Western blotting, however, shows no or very weak reactions (Hilvo et al., 2004).

2.2.3 CA IX in cancers

One of the interesting features of CA IX is its overexpression in human epithelial tumors originating from the tissues with normally absent or low CA IX expression (Pastoreková et al., 1997). In tumors high catalytic activity of CA IX has been suggested to act in acidification of an extracellular microenvironment facilitating tumor invasion, whereas ectopic CA IX –mediated adhesion may lead to perturbation of normal cell-cell signalling (Ortova Gut et al., 2002). CA IX has a capacity to modulate E-cadherin –mediated cell adhesion, which could be important in hypoxia-induced tumor progression (Svastová et al., 2003). Also another interesting feature of CA IX is that it is usually absent or diminished in tumors originating from CA IX-positive tissues. The intestinal metaplasia of the gastric mucosa shows a positive staining for CA IX, which, however, is considerably weaker than in normal stomach mucosa (Pastoreková et al., 1997). In adenomas CA IX expression is decreased significantly towards the high grade dysplasia. The expression level is normal in well differentiated adenocarcinomas, while in carcinomas with less differentiation it is decreased. This indicates that CA IX expression is sustained in several types of gastric tumors (Leppilampi et al., 2003).

CA IX is overexpressed in von Hippel-Lindau (VHL)-defective tumors and under hypoxic conditions. In the von Hippel-Lindau (VHL) tumor suppressor defective renal carcinoma cells (RCC), up-regulation of CA IX is associated with the loss of regulation by hypoxia (Wykoff et al., 2000). CA IX expression is detected in all primary renal cell carcinomas (RCCs), cystic RCCs, metastatic RCCs and aspiration cytological smears. It is suggested that CA IX expression might have a clinical importance in the early detection and treatment of RCC and may also be used as a diagnostic biomarker of metastatic clear cell RCC (ccRCC) (Liao et al., 1997).

CA IX expression is detected in colorectal carcinomas. The hyperplastic polyps show a weak or moderate reaction for CA IX in the cryptal area, when the adenomas show reaction mainly in the superficial part of the mucosa. The most prominent reaction is observed in the tumors with a mucinous component. CA IX is abnormally expressed in colorectal neoplasms suggesting its involvement in their pathogenesis. CA IX expression in areas with high proliferative capacity and the co-occurrence of CA IX and Ki-67 cell proliferation marker in the same tumor cell indicates its potential for use as a marker of increased proliferation in the colorectal mucosa (Saarnio et al., 1998a). CA IX expression is also very high in hereditary nonpolyposis colorectal cancer (HNPCC)

suggesting that CA IX could be a potential diagnostic and therapeutic target in HNPCC (Niemelä et al., 2007).

All of the esophageal squamous cell carcinomas show CA IX-positive staining and the pattern of staining is mainly membranous. However, the degree of CA IX staining does not correlate with the pathological features of the tumors. In Barrett's epithelium CA IX shows positivity in all types of metaplastic cells and shows no difference in dysplastic epithelium. 80% of esophageal adenocarcinomas are positive for CA IX, but the degree of the expression is inversely correlated with histological tumor differentiation. It is suggested that the tumor-associated CA IX might play a role in proliferation and regeneration in esophageal squamous epithelium and loss of CA IX expression may be related to cancer progression in Barrett's-associated adenocarcinomas (Turner et al., 1997). CA IX is mainly localized at the basolateral surface of the epithelial cells in the biliary epithelial tumors. All non-invasive dysplastic lesions and 57% of invasive lesions of gall-bladder express CA IX. In liver 78% of cholangiocellular malignant lesions show a positive reaction for CA IX, whereas only 33% of hepatocellular carcinomas show a weak immunoreaction. It is suggested that abnormal expression of CA IX may be linked to malignant transformation of hepatobiliary cells and it might be a promising marker for biliary differentiation in hepatobiliary neoplasms (Saarnio et al., 2001).

Immunostaining of CA IX is positive in 80% of the lung tumors and the staining is more often focal than diffuse. Carcinoma in situ and microinvasive epithelioma also show CA IX expression. It is suggested that CA IX is a useful marker for the differentiation between preneoplastic lesions and bronchial non-small cell lung cancers (Vermylen et al., 1999). CA IX is detected in 46% of malignant breast tissues and in 11% of benign lesions and the staining is mostly confined to plasma membranes of abnormal epithelial cells. In addition *CA9* mRNA is detected in 64% of malignant breast tumors and in 33% of benign lesions. *CA9* positivity and breast tumor marker c-erbB2 overexpression are also related to each other. It is suggested that CA IX is important in breast carcinogenesis and might have potential use as a breast tumor marker (Bartosová et al., 2002). The hyperplastic ductal epithelium in pancreatic carcinoma specimens shows an increased staining for CA IX suggesting that it may contribute to the pancreatic tumorigenesis (Kivelä et al., 2000b). Significant immunoreactivity of CA IX is also observed in cervical intraepithelial neoplasias, adenocarcinoma in situ and frank carcinomas both squamous cell and adenocarcinoma (Liao et al., 1994).

CA IX is induced by hypoxia in head and neck squamous cell carcinoma (HNSCC) cell lines and is overexpressed in HNSCC tumors. Overexpression of CA IX in the perinecrotic area of the tumor may help maintaining the intracellular pH giving tumor cells a survival advantage and enhancing resistance to radiotherapy and chemotherapy. CA IX is a potential therapeutic target in HNSCC (Beasley et al., 2001). CA IX is up-regulated in intrinsic and metastatic brain tumors. Hemangioblastomas from von Hippel-Lindau patients also display high expression levels of CA IX. It is suggested that CA IX may contribute to the management of tumor-specific acid load and provide a therapeutic target (Proescholdt et al., 2005). 80% of the oligodendroglial brain tumors also show CA IX expression and tumors with moderate or strong CA IX expression have decreased level of cell proliferation. CA IX is an independent prognostic indicator in oligodendroglial brain tumors and it predicts poor prognosis. It is suggested that because CA IX is mainly expressed in tumor tissue CA IX could serve as a target molecule for anticancer treatments (Järvelä et al., 2008). In addition CA IX expression is observed in 78% of human diffusely infiltrating astrocytomas. The CA IX immunoreactivity shows strong association with tumormalignancy grades. It is suggested that CA IX is a useful biomarker for predicting poor prognosis also in astrocytic tumors (Haapasalo et al., 2006). CA IX may be a promising target for the therapeutic interventions in the brain.

2.2.4 Regulation of CA IX expression

CA IX promoter was detected by deletion analysis and it contains five protected regions (PRs). Four of the PRs are *cis*-regulatory elements critically affecting the expression of CA IX and a novel protected region 4 (PR4) is a silencer element. PR1 and PR2 are crucial for transcriptional activity of CA IX, when PR4 is affecting negatively to transcription and functions as a promoter-, position-, and orientation-independent silencer element (Kaluz et al., 1999). Another analysis indicated binding sites for activator protein 1 (AP1) and specificity protein (SP) transcription factors in PR1 and PR2. Detailed deletion analysis proved that PR1-PR2 account for 90% of the CA IX promoter activity but neither one of the PR1 or PR2 are sufficient for transactivation on their own. Thus, synergistic co-operation between SP and AP1 factors bound to the adjacent PR1 and PR2 is necessary for CA IX transcriptional activity (Kaluzová et al., 2001).

However, *CA9* promoter is most importantly regulated by a hypoxia response element (HRE) close to its transcriptional start site and it is recognized by hypoxia-inducible factor-1 (HIF-1). The transcriptional complex HIF-1 is an important mediator of gene expression patterns in tumors and

the tumor-associated CA IX is tightly regulated by this system. *CA9* is strongly induced by reduced oxygen levels (hypoxia) in a range of tumor cell lines. The von Hippel-Lindau (VHL) tumor suppressor gene appears to play a critical role in this process as the clear cell renal carcinoma cell (ccRCC) lines with mutant VHL express CA IX constitutively. Reintroduction of the wild-type VHL gene into the same cell lines results in down-regulation of CA IX in normal oxygen levels (normoxia), with a restoration of the hypoxic response. (Wykoff et al., 2000)

In tumors other than ccRCC, pVHL plays a critical role as an upstream negative regulator of an α-subunit of the HIF-1. HIF-1 assembles under hypoxic conditions from constitutive β-subunit and oxygen-regulated α-subunit. In normoxia pVHL binds an HIF-1α subunit modified by the proline hydroxylases and targets it to proteasomal degradation, which results in HIF-1α destruction. Hypoxia occurs frequently in tumors as a result of aberrant vasculature. In hypoxia HIF-1α escapes the hydroxylation and pVHL fails to recognize it. This leads to HIF-α stabilization, traslocation to nucleus, hetrodimerization with HIF-β subunit, formation of the HIF transcription complex and finally transcriptional activity. HIF-1 activates a large spectrum of genes involved in the response of tumor cells to hypoxia, including those triggering neoangiogenesis (VEGF), erythropoesis (EPO-1), glucose transport (GLUT-1, GLUT-3), glycolysis (LDHA) and other adaptive processes. (Pastoreková & Pastorek, 2004; Pastoreková & Závada, 2004)

2.2.5 CA IX knock-out –mice

Ortova Gut et al. (2002) obtained CA IX -deficient mice with null mutation of the *Car9* gene (*Car9*$^{-/-}$) by targeted gene disruption. The first exon of *Car9* gene was interrupted by a replacement of a 14 bp region with a pgk-neo cassette in a reverse orientation, which was designed to cause a shift in the reading frame to prevent its correct translation. The CA IX -deficient mice were healthy and fertile and showed no visible deviations from their wild-type littermates. However, homozygous mice developed gastric hyperplasia of the glandular epithelium with numerous pathological cysts. The first changes of gastric mucosa were observed in the newborn animals and the hyperplasia became prominent in 4-week-old mice. No morphological and histological abnormalities were observed in lung, spleen, liver, kidney, jejunum, ileum and colon. Loss of CA IX led to overproduction of mucus-secreting pit cells and reduction of chief cells, and the proportion of parietal cells was significantly decreased but their number was not reduced. CA IX -deficient mice showed normal gastric pH, acid secretion and serum gastrin levels. The function of CA IX in the gastric mucosa is

still unclear but these phenotypic features demonstrate the importance of CA IX in morphogenesis and homeostasis of the glandular gastric epithelium. (Ortova Gut et al., 2002)

2.3 Carbonic anhydrases in brain tissue

Carbonic anhydrases (CA) were demonstrated in the mammalian brain over 60 years ago (Ashby, 1943), where they have numeral physiological roles. Nowadays several CA isozymes are expressed in the central nervous system (CNS) where each of them has a characteristic distribution patterns. CAs participates in CNS in regulating cerebrospinal fluid (CSF) secretion, blood-brain barrier and glial cell function. Expression of CA isozymes in the brain is illustrated in Table 2.1.

Table 2.1. The expression of CA isoforms in brain. (Ghandour et al., 1992 & 2000; Hilvo et al., 2004 & 2005; Ivanov et al., 2001; Kida et al., 2006; Lakkis et al., 1997; Lehtonen et al., 2004a; Nógrádi et al., 1993; Parkkila et al., 2001; Taniuchi et al., 2002)

Isozyme	**Positive regions**	**Cell types**
II	Whole brain	Neurons, Astrocytes, Oligodentrocytes, Myelin
III	Choroid plexus	Microglial cells
IV	Whole brain	Endothelial cells
V	Whole brain	Astrocytes, Neurons
VII	Choroid plexus, Pia, Thalamus, Hippocampus, Cerebellum	Neurons, Purkinje cells
CA-RP VIII	Choroid plexus, Pia, Thalamus, Hippocampus, Cerebellum	Neurons, Purkinje cells
IX	Choroid plexus	Neuronal axons, Purkinje cells, Ventricular lining cells
CA-RP X		Myelin
CA-RP XI		Astrocytes, Neurons
XII	Pituitary glands, Remnant of Rathke's pouch, Choroid plexus	Ganglion cells
XIII		Oligodentrocytes, Neurons, Myelin
XIV	Hippocampus, Corpus callosum, Cerebellar white matter, Pyramidal tract, Choroid plexus, Cerebral cortex, Cerebellum	Neurons
XV	Expression pattern still unknown	

CA II was the first isozyme found in mammalian brain. In the human brain CA II is localized to oligodendrocytes, myelin and Choroid plexus epithelium. CA II is present in a subset of neurons, in a few astrocytes and transiently during brain development in the endothelial cells of microvessels. CA II is also present in oligodendrocyte processes in contact with myelinating axons, myelin sheaths and axolemma. It is suggested that CA II is involved in many of biologic processes in the developing and adult human brain and may contribute to better understanding of the pathogenesis of cerebral calcifications and mental retardation caused by CA II-deficiency (Kida et al., 2006). CA III is expressed in the epithelial cells of the developing and mature Choroid plexuses. CA II and CA III are present in active brain macrophages, whereas resting microglial cells express only CA III. (Nógrádi et al., 1993)

CA IV is expressed in luminal surface of the endothelial cells of the cerebral capillaries. It is indicated that CA IV might be a cytochemical marker associated with the blood-brain barrier and have an important role in CO_2 and HCO_3^- homeostasis in brain (Ghandour et al., 1992). Thus, CA IV contributes to extracellular buffering in the central nervous system; however, it appears to be more important in the hippocampus (Shah et al., 2005). CA VB is expressed in astrocytes and neurons throughout the central nervous system, in cerebral cortex, hippocampus, cerebellum, spinal cord and sciatic nerve. The expression of CA V in astrocytes and neurons suggests that this isozyme has a cell-specific physiological role in the nervous system. In astrocytes CA V may play an important role in gluconeogenesis by providing bicarbonate ions for the pyruvate carboxylase. CA V in neurons may be involved in the regulation of the intramitochondrial calcium level. CA V may also participate in bicarbonate ion-induced GABA responses by regulating the bicarbonate homeostasis in neurons (Ghandour et al., 2000).

Membrane-associated CA IX is expressed in some neuronal axons and Purkinje cells of moure brain tissue (Hilvo et al., 2004). The normal human brain tissue shows only low or no expression of CA IX. In the peripheral and central nervous systems CA IX expression is detected in the ventricular lining cells and the Choroid plexus (Ivanov et al., 2001). The membrane-bound CA activity is suggested to participate in the production of an alkaline shift linked to neuronal signal transduction (Parkkila et al., 2001). CA IX is present in the developing brain during organogenesis of a mouse embryo. The brain tissue is stained moderately and some positive staining is also observed in the mesenchymal cells beneath the developing brain. Moderate staining is also detected in the nerve ganglia and Choroid plexus of the developing brain (Kallio et al., 2006). In the peripheral and central nervous system the CA XII is expressed in the posterior lobe of the pituitary glands, remnant

of Rathke's pouch, the Choroid plexus and limited numbers of ganglion cells in the cortex (Ivanov et al., 2001). CA XII is also expressed in the brain and nerve ganglia during organogenesis, most prominently in the Choroid plexus at the time when the developing Choroid plexus becomes visible (Kallio et al., 2006).

CA XIII is expressed in the oligodendrocytes and positive signal is also detected with the nerve fiber bundles. The latter finding also indicates its location in myelin. (Lehtonen et al., 2004a) CA XIV shows a unique distribution in neurons of mammalian brain. CA XIV is expressed on neuronal membranes and axons involved in motor function and coordination in both mouse and human brain. The expression of CA XIV is highest on large neuronal bodies and axons in the anterolateral part of pons and medulla oblongata. In addition it is expressed in the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract and Choroid plexus. In mouse brain it is also expressed in the molecular layer of the cerebral cortex and granular cellular layer of the cerebellum. These findings suggest that CA XIV may have an important role in modulating excitatory synaptic transmission in brain (Parkkila et al., 2001). CA XIV also contributes to extracellular buffering in the central nervous system (Shah et al., 2005). RT-PCR and *in situ* –hybridization in mice tissues shows also positive expression of CA XV in the brain (Hilvo et al., 2005), but the expression pattern is still unknown.

CA VII and CA-RP VIII expression is detected in the cerebrum in pia, Choroid plexus and neurons of the cortical layer, thalamus and medial habenulae. They are also expressed in the pyramidal and granular cells of the hippocampus. In the cerebellum both are expressed in different degrees in the Purkinje cells and a lower expression level occurred in the molecular and granular cell layers (Lakkis et al., 1997). CA-RP X is expressed in the myelin sheaths and CA-RP XI is expressed in the neural cell bodies, neurites and astrocytes in limited regions of the brain. In the fetal brain CA-RP VIII and XI are expressed in the neuroprogenitor cells in the subventricular zone and detected in the neural cells migrating to the cortex. In the fetal brain CA-RP X is detected in the neural cells in the cortex. In both adult and fetal brains CA-RP VIII and XI are expressed in the epithelial cells of Choroid plexus. It is suggested that these CA-RPs play roles in various biological process of the CNS (Taniuchi et al., 2002).

2.4 Principles of phenotype analysis in the brain

For accurate phenotype analysis in all species it is essential to ensure that the initial stages of assessment are always performed impartially and that none of the features are missed. It is important that findings are not anticipated or biased by making premature assumptions about the results. Methods for behavioural, functional and morphological characterization should also be directly comparable between different groups. For an understanding of the mouse mutations and identification of phenotype variations a full clinical and pathological characterization of the animals is needed. (Rogers et al., 1997)

Although some sophisticated techniques have been used to evaluate behaviour, the majority of these analyses have been qualitative rather than quantitative. There has not been any comprehensive routine for screening and testing protocol designed to identify and characterize phenotype variation or disorders associated with the mouse genome. Rogers et al. (1997) invented a systematic, objective protocol for phenotype analysis called SHIRPA (Figure 2.1). It is a semi-quantitative three-stage protocol designed as a series of individual tests that provide quantitative data about an individual performance (Table 2.2). Test-specific performances are directly comparable between animals and their performances over time and between groups. The tests give an opportunity to define abnormalities or variations in the mouse providing information about the pattern of function of a particular system for example the brain and neuromuscular system. The SHIRPA primary screen utilizes standard methods to provide a behavioural and functional profile by observational assessment. Observation of the mice includes gait, grooming and other normal behaviours and also measurement of a range of parameters like body weight. The secondary and tertiary stages of SHIRPA are tailored to specific biological systems. The secondary screen consists of a comprehensive behavioural assessment battery and pathological analysis. This phenotype analysis is suitable for a wide range of applications including the characterization of spontaneous and induced mutants, the analysis of transgenic and gene-targeted phenotypes and the definition of variation between strains. The tertiary screening stage is tailored to evaluate the existing or potential models of neurological disease. It is also used to evaluate the phenotypic variability that may be the result of unknown genetic influences. SHIRPA utilizes standardized protocols for behavioural and functional assessment that provide a sensitive measure for quantifying phenotype expression in the mouse. These analyses can test the function of specific neural pathways, which contributes to a greater understanding of neurological disorders. (Rogers et al., 1997)

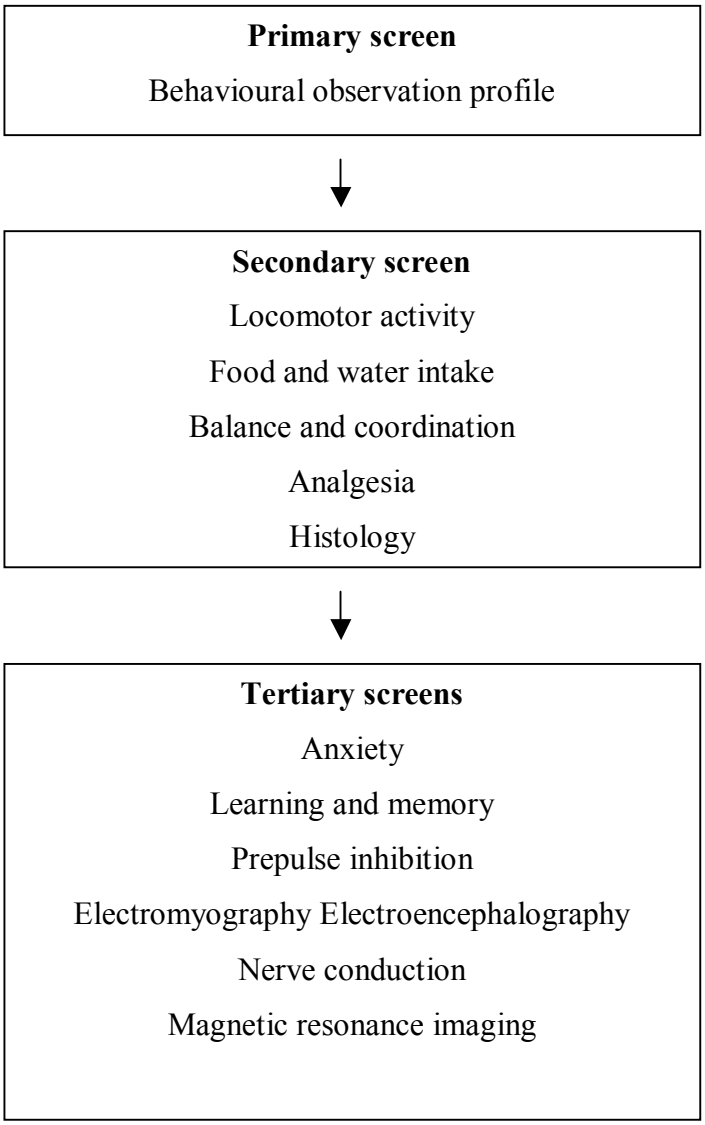

Figure 2.1. SHIRPA three-stage protocol for phenotype analysis. The figure is adapted from (Rogers et al., 1997).

The SHIRPA screening battery and discriminant analysis of the data have enabled determining the relevant contribution of behavioural measurements to the observed differences in phenotype of mouse strains. This data has confirmed the importance of carrying out a comprehensive profile in order to accurately characterise the phenotype of gene-targeted and transgenic mice (Rogers et al., 1999). Despite punctual attention to experimental protocol attention to environmental factors is essential. Differences in noise, light, home cage environment, handling and diet can alter behaviour. Behavioural testing rooms should be located in an area removed from distractions that may interfere with behavioural studies. Furthermore by keeping these tests in the same place will help to reduce the variability in mouse behaviour (Bailey et al., 2006).

Table 2.2. The individual tests contributing to the assessment of specific functions in the SHIRPA protocol. Data obtained from (Rogers et al., 1997).

Muscle and lower motor neurone function		
Body position	Spontaneous activity	Locomotor activity
Gait	Tail elevation	Limb position
Positional passivity	Visual placing	Body tone
Wire maneuver	Limb tone	Abdominal tone
Righting reflex	Passivity	Urination and defecation
Motor performance	Balance and coordination	
Spinocerebellar function		
Body position	Locomotor activity	Gait
Tail elevation	Limb position	Visual placing
Grip strength	Body tone	Limb tone
Abdominal tone	Righting reflex	Motor performance
Balance and coordination		
Sensory function		
Transfer arousal	Gait Limb	position
Touch escape	Visual placing	Pinna reflex
Corneal reflex	Toe pinch	Righting reflex
Analgesia		
Neuropsychiatric function		
Body position	Spontaneous activity	Bizarre behavior
Transfer arousal	Locomotor activity	Food and water intake
Startle response	Touch-escape	Positional passivity
Body tone	Righting reflex	Catalepsy
Fear	Irritability	Aggression
Anxiety	Vocalization	Prepulse inhibition
Learning and memory		
Autonomic function		
Palpebral closure	Piloerection	Startle response
Tail elevation	Skin color	Salivation
Temperature	Food and water intake	Respiration rate
Heart rate	Urination and defecation	

Mouse mutations provide valuable models of human disease allowing us to study developmental and pathological processes. Mouse models are very useful for the study of progressive late onset neurological disorders. However, it has to be remembered that the models are only as useful as our ability to define their phenotype and mouse models of neurological abnormalities are only valuable if accurately assessed. The three-stage SHIRPA procedure has been reported in a high throughput experiment, in which different mutants were ascertained at one age point using stage 1 of the protocol. In a study, of which duration was 16 months, SHIRPA was validated by using a large group with one single mutation legs at odd angles (*Loa*) that causes neurological dysfunction. This study was the first longitudinal SHIRPA analysis. (Rogers et al., 2001)

3. Aims of the Research

The main goal of this research was to investigate the role of carbonic anhydrase IX in the brain. To reach this goal we analysed the *Car9*$^{-/-}$ knock-out mice brain phenotype using behavioural tests, morphological analysis and cDNA microarray. Our aim was to find out in what time scale the alterations previously seen in the *Car9*$^{-/-}$ knock-out mice brain tissue become visible by morphological analysis and if the previously observed behavioural changes can be detected in the specific behavioural tests.

4. Methods

4.1 Behavioural tests

The generation of *Car9*$^{-/-}$ mice by targeted disruption of the *Car9* gene has been described earlier by Ortova Gut et al. (2002). These *Car9*$^{-/-}$ knock-out C57BL/6 mice were produced and genotyped in the animal facility of the University of Oulu and then delivered to the animal facility of the University of Tampere. Behavioural tests were performed by using slightly modified SHIRPA protocol (Roges et al., 1997) on 14 wild-type C57BL/6 mice (7 male and 7 female) and on 14 *Car9*$^{-/-}$ knock-out C57BL/6 mice (7 male and 7 female). These tests were performed always between 9 - 12 am in every two months starting from two months to 12 months old mice. The mice were tattooed for a clear recognition. The following Table 4.1 shows animals tested at different time points. One wild-type mouse had to be sacrificed before testing at the eight month time point because of the paw injury. The tests were approved by the institutional animal care committee (University of Tampere).

Table 4.1. Animals tested in the behavioural tests at different time points.

Age	**Wild-type**	***Car*$^{-/-}$**
2 months	14	14
4 months	13	13
6 months	12	12
8 months	10	11
10 months	9	10
12 months	4	4

4.1.1 Sensory functions and basic characteristics

In every time point the mice were weighed first and then each mouse was placed on the testing arena (50 x 70 cm), where all the observations were recorded. Simultaneously during the 60 second observation period mice's overall well-being, condition of the fur**,** transfer arousal, body position, spontaneous activity, tremor, palpebral closure, urination, defecation, tail elevation and locomotor

activity were recorded. After this observation period mice were tested in positional passivity, limb grasping, visual placing, startle response, touch escape, corneal reflex and tactile sense.

In transfer arousal we observed the mice's immediate reaction when placed onto the arena for the first time. We recorded whether the mice had a prolonged freeze (over 5 seconds), brief freeze (few seconds) then active movement, no freeze and immediate movement or if the mice were extremely excited (manic). We also observed the mice's body positions, whether they were completely flat, lying on their side, lying prone, sitting or standing, rearing on hind legs or if the mice were repeatedly leaping vertically. In spontaneous activity we observed the mice's normal or abnormal activity, weather they were doing nothing and just resting, had a casual scratching and slow movement, had a vigorous scratching and moderate movement, had a vigorous rapid movement or had a extremely vigorous rapid movement on the arena. While testing the mice we also recorded if the mice's tails were dragging, horizontally extended or elevated.

In positional passivity test we observed the mice's struggle response to sequential handling. We recorded if the mice struggled when held by tail or neck, when laid supine (on back) or if the mice did not struggle at all. We also observed the extension of forelimbs when mice were lowered by the base of tail from a 15 centimetre height in visual placing test. We recorded whether the extension was upon a nose contact, upon a vibrasse contact, before vibrasse contact, early vigorous or if mice had no extension of forelimbs at all. To test the startle response we played a loud sound 15 centimetres above the arena. The mice's initial reaction was recorded, whether it was preyer reflex (backwards flick of pinnae), jump up or other movement or if the mice had no reaction at all. In touch escape test we observed the mice's initial reaction to a finger coming from front above. We recorded if the mice had no reaction to touching, had reaction to touching or if they escaped before touching.

4.1.2 Locomotor activity

Locomotor activity was tested on the arena divided into 10 x 10 centimetre squares. Mice were placed always on the same spot on the arena and observed for 30 seconds. Locomotor activity was recorded by counting the squares mice visited by all four feet during the observation time. The arena was cleaned with 70% ethanol after each test.

4.1.3 Balance beam

Balance of the mice was studied using three wooden beams with different diameters (1cm, 1.5cm and 2cm). The beam was placed one metre above padded surface. Dark box (home-base) was placed on the other end of the beam. During the testing the mice were put on free end of the beam their tail facing to the home-base. We recorded if the mice turned around to face the home-base, distance they walked on the beam and the time they used to reach home-base. If the mouse fell down the position and time spent on the beam was recorded. Observation time was 60 seconds. The beams and home-base were cleaned with 70% ethanol after each test.

4.1.4 Open/dark –field

Open/dark –field box (30 x 60 cm) was divided in two compartments of same size, black box and open field connected by an opening (5 x 6 cm). The mice were placed into the black box and were observed for 60 seconds. Open field was divided into nine squares. During the testing we recorded how many times the mouse peeked through the doorway and entered the open field. In the latter case we also counted the squares visited. The Open/dark –field box was cleaned with 70% ethanol after each test.

4.1.5 T-maze

T-maze was a black T-shaped box, which was open on the top and had a home-base in the end of the one arm. The arms of the T were each 20 centimetres long, 6 centimetres wide and walls were 10 centimetres high. The home-base was 10 x 6 cm and had opening (5 x 6cm) to the arm. The mice were placed into the home-base and the time when the mouse left the home-base was recorded. The observation time was 60 seconds and visits to the arms of the T-maze box were counted (right, left and down to home-base). The T-maze box was cleaned with 70% ethanol after each test.

4.1.6 Morris water maze

Morris water maze swimming pool (50 x 70 cm) was divided into four quarters and hidden underwater platform was placed into one of the quarters. Water temperature was 24˚C and the platform was about one centimetre under water. The place of the platform was same every time. In pretraining the mice were first placed on the hidden platform and then waited whether they started to swim. If the mice did not jump into the water, they were gently helped into water. Timing started as the mouse left the platform. Following pretraining were training trials where the mice were placed into water facing a wall on the other side of the pool than the platform. The finding of the platform was timed. The observation time was 30 seconds and if the mice did not find the platform they were placed on the platform. These procedures were done altogether eight times including the first pretraining. After training trials a probe trial was performed where the underwater platform was removed and mice were placed into water. The observation time was 60 seconds. Quarter visits were counted in total and times mice visited in the platform quarter. The time spend on the platform quarter was also timed.

4.2 Tissue analysis

4.2.1 Tissue preparation

Brain tissue samples were obtained from the wild-type and the knock-out mice at every time point after the behavioural tests. One mouse from both groups was sacrificed at 2, 4, 6 and 8 months, 5 wild-type and 6 knock-out mice at 10 months and 4 mice from both groups at 12 months. For RNA analysis small portion of the brain was placed into RNA Later (Ambion, Austin, TX, USA) and frozen at - 80˚C. Remaining tissue was fixed in 4 % paraformaldehyde, embedded in paraffin and used for morphological analysis. Mouse tissue sections were cut at 5 µm and placed on microscope slides.

4.2.2 Tissue staining

Tissue samples were stained with Haematoxylin-Eosin method. First the microscope slides were transported through xylene and descending ethanol series and placed into dH2O until staining. In the first step of the staining, the sections were immersed in Mayer's haematoxylin for 7 minutes. After that they were washed in running water for 2 minutes, rinsed with dH2O for one and half minutes and then treated with 70% ethanol for 15 seconds. After the staining with Eosin Y for 15 seconds the sections were treated with 96% ethanol twice for 30 seconds and then twice with absolute ethanol for one minute. Finally the slides were placed into xylene for a few minutes before applying the cover-slip on the slides.

4.2.3 Electron microscopy

For Electron microscopy the brain tissue samples were obtained from mice sacrificed at 10 month time point. Tissues were processed in the Electron Microscopy Laboratory of Tampere University Medical School by Ms Raija Hukkila, according to the following protocol.

Mouse brain tissues were fixed in 2.5% glutaraldehyde overnight. Glutaraldyhyde was diluted with 0.1 M phosphatebuffer (pH: 7.4). On the following day the tissue samples were washed with 0.1 M phosphatebuffer (pH: 7.4) and were kept there over night. Post fixation was done for an hour with 1% osmiumtetroxide (OsO4), which was also diluted with 0.1M phosphatebuffer. After the fixations the tissue samples were washed with dH2O. Dehydration was performed in ascending acetone series. After dehydration the tissue samples were placed into covered tube containing acetone:Epon (1:1) for one hour. Then the tissue samples were placed into open tube containing Epon overnight. Next day the tissue samples were mounted in Epon, which was polymerised for two days at 60˚C. Half thin sections were cut at 1.5 μm with a microtome and stained on microscope slides with Toluidine Blue. After microscopy the exact regions for final sections were chosen. The final sections were cut at 70nm with ultramicrotome and diamond knife and placed into 300 mesh copper grids. After cutting the samples were stained in uranylacetate for 30 minutes and for 5 minutes in leadcitrate. Electron microscopy was performed by Doctor Raija Sormunen in the Electron microscopy Laboratory of Biocenter Oulu.

4.3 Microarray

For the cDNA microarray the brain tissue samples were obtained from 6 wild-type and 6 $Car9^{-/-}$ knock-out mice. Total RNA extraction from the brain tissue was performed by using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. DNase treatment for the samples was done using RNase-free DNase (Qiagen). RNA concentration and purity were determined using optical density (OD) measurements at 260 and 280 nm. All the samples had an OD260/OD280 ratio of 1.95 or higher.

Microarray was performed at Turku Center for Biotechnology in Finnish DNA-Microarray Centre with Illumina™ Sentrix BeadArray technology. Microarray analysis was performed by Mr Mika Hilvo in the Institute of Medical Technology by Genespring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA, USA). The fold –change values were calculated for each gene using the same software. The microarray data was filtered according to a fold –change of 1.40 and -1.40 for up and down-regulated expression.

4.4 Statistical tests

T-test was performed for Morris water maze and T –maze. A non-parametric Mann-Whitney U and Kolmokorov-Smirnov tests were performed for Locomotor activity and partly for Morris water maze to evaluate if the findings were statistically significant because the data was not normally distributed. P –values < 0.05 were considered significant.

5. Results

5.1 Behavioural analysis

5.1.1 Sensory functions and basic characteristics

We were not able to observe any significant differences between the *Car9*$^{-/-}$ knock-out mice and the wild-type mice while recording mice's overall well-being, weight, condition of the fur, body position, tremor, palpebral closure, urination, defecation, tail elevation, positional passivity, limb grasping, visual placing, startle response, touch escape, corneal reflex and tactile sense.

The mice's overall well-being was very good throughout the testing period. Only one wild-type mouse had to be killed at eight months because of the detected paw injury. Condition of the mice's furs was also very good excluding few exceptions. There was no tremor or problems with palpebral closure, corneal reflex and tactile sense in either one of the groups. We could not observe any differences in urination or in defecation. The knock-out mice males weighted slightly more than the wild-type mice males throughout the tests but same difference could not be observed in the female mice. The body position of the mice from both groups was always normal, thus they were sitting or standing and rearing on hind legs. At two month time point the knock-out mice had a tendency to have tail elevated. 42.9% of the knock-out mice had an elevated tail when 100% of the wild-type mice had a horizontally extended tail. At four month time point still 23.1% of the knock-out mice had an elevated tail and 100% of the wild-type mice had a horizontally extended tail. After this time point no difference in tail elevation could be observed. Tail elevation reflects fear and anxiety.

In positional passivity test none of the mice from either group could be laid on their backs. First most of them struggled when held by tail and neck. As the mice became older and got used to the handling, the struggling degreased when held by tail and neck, but still the mice could not be laid on their backs. Same tendency could be observed in touch escape where the majority of the mice first escaped the touch but older they got used to handling and did not escape and only reacted to touching. Noteworthy was that every mice reacted to touching. We could not observe any difference

in limb grasping. Both groups could perform this task well excluding few exceptions with chubby older mice to whom it was physically impossible task. No difference could be observed in visual placing and startle response.

In transfer arousal test we recorded the mice's immediate reaction to new environment, and in spontaneous activity we observed their normal or abnormal activity. When testing transfer arousal and spontaneous activity we were able to note that the *Car9*$^{-/-}$ knock-out mice had a tendency to be more active in their response than the wild-type mice, especially when they became older. At eight month time point 70% of the wild-type mice had a prolonged freeze in transfer arousal as only 36.4% of the knock-out mice had a prolonged freeze. 54.5% of the knock-out mice moved immediately, whereas only 10% of the wild-type mice showed immediate movement. At ten month time point 66.7% of the wild-type mice had a prolonged freeze and 22.2% showed immediate movement, while 30% of the knock-out mice had a prolonged freeze and 60% showed immediate movement. No difference could be observed in the spontaneous activity at eight month time point, but at ten months 55.6% of the wild-type mice were resting and did nothing, while 50% of the knock-out mice had moderate movement and 30% had slow movement. However, at 12 month time point no significant difference could be observed due to small test group.

5.1.2 Locomotor activity

The locomotor activity was recorded as amount of squares each mouse visited during the 30 second recording time in the arena. We found no significant difference between the wild-type mice and the *Car9*$^{-/-}$ knock-out mice as a whole group (p –value = 0.073 with Mann-Whitney U test), but a difference between them was seen when observing the individual tests at each time point. First it was found that at two month time point the wild-type mice were more active in their response than the knock-out mice. At four month time point the mice's activity was about the same. However, after this time point the *Car9*$^{-/-}$ knock-out mice were more active than the wild-type mice. At the eight month time point the knock-out mice were significantly more active. All the observations are illustrated in Figure 5.1.

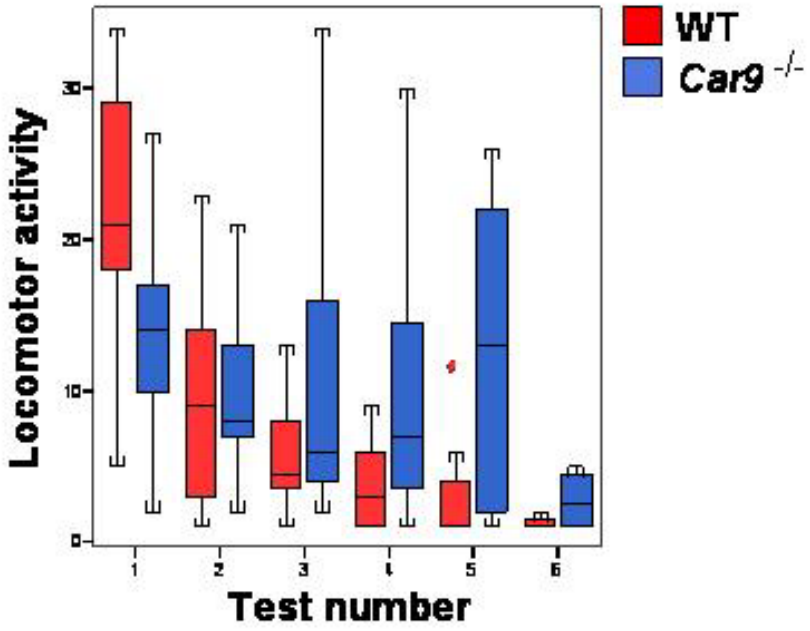

Figure 5.1. Locomotor activity. Squares in total during 30 second observation time (p – value=0.073). X-axis shows the number of tests (1 = 2 months, 2 = 4 months, 3 = 6 months, 4 = 8 months, 5 = 10 months and 6 = 12 months). Y-axis shows the number of squares the mice visited. Genotype 1 and 2 represent the wild-type mice (red) and knock-out mice (blue), respectively.

5.1.3 Balance beam

We were not able to observe any significant difference while recording balance beam between the wild-type mice and the *Car9*$^{-/-}$ knock-out mice. The mice from both groups were able to perform this task equally well. Mice from both groups used on an average same time to reach the home-base and fell down about equal amounts. In addition no difference could be observed in the turning of the mice. The balance beam -test showed that the *Car9*$^{-/-}$ knock-out mice had a similar balance and coordination as the wild-type mice.

5.1.4 Open/dark –field

We were not able to observe any significant difference while recording Open/dark –field task between the wild-type mice and the *Car9*$^{-/-}$ knock-out mice. However, the wild-type mice had a tendency be braver and they came out to the open field more often and visited more squares than the knock-out mice at two and four month time points. This difference disappeared at six months old

mice. After this time point the knock-out mice were slightly more active and visited more squares than the wild-type mice. The number of times mice came out to the open field was about the equal after the four month time point. No difference was observed in the number of times mouse peeked through the doorway.

5.1.5 T-maze

In the T-maze, when the mice left the home-base, a same kind of trend was seen as in the locomotor activity. First the wild-type mice were more active and left the home-base more rapidly than the *Car9*$^{-/-}$ knock-out mice. This difference evened out with 6 months old mice, but at 10 and 12 months the wild-type mice were again slightly more active than the knock-out mice. This is shown in Figure 5.2 (p –value = 0.001 with T-test). Other aspect observed in T-maze was the number of arms the mice visited. We were not able to observe any significant difference in this aspect while recording (p –value = 0.051 with Mann-Whitney U test). However, we observed that the wild-type mice had a tendency to visit more arms than the knock-out mice throughout the whole testing period (data not shown).

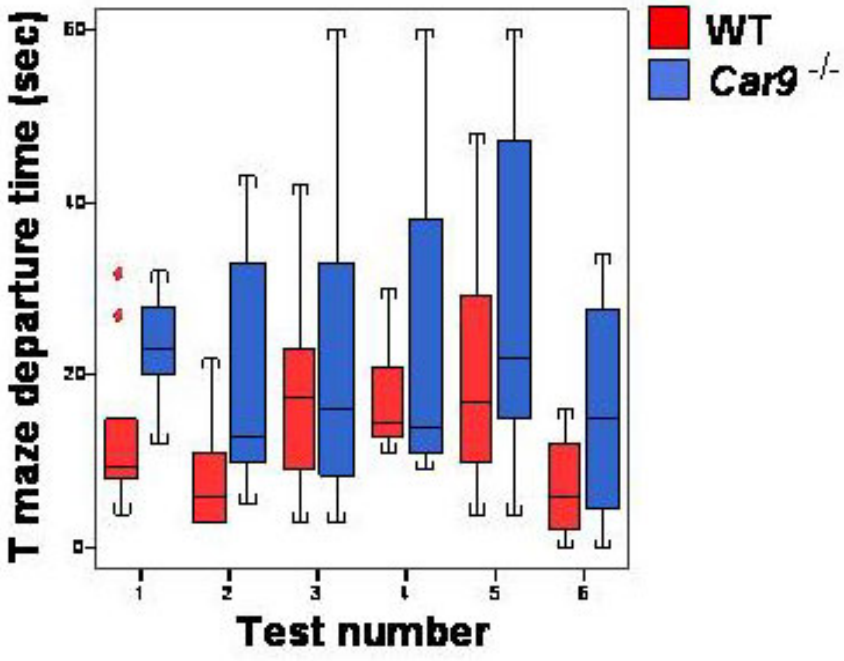

Figure 5.2. T-maze. The time, when mice left the home-base (p –value=0.001). X-axis shows the number of tests (1 = 2 months, 2 = 4 months, 3 = 6 months, 4 = 8 months, 5 = 10 months and 6 = 12 months). Y-axis shows the time (sec), when the mice left the home-base. Genotype 1 and 2 represent the wild-type mice (red) and knock-out mice (blue), respectively.

5.1.6 Morris water maze

In Morris water maze we observed distinctive difference in learning and visual memory between the wild-type mice and the *Car9*$^{-/-}$ knock-out mice. In Figure 5.3 the time spent altogether in learning using visual clues during pretraining and training trials shows that the knock-out mice were poorer to learn at every time point than the wild-type mice (p –value = 0.004 with T-test). Same trend is seen in Figure 5.4 as the wild-type mice spent more time on finding the removed platform than the knock-out mice (p –value = 0.000 with Mann-Whitney U test). This indicates that the wild-type mice have a better memory function than the knock-out mice and they also learn more rapidly.

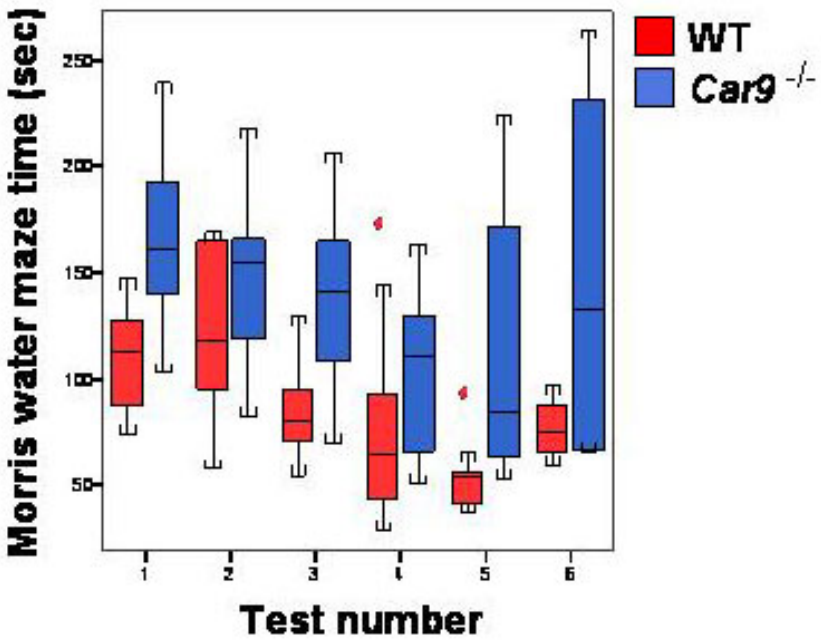

Figure 5.3. Morris water maze. The time spent altogether for learning in pretraining and training trials (p –value=0.004). X-axis shows the number of tests (1 = 2 months, 2 = 4 months, 3 = 6 months, 4 = 8 months, 5 = 10 months and 6 = 12 months). Y-axis shows the time (sec) the mice spent altogether for learning in pretraining and training trials. Genotype 1 and 2 represent the wild-type mice (red) and knock-out mice (blue), respectively.

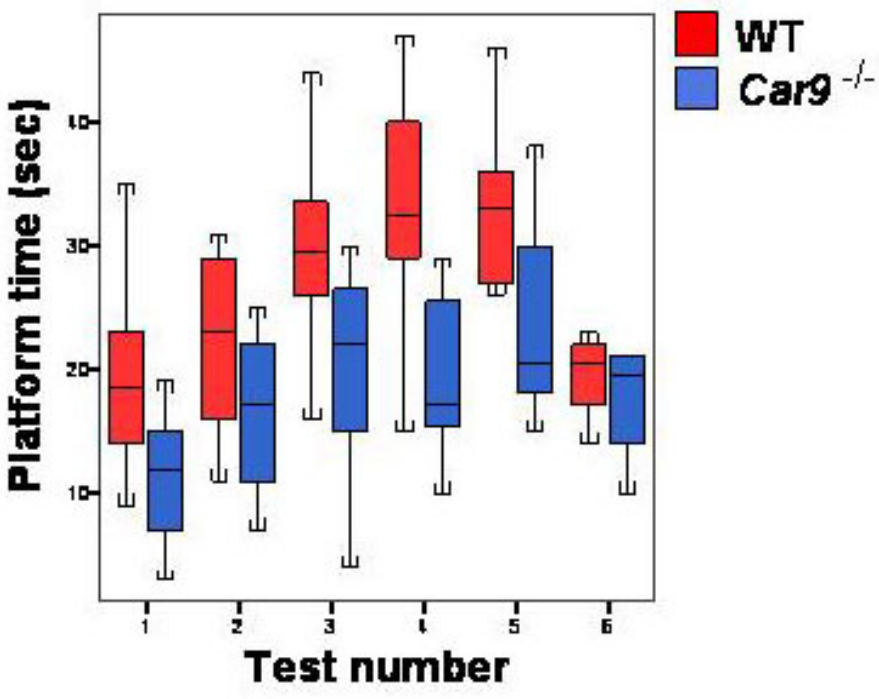

Figure 5.4. Morris water maze. The time spent in the quarter, where the hidden platform was earlier (p –value=0.000). X-axis shows the number of tests (1 = 2 months, 2 = 4 months, 3 = 6 months, 4 = 8 months, 5 = 10 months and 6 = 12 months). Y-axis shows the time (sec) the mice spent in the quarter, where the hidden platform was earlier. Genotype 1 and 2 represent the wild-type mice (red) and knock-out mice (blue), respectively.

5.2 Tissue analysis

5.2.1 HE –staining

As expected we observed alterations in the morphology of *Car9*$^{-/-}$ knock-out mouse brain tissue that can be interpreted as a vacuolar change. These vacuolar alterations can be seen in 8 months old *Car9*$^{-/-}$ knock-out mice and in 10 months old knock-out mice 50% of the brain samples showed this alteration. These vacuolar changes were detected in all regions of the brain including the cortex and deeper white matter. Figure 5.6 shows a vacuolar brain tissue sample obtained from 10 months old knock-out mice. As a control we used a brain tissue sample obtained from 12 months old wild-type mouse (Figure 5.5).

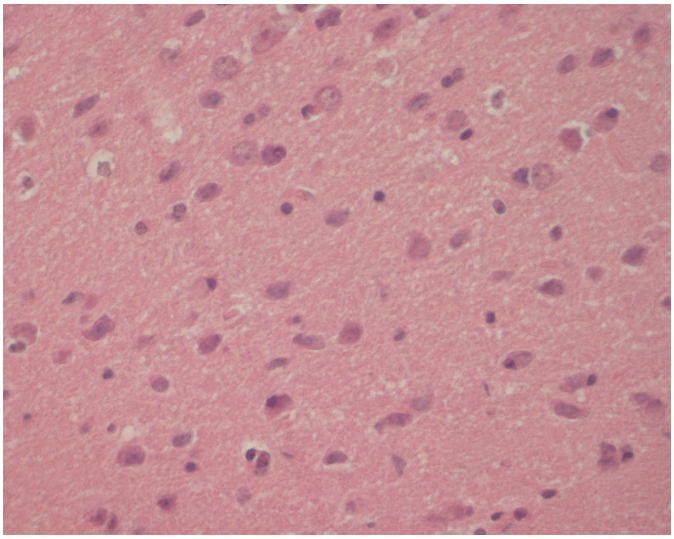

Figure 5.5. Mouse brain tissue obtained from 12 months old wild-type mouse.

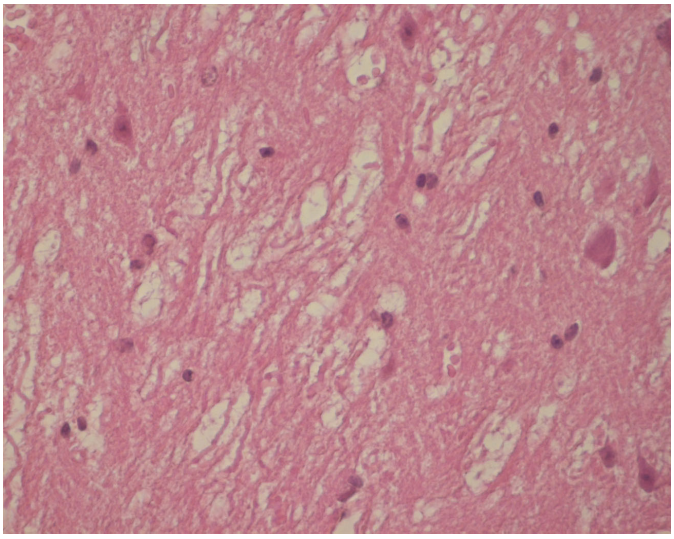

Figure 5.6. Mouse brain tissue obtained from 10 months old *Car9*$^{-/-}$ knock-out mouse.

5.2.2 Electron microscopy

In electron microscopy, we observed same alterations as in HE –staining. In Figures 5.7 and 5.8 control brain tissue samples are obtained from 10 months old wild-type mice. The vacuolar changes can be seen in the brain tissue sample in Figures 5.9. and 5.10, which were obtained from 10 months old *Car9*$^{-/-}$ knock-out mice. We also observed myelin degeneration in the knock-out mice brain tissue samples. This can be seen in Figures 5.10 and 5.11. In addition in the *Car9*$^{-/-}$ knock-out mouse brain tissue samples we observed neuron degeneration and empty axons/dendrites, which can be seen in Figure 5.12. We also observed decomposed cells as seen in Figure 5.13. These alterations were detected all over the brain, in the cortex and also in deeper regions of the brain.

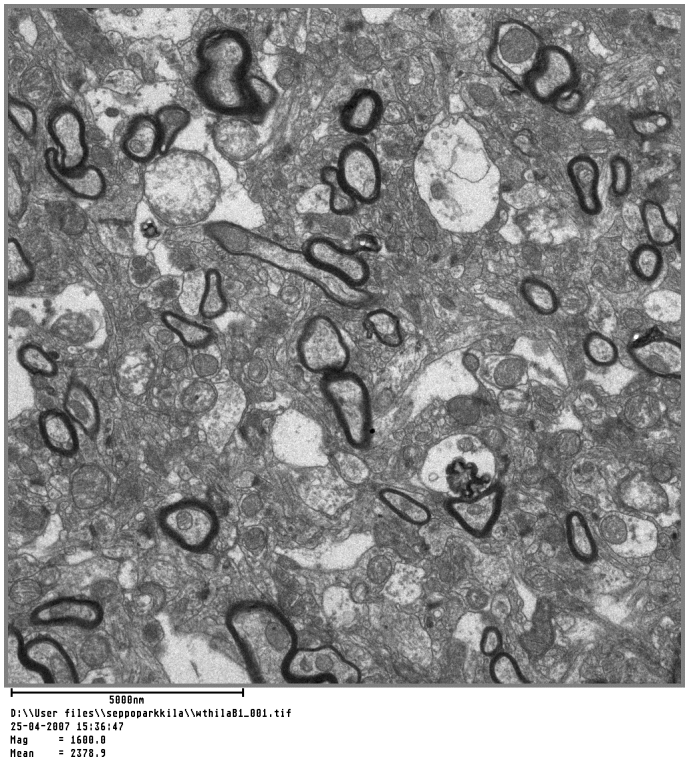

Figure 5.7. Mouse brain tissue obtained from 10 months old wild-type mouse.

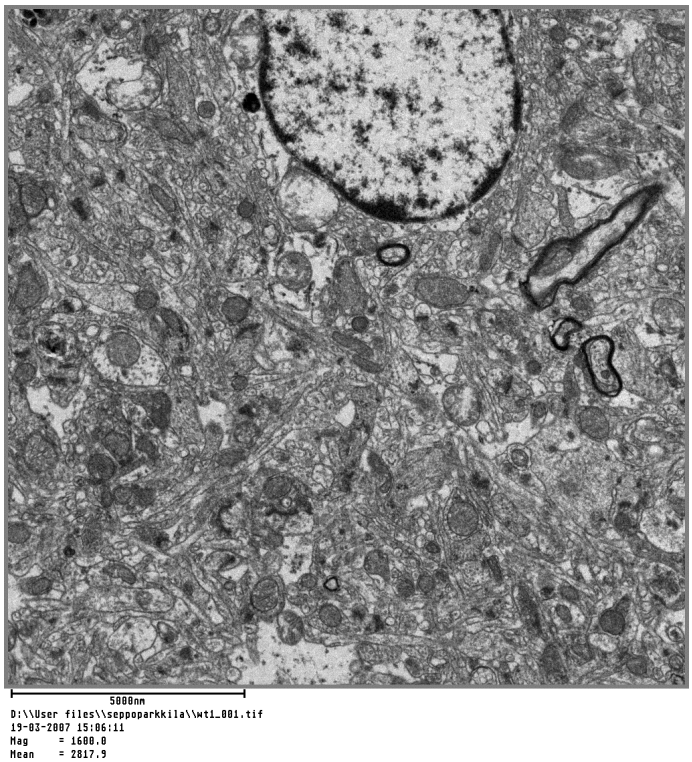

Figure 5.8. Mouse brain tissue obtained from 10 months old wild-type mouse.

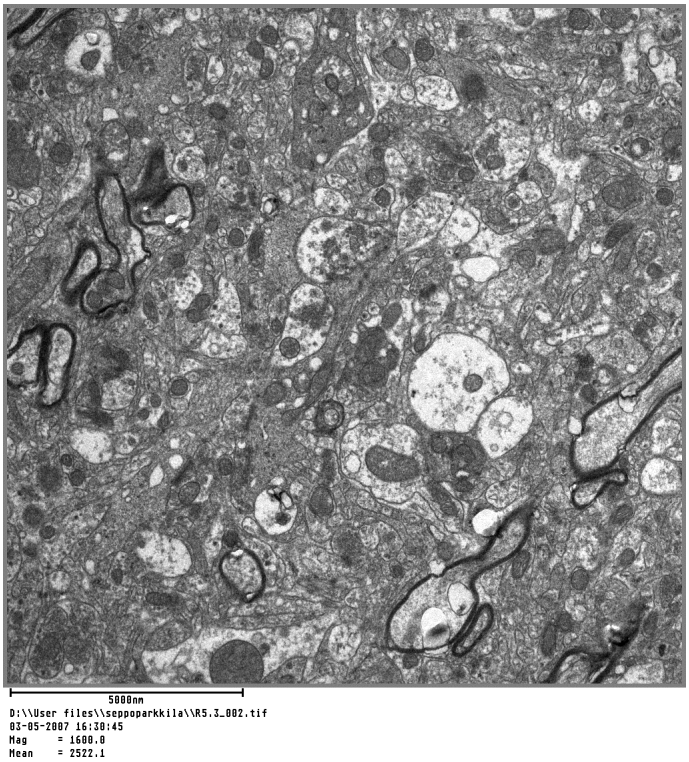

Figure 5.9. Mouse brain tissue obtained from 10 months old *Car9*$^{-/-}$ knock-out mouse. Vacuolar changes can be seen in the tissue sample.

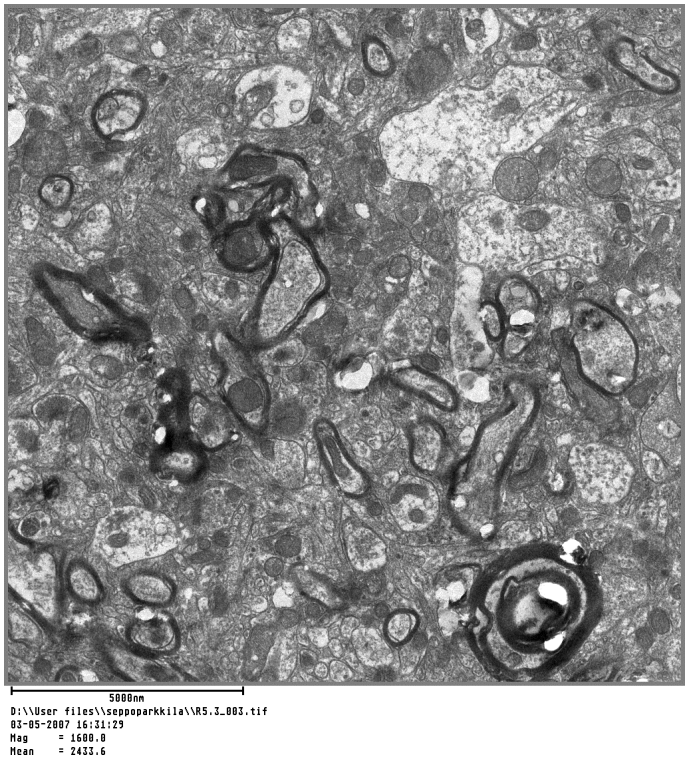

Figure 5.10. Mouse brain tissue obtained from 10 months old *Car9*$^{-/-}$ knock-out mouse. Vacuolar changes and myelin degeneration can be seen in the tissue sample.

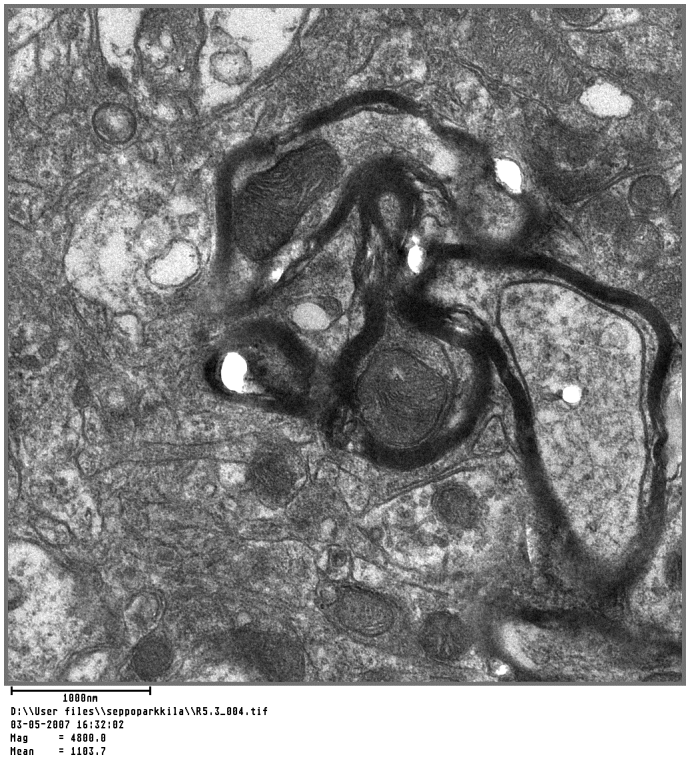

Figure 5.11. Mouse brain tissue obtained from 10 months old *Car9*$^{-/-}$ knock-out mouse. Myelin degeneration can be seen in the tissue sample.

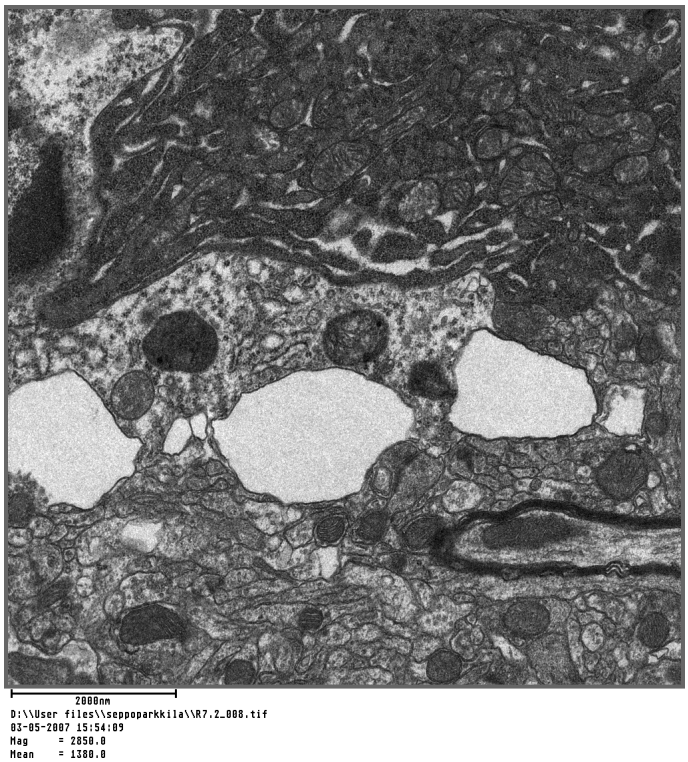

Figure 5.12. Mouse brain tissue obtained from 10 months old *Car9*$^{-/-}$ knock-out mouse. Degenerative neurons and empty axons/dentrites can be seen in the tissue sample.

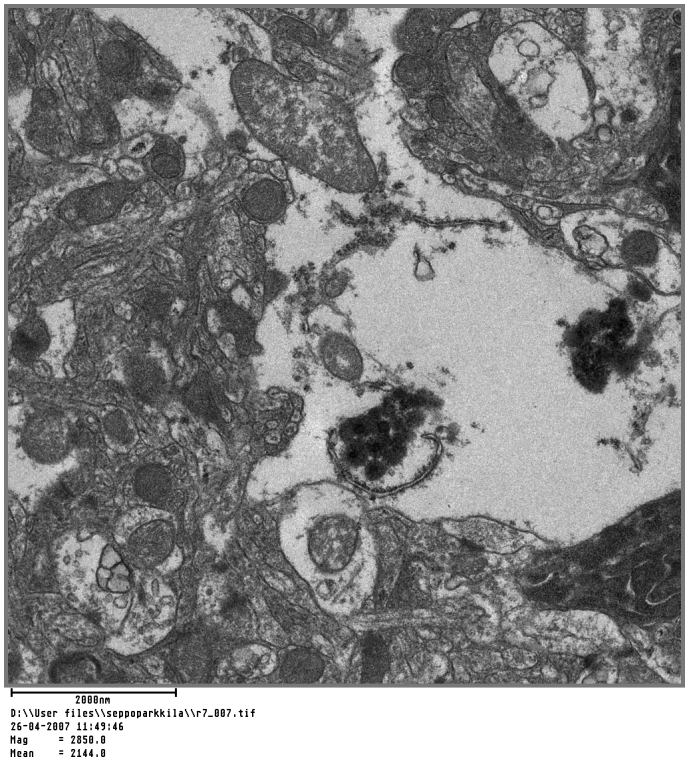

Figure 5.13. Mouse brain tissue obtained from 10 months old *Car9*$^{-/-}$ knock-out mouse. A decomposed cell can bee seen in the tissue sample.

5.3 Microarray

In cDNA microarray, we obtained 11 up-regulated genes with a fold –change over 1.40 (Table 5.1) and 12 down-regulated genes with a fold –change under -1.40 (Table 5.2). The data was obtained from 4 *Car9*$^{-/-}$ knock-out mice and 4 wild-type mice. NeoR cassette was used to create the null mutation of *Car9*$^{-/-}$ knock-out, and thus the corresponding gene was highly overexpressed in the microarray. Although positive signal was observed for CA IX mRNA, CA IX protein is not produced in these mice (data not shown). Therefore, the signal most probably reflects the fact that the probe used in the microarray analysis detects a fragment of CA IX mRNA that is not translated.

Car9 gene is located in chromosome 4qB1. One up-regulated gene is located at the same loci (Table 5.1). In addition six of the down-regulated genes are located in the chromosome 4qA5 (Table 5.1), which is next to 4qB1. In total there were 11 down-regulated genes (data not shown), which were located in the chromosome 4, suggesting that a larger disruption of this chromosomal region may have occurred during the production of the knock-out mice.

Table 5.1. Up-regulated *Car9*$^{-/-}$ knock-out mice genes. Data obtained with 4 *Car9*$^{-/-}$ knock-out mice and 4 wild-type mice. Genes with a fold –change over 1.40 are included to the table.

Definition	**Symbol**	**Accession**	**Fold – change**	**p – value**	**Location in Chromosome 4**
NeoR			8,87	0,0001	
Mus musculus S100 calcium binding protein A8 (calgranulin A) (S100a8), mRNA.	S100a8	NM_013650.1	2,26	0,0240	
Mus musculus 2 days neonate thymus thymic cells cDNA, RIKEN full-length enriched library, clone: E430002C01 product: hypothetical protein, full insert sequence	E130306D19Rik	AK153880	2,16	0,0000	qB1
Mus musculus carbonic anhydrase 9 (Car9), mRNA.	Car9	NM_139305.1	1,91	0,0000	qB1
Mus musculus WD repeat domain 9 (Wdr9), mRNA.	Wdr9	NM_145125.1	1,68	0,0014	
Mus musculus WD repeat domain 9 (Wdr9), mRNA.	Wdr9	NM_145125.1	1,62	0,0011	
Mus musculus heterogeneous nuclear ribonucleoprotein D-like (Hnrpdl), mRNA.	Hnrpdl	NM_016690.2	1,59	0,0130	
Mus musculus programmed cell death 4 (Pdcd4), mRNA.	Pdcd4	NM_011050	1,46	0,0288	
Mus musculus WD repeat and SOCS box-containing 1 (Wsb1), mRNA.	Wsb1	NM_019653.2	1,43	0,0289	
Mus musculus carbonic anhydrase 9 (Car9), mRNA.	Car9	NM_139305.1	1,42	0,0007	
Mus musculus expressed sequence AI448196 (AI448196), mRNA.	AI448196	XM_136041.3	1,40	0,0221	

Table 5.2. Down-regulated *Car9*$^{-/-}$ knock-out mice genes. Data obtained with 4 *Car9*$^{-/-}$ knock-out mice and 4 wild-type mice. Genes with a fold –change under -1.40 are included to the table.

Definition	**Symbol**	**Accession**	**Fold – change**	**p – value**	**Chromosome location**
Mus musculus 12 days embryo spinal ganglion cDNA, RIKEN full-length enriched library, clone:D130052N13 product: hypothetical protein, full insert sequence		AK051496	-3,37	0,0057	qA5
Mus musculus chemokine (C-C motif) ligand 21c (leucine) (Ccl21c), mRNA.	CKb9; exodus-2; Scya21c; SLC; TCA4	NM_023052	-3,04	0,0005	qA5
Mus musculus hypothetical LOC331139 (LOC331139), mRNA.		XM_284587.2	-2,59	0,0090	qA5
Mus musculus chemokine (C-C motif) ligand 21b (serine) (Ccl21b), mRNA.	6CKBAC2; 6Ckine; ALP; CKb9; plt; Scya21; Scya21b; SLC; Tca4	NM_011124.3	-2,04	0,0040	qA5
Mus musculus chemokine (C-C motif) ligand 21a (leucine) (Ccl21a), mRNA.	6CKBAC1; 6Ckine; CKb9; Scya21; Scya21a; SLC; TCA4	NM_011335.1	-1,98	0,0075	qA5
Mus musculus early growth response 4 (Egr4), mRNA.	NGF1-C; NGFI-C; NGFIC; pAT133	NM_020596.1	-1,88	0,0496	
Mus musculus ciliary neurotrophic factor receptor (Cntfr), mRNA.	Cntfralpha	NM_016673.1	-1,59	0,0193	qA5
Mus musculus cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a), mRNA.	CAP20; CDKI; Cdkn1; CIP1; mda6; P21; p21Cip1; p21WAF; SDI1; Waf1	NM_007669.2	-1,52	0,0378	
Mus musculus calcium/calmodulin-dependent protein kinase kinase 2, beta (Camkk2), mRNA.	6330570N16Rik; mKIAA0787	NM_145358.1	-1,45	0,0056	
Nuclear factor I/C (Nfic), mRNA		NM_008688	-1,44	0,0292	
Mus musculus cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a), mRNA.	CAP20; CDKI; Cdkn1; CIP1; mda6; P21; p21Cip1; p21WAF; SDI1; Waf1	NM_007669.2	-1,43	0,0402	
Mus musculus RIKEN cDNA 6430704M03 gene (6430704M03Rik), mRNA.		XM_131434	-1,40	0,0081	qB3

6. Discussion

Ortova Gut et al. (2002) obtained CA IX -deficient mice with null mutation of the *Car9* gene (*Car9*$^{-/-}$) by targeted gene disruption. A group of *Car9*$^{-/-}$ knock-out mice (C57BL6) were produced for research purposes and during the maintenance period we observed a slightly abnormal activity. The *Car9*$^{-/-}$ knock-out mice were more aggressive and active than their wild-type littermates. Thus, a decision of taking brain tissue samples was made. Unexpectedly, these brain tissue samples showed vacuolar degeneration. Therefore we decided to study *Car9*$^{-/-}$ knock-out mice using behavioural analysis and brain morphological analysis. Our aim with these studies was to find out when these vacuolar changes become visible in brain and if the behavioural abnormalities can be detected in specific behavioural analyses.

The phenotype analysis was a longitudinal 12 month study using slightly modified SHIRPA protocol. The *Car9*$^{-/-}$ knock-out mice were tested at every two months. The *Car9*$^{-/-}$ knock-out mice were in good condition during the entire testing period and direct physical alterations could not be observed. The behavioural analysis results showed that *Car9*$^{-/-}$ knock-out mice were indeed more active in their response than the wild-type mice. This was seen in locomotor activity, in transfer arousal and in spontaneous activity as the 8 months old *Car9*$^{-/-}$ knock-out mice showed a distinct difference in motor activity, in initial reaction to new place and in normal mouse activity, respectively. The motor activity difference could be observed already at 6 months old knock-out mice in locomotor activity test but in spontaneous activity the difference was not seen until at 10 month time point. Majority of the knock-out mice moved immediately when placed to new location in transfer arousal at 8 and 10 months time point. Same kind of results could be observed in Open/dark –field test as the knock-out mice were more active counted as squares visited at 8 month time point. These results indicate that *Car9*$^{-/-}$ knock-out mice have a major need to move. This observation becomes obvious at 8 month time point, while the ageing wild-type mice have normally slower movement.

Open/dark –field test also showed that the wild-type mice were slightly braver at first two testing times, but this difference became even in 6 months old mice. This was observed in times mice came out to the open field. T-maze test showed same kind of results as the wild-type mice were distinctively braver and left the home-base more rapidly at 2 and 4 month time points. At 6 and 8

month time points the difference disappeared and became apparent again at 10 month time point. Tail elevation showed that the *Car9*$^{-/-}$ knock-out mice were more anxious and had their tails elevated more often than the wild-type mice at first two testing times, which could relate to this tendency of the wild-type being braver.

A distinct difference in learning and visual memory was also seen in behavioural tests between the *Car9*$^{-/-}$ knock-out mice and the wild-type mice as the knock-out mice showed slower development than the wild-type mice. These alterations were observed in Morris water maze -test throughout the whole testing period. The Morris water maze -test showed that *Car9*$^{-/-}$ knock-out mice had difficulties to learn and they had also poorer memory function. Largest differences were observed at 6 and 8 months time points.

When performing this kind of behavioural tests it has to be considered that human error is always present. Tests have to be performed every time exactly the same way to minimalise the error marginals by the tester. For the same reason the order of the tests and the testing time need to remain constant. We minimalised these errors by filming most of the tests and by counting the results always at least two times. In observational tests the evaluation of the mice behaviour is difficult but by using the same tester every time the observational differences are excluded or at least minimalised.

Both HE –staining and electron microscopy showed vacuolar changes in the brain tissues of *Car9*$^{-/-}$ mice, however, the changes were minor than the original changes, which led us to this study. The minor vacuolar changes might be due to a fact that our mice were younger than the original mice and also our tissue preparation methods might have been more accurate. The vacuolar changes are always a sign of tissue degeneration. The cell adhesion properties of CA IX may be involved in maintenance of mucosal integrity contributing to proper intercellular contacts and communication (Ortova Gut et al., 2002), which might also be the case in brain. Another possibility is that there might be lipid accumulations, which, however, could not be shown in special staining from ice section (data not shown). Cell decomposition was seen in electron microscopy and rapid cell death or degeneration could be the cause of the vacuolarization in the brain tissue. Electron microscopy also showed neuron and axon/dentrite degeneration. One possibility, which also have to be considered, is that the vacuolarization seen in *Car9*$^{-/-}$ knock-out mice may be generated as an artefact during the tissue preparation. However, the brain tissue preparations were identical in both groups and similar vacuolar changes could not be seen in control animals.

In cDNA microarray we obtained 12 down-regulated genes and 11 up-regulated genes. *Car9* gene is located in chromosome 4qB1 and one up-regulated gene was located at the same loci. In addition six of the down-regulated genes were located in the chromosome 4qA5, which is next to loci qB1. All of the down-regulated 11 genes were located in chromosome 4, which indicates that a larger disruption of this chromosomal region may have occurred during the production of the knock-out mice. When producing the *Car9*$^{-/-}$ knock-out there might have occurred deletion of an important promoter or enhancer, which affects all of these down-regulated genes. Thus, further investigations are needed to define what has happened in chromosome 4 and if this brain phenotype is due to this major alteration of several genes or due to a *Car9*$^{-/-}$ gene.

In all of these analyses the abnormalities in the *Car9*$^{-/-}$ knock-out mice appeared at 6 to 8 months old and the 8 month time point seemed to be the breaking point in every test. In almost all of the behavioural tests, which indicated difference between the knock-out mice and the wild-type mice, the difference became obvious at 8 month time point. Also the vacuolar changes became visible in 8 months old knock-out mice. In electron microscopy, which was performed at 10 month time point distinct abnormalities could be observed. These results suggest that the alterations became permanent in brain at eight months old mice.

The expression of CA IX is low in brain tissue and its exact mechanism there is yet unknown. We observed in close proximity of *Car9* gene down-regulation of several genes. The alteration affecting all of the down-regulated genes might have shown up in the production of the *Car9*$^{-/-}$ knock-out mice, which is why further investigations is needed to find out if our findings are related solely to CA IX -deficiency. After these defects are solved this mouse model could function as a study tool for degenerative brain disease.

7. Conclusions

We have identified a mouse strain, which has alterations in behaviour and brain tissue. The *Car9*$^{-/-}$ knock-out mice had degenerative changes in their brain tissue stated as vacuolar. Electron microscopy showed also myelin and neuron degeneration, empty axons/dentrites and degenerative cells. These alterations in the brain became apparent at 8 months old mice as seen in HE –staining and the electron microscopy showed degenerative cell alterations in 10 months old mice. According to the behavioural tests the *Car9*$^{-/-}$ knock-out mice had changes in their motor activity, thus they were more active. The knock-out mice had also difficulties to learn in Morris water maze task and they showed also poorer memory functions. The behavioural changes became also obvious at 8 month time point. This indicates that these alterations in the brain phenotype of the *Car9*$^{-/-}$ knock-out mice became permanent at 8 months old mice. Because several genes were down-regulated in cDNA microarray in the close proximity of *Car9*$^{-/-}$ gene further investigations are necessary to find out if our findings are related to CA IX -deficiency.

8. References

Ashby W. Carbonic anhydrase in mammalian tissue. J Biol Chem 1943;151:521-527.

Bailey KR, Rustay NR, Crawley JN. Behavioral phenotyping of transgenic and knockout mice: practical concerns and potential pitfalls. ILAR J 2006;47:124-131.

Barnea G, Silvennoinen O, Shaanan B, Honegger AM, Canoll PD, D'Eustachio P, Morse B, Levy JB, Laforgia S, Huebner K, et al. Identification of a carbonic anhydrase-like domain in the extracellular region of RPTP gamma defines a new subfamily of receptor tyrosine phosphatases. Mol Cell Biol 1993;13:1497-1506.

Bartosová M, Parkkila S, Pohlodek K, Karttunen TJ, Galbavý S, Mucha V, Harris AL, Pastorek J, Pastoreková S. Expression of carbonic anhydrase IX in breast is associated with malignant tissues and is related to overexpression of c-erbB2. J Pathol 2002;197:314-321.

Beasley NJ, Wykoff CC, Watson PH, Leek R, Turley H, Gatter K, Pastorek J, Cox GJ, Ratcliffe P, Harris AL. Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. Cancer Res 2001;61:5262-5267.

Brady HJ, Lowe N, Sowden JC, Edwards M, Butterworth PH. The human carbonic anhydrase I gene has two promoters with different tissue specificities. Biochem J 1991;277:903-905.

Brown D, Zhu XL, Sly WS. Localization of membrane-associated carbonic anhydrase type IV in kidney epithelial cells. Proc Natl Acad Sci U S A 1990;87:7457-7461.

Cabiscol E, Levine RL. The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation. Proc Natl Acad Sci U S A 1996;93:4170-4174.

Cammer W, Zhang H. Comparison of immunocytochemical staining of astrocytes, oligodendrocytes, and myelinated fibers in the brains of carbonic anhydrase II-deficient mice and normal littermates. J Neuroimmunol 1991;34:81-86.

Chegwidden WR, Carter ND. Introduction to the carbonic anhydrases. In: Chegwidden WR, Carter ND and Edwards YH (eds), The Carbonic Anhydrases: New Horizons, Birhkhäuser Verlag, Basel, 2000, pp. 13-28.

Decker B, Sender S, Gros G. Membrane-associated carbonic anhydrase IV in skeletal muscle: subcellular localization. Histochem Cell Biol 1996;106:405-411.

Earnhardt JN, Qian M, Tu C, Lakkis MM, Bergenhem NC, Laipis PJ, Tashian RE, Silverman DN. The catalytic properties of murine carbonic anhydrase VII. Biochemistry 1998;37:10837-10845.

Fleming RE, Crouch EC, Ruzicka CA, Sly WS. Pulmonary carbonic anhydrase IV: developmental regulation and cell-specific expression in the capillary endothelium. Am J Physiol 1993;265:627-635.

Fleming RE, Parkkila S, Parkkila AK, Rajaniemi H, Waheed A, Sly WS. Carbonic anhydrase IV expression in rat and human gastrointestinal tract regional, cellular, and subcellular localization. J Clin Invest 1995;96:2907-2913.

Fraser P, Cummings P, Curtis P. The mouse carbonic anhydrase I gene contains two tissue-specific promoters. Mol Cell Biol 1989;9:3308-3313.

Fujikawa-Adachi K, Nishimori I, Taguchi T, Onishi S. Human mitochondrial carbonic anhydrase VB. cDNA cloning, mRNA expression, subcellular localization, and mapping to chromosome x. J Biol Chem 1999a;274:21228-21233.

Fujikawa-Adachi K, Nishimori I, Taguchi T, Onishi S. Human carbonic anhydrase XIV (CA14): cDNA cloning, mRNA expression, and mapping to chromosome 1. Genomics 1999b;61:74-81.

Ghandour MS, Langley OK, Zhu XL, Waheed A, Sly WS. Carbonic anhydrase IV on brain capillary endothelial cells: a marker associated with the blood-brain barrier. Proc Natl Acad Sci U S A 1992;89:6823-6827.

Ghandour MS, Parkkila AK, Parkkila S, Waheed A, Sly WS. Mitochondrial carbonic anhydrase in the nervous system: expression in neuronal and glial cells. J Neurochem 2000;75:2212-2220.

Haapasalo JA, Nordfors KM, Hilvo M, Rantala IJ, Soini Y, Parkkila AK, Pastoreková S, Pastorek J, Parkkila SM, Haapasalo HK. Expression of carbonic anhydrase IX in astrocytic tumors predicts poor prognosis. Clin Cancer Res 2006;12:473-477.

Hageman GS, Zhu XL, Waheed A, Sly WS. Localization of carbonic anhydrase IV in a specific capillary bed of the human eye. Proc Natl Acad Sci U S A 1991;88:2716-2720.

Hewett-Emmet D. Evolution and distribution of the carbonic anhydrase gene families. In: Chegwidden WR, Carter ND and Edwards YH (eds), The Carbonic Anhydrases: New Horizons, Birhkhäuser Verlag, Basel, 2000, pp. 29-76

Hewett-Emmett D, Tashian RE. Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. Mol Phylogenet Evol 1996;5:50-77.

Hilvo M, Rafajová M, Pastoreková S, Pastorek J, Parkkila S. Expression of carbonic anhydrase IX in mouse tissues. J Histochem Cytochem 2004;52:1313-1322.

Hilvo M, Tolvanen M, Clark A, Shen B, Shah GN, Waheed A, Halmi P, Hänninen M, Hämäläinen JM, Vihinen M, Sly WS, Parkkila S. Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. Biochem J 2005;392:83-92.

Hilvo M, Innocenti A, Monti SM, De Simone G, Supuran CT, Parkkila S. Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. Curr Pharm Des 2008;14:672-678.

Ivanov SV, Kuzmin I, Wei MH, Pack S, Geil L, Johnson BE, Stanbridge EJ, Lerman MI. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc Natl Acad Sci U S A 1998;95:12596-12601.

Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Závada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 2001;158:905-919.

Järvelä S, Parkkila S, Bragge H, Kähkönen M, Parkkila AK, Soini Y, Pastoreková S, Pastorek J, Haapasalo H. Carbonic anhydrase IX in oligodendroglial brain tumors. BMC Cancer 2008;8:1.

Kallio H, Pastoreková S, Pastorek J, Waheed A, Sly WS, Mannisto S, Heikinheimo M, Parkkila S. Expression of carbonic anhydrases IX and XII during mouse embryonic development. BMC Dev Biol 2006;6:22.

Kaluz S, Kaluzová M, Opavský R, Pastoreková S, Gibadulinová A, Dequiedt F, Kettmann R, Pastorek J. Transcriptional regulation of the MN/CA 9 gene coding for the tumor-associated carbonic anhydrase IX. Identification and characterization of a proximal silencer element. J Biol Chem 1999;274:32588-32595.

Kaluzová M, Pastoreková S, Svastová E, Pastorek J, Stanbridge EJ, Kaluz S. Characterization of the MN/CA 9 promoter proximal region: a role for specificity protein (SP) and activator protein 1 (AP1) factors. Biochem J 2001;359:669-677.

Karhumaa P, Parkkila S, Türeci O, Waheed A, Grubb JH, Shah G, Parkkila A, Kaunisto K, Tapanainen J, Sly WS, Rajaniemi H. Identification of carbonic anhydrase XII as the membrane isozyme expressed in the normal human endometrial epithelium. Mol Hum Reprod 2000;6:68-74.

Karhumaa P, Kaunisto K, Parkkila S, Waheed A, Pastoreková S, Pastorek J, Sly WS, Rajaniemi H. Expression of the transmembrane carbonic anhydrases, CA IX and CA XII, in the human male excurrent ducts. Mol Hum Reprod 2001a;7:611-616.

Karhumaa P, Leinonen J, Parkkila S, Kaunisto K, Tapanainen J, Rajaniemi H. The identification of secreted carbonic anhydrase VI as a constitutive glycoprotein of human and rat milk. Proc Natl Acad Sci U S A 2001b;98:11604-11608.

Kato K. Sequence of a novel carbonic anhydrase-related polypeptide and its exclusive presence in Purkinje cells. FEBS Lett 1990;271:137-40.

Kaunisto K, Parkkila S, Rajaniemi H, Waheed A, Grubb J, Sly WS. Carbonic anhydrase XIV: luminal expression suggests key role in renal acidification. Kidney Int 2002;61:2111-2118.

Kida E, Palminiello S, Golabek AA, Walus M, Wierzba-Bobrowicz T, Rabe A, Albertini G, Wisniewski KE. Carbonic anhydrase II in the developing and adult human brain. J Neuropathol Exp Neurol 2006;65:664-674.

Kim G, Selengut J, Levine RL. Carbonic anhydrase III: the phosphatase activity is extrinsic. Arch Biochem Biophys 2000;377:334-340.

Kim G, Lee TH, Wetzel P, Geers C, Robinson MA, Myers TG, Owens JW, Wehr NB, Eckhaus MW, Gros G, Wynshaw-Boris A, Levine RL. Carbonic anhydrase III is not required in the mouse for normal growth, development, and life span. Mol Cell Biol 2004;24:9942-9947.

Kivelä J, Parkkila S, Parkkila AK, Leinonen J, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. J Physiol 1999;520:315-320.

Kivelä A, Parkkila S, Saarnio J, Karttunen TJ, Kivelä J, Parkkila AK, Waheed A, Sly WS, Grubb JH, Shah G, Türeci O, Rajaniemi H. Expression of a novel transmembrane carbonic anhydrase isozyme XII in normal human gut and colorectal tumors. Am J Pathol 2000a;156:577-584.

Kivelä AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivelä J, Parkkila AK, Pastoreková S, Pastorek J, Waheed A, Sly WS, Rajaniemi H. Expression of transmembrane carbonic anhydrase isoenzymes IX and XII in normal human pancreas and pancreatic tumours. Histochem Cell Biol 2000b;114:197-204.

Kivelä AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Bartosová M, Mucha V, Novak M, Waheed A, Sly WS, Rajaniemi H, Pastoreková S, Pastorek J. Expression of von Hippel-Lindau tumor suppressor and tumor-associated carbonic anhydrases IX and XII in normal and neoplastic colorectal mucosa. World J Gastroenterol 2005;11:2616-2625.

Krueger NX, Saito H. A human transmembrane protein-tyrosine-phosphatase, PTP zeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. Proc Natl Acad Sci U S A 1992;89:7417-7421.

Kummola L, Hämäläinen JM, Kivelä J, Kivelä AJ, Saarnio J, Karttunen T, Parkkila S. Expression of a novel carbonic anhydrase, CA XIII, in normal and neoplastic colorectal mucosa. BMC Cancer 2005;5:41.

Lakkis MM, Bergenhem NC, Tashian RE. Expression of mouse carbonic anhydrase VII in E. coli and demonstration of its CO2 hydrase activity. Biochem Biophys Res Commun 1996;226:268-72.

Lakkis MM, O'Shea KS, Tashian RE. Differential expression of the carbonic anhydrase genes for CA VII (Car7) and CA-RP VIII (Car8) in mouse brain. J Histochem Cytochem 1997;45:657-62.

Lehtonen J, Shen B, Vihinen M, Casini A, Scozzafava A, Supuran CT, Parkkila AK, Saarnio J, Kivelä AJ, Waheed A, Sly WS, Parkkila S. Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. J Biol Chem. 2004a;279:2719-2727.

Lehtonen JM, Parkkila S, Vullo D, Casini A, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors. Inhibition of cytosolic isozyme XIII with aromatic and heterocyclic sulfonamides: a novel target for the drug design. Bioorg Med Chem Lett 2004b;14:3757-3762.

Leppilampi M, Saarnio J, Karttunen TJ, Kivelä J, Pastoreková S, Pastorek J, Waheed A, Sly WS, Parkkila S. Carbonic anhydrase isozymes IX and XII in gastric tumors. World J Gastroenterol 2003;9:1398-1403.

Levy JB, Canoll PD, Silvennoinen O, Barnea G, Morse B, Honegger AM, Huang JT, Cannizzaro LA, Park SH, Druck T, et al. The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system. J Biol Chem 1993;268:10573-10581.

Liao SY, Brewer C, Závada J, Pastorek J, Pastoreková S, Manetta A, Berman ML, DiSaia PJ, Stanbridge EJ. Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Am J Pathol 1994;145:598-609.

Liao SY, Aurelio ON, Jan K, Závada J, Stanbridge EJ. Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res 1997;57:2827-2831.

Lindskog S, Silverman DN. The catalytic mechanism of mammalian carbonic anhydrases. In: Chegwidden WR, Carter ND and Edwards YH (eds), The Carbonic Anhydrases: New Horizons, Birkhäuser Verlag, Basel, 2000, pp. 175-195.

Lovejoy DA, Hewett-Emmett D, Porter CA, Cepoi D, Sheffield A, Vale WW, Tashian RE. Evolutionarily conserved, "acatalytic" carbonic anhydrase-related protein XI contains a sequence motif present in the neuropeptide sauvagine: the human CA-RP XI gene (CA11) is embedded between the secretor gene cluster and the DBP gene at 19q13.3. Genomics 1998;54:484-493.

Montgomery JC, Venta PJ, Eddy RL, Fukushima YS, Shows TB, Tashian RE. Characterization of the human gene for a newly discovered carbonic anhydrase, CA VII, and its localization to chromosome 16. Genomics 1991;11:835-848.

Mori K, Ogawa Y, Ebihara K, Tamura N, Tashiro K, Kuwahara T, Mukoyama M, Sugawara A, Ozaki S, Tanaka I, Nakao K. Isolation and characterization of CA XIV, a novel membrane-bound carbonic anhydrase from mouse kidney. J Biol Chem 1999;274:15701-15705.

Nagao Y, Platero JS, Waheed A, Sly WS. Human mitochondrial carbonic anhydrase: cDNA cloning, expression, subcellular localization, and mapping to chromosome 16. Proc Natl Acad Sci U S A 1993;90:7623-7627.

Niemelä AM, Hynninen P, Mecklin JP, Kuopio T, Kokko A, Aaltonen L, Parkkila AK, Pastoreková S, Pastorek J, Waheed A, Sly WS, Orntoft TF, Kruhøffer M, Haapasalo H, Parkkila S, Kivelä AJ. Carbonic anhydrase IX is highly expressed in hereditary nonpolyposis colorectal cancer. Cancer Epidemiol Biomarkers Prev 2007;16:1760-1766.

Nishimori I, Innocenti A, Vullo D, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors: the inhibition profiles of the human mitochondrial isoforms VA and VB with anions are very different. Bioorg Med Chem 2007;15:6742-6747.

Nógrádi A, Kelly C, Carter ND. Localization of acetazolamide-resistant carbonic anhydrase III in human and rat choroid plexus by immunocytochemistry and in situ hybridisation. Neurosci Lett 1993;151:162-165.

Opavský R, Pastoreková S, Zelník V, Gibadulinová A, Stanbridge EJ, Závada J, Kettmann R, Pastorek J. Human MN/CA9 gene, a novel member of the carbonic anhydrase family: structure and exon to protein domain relationships. Genomics 1996;33:480-487.

Ortova Gut M, Parkkila S, Vernerová Z, Rohde E, Závada J, Höcker M, Pastorek J, Karttunen T, Gibadulinová A, Závadová Z, Knobeloch KP, Wiedenmann B, Svoboda J, Horak I, Pastoreková S. Gastric hyperplasia in mice with targeted disruption of the carbonic anhydrase gene Car9. Gastroenterology 2002;123:1889-1903.

Parkkila S, Parkkila AK, Juvonen T, Waheed A, Sly WS, Saarnio J, Kaunisto K, Kellokumpu S, Rajaniemi H. Membrane-bound carbonic anhydrase IV is expressed in the luminal plasma membrane of the human gallbladder epithelium. Hepatology 1996;24:1104-1108.

Parkkila S, Parkkila AK, Lehtola J, Reinilä A, Södervik HJ, Rannisto M, Rajaniemi H. Salivary carbonic anhydrase protects gastroesophageal mucosa from acid injury. Dig Dis Sci 1997;42:1013-1019.

Parkkila S, Parkkila AK, Rajaniemi H, Shah GN, Grubb JH, Waheed A, Sly WS. Expression of membrane-associated carbonic anhydrase XIV on neurons and axons in mouse and human brain. Proc Natl Acad Sci U S A 2001;98:1918-1923.

Parkkila S, Kivelä AJ, Kaunisto K, Parkkila AK, Hakkola J, Rajaniemi H, Waheed A, Sly WS. The plasma membrane carbonic anhydrase in murine hepatocytes identified as isozyme XIV. BMC Gastroenterol 2002;2:13.

Pastorek J, Pastoreková S, Callebaut I, Mornon JP, Zelník V, Opavský R, Zat'ovicová M, Liao S, Portetelle D, Stanbridge EJ, et al. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. Oncogene 1994;9:2877-2888.

Pastoreková S, Závadová Z, Kostál M, Babusíková O, Závada J. A novel quasi-viral agent, MaTu, is a two-component system. Virology 1992;187:620-626.

Pastoreková S, Parkkila S, Parkkila AK, Opavský R, Zelník V, Saarnio J, Pastorek J. Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts. Gastroenterology 1997;112:398-408.

Proescholdt MA, Mayer C, Kubitza M, Schubert T, Liao SY, Stanbridge EJ, Ivanov S, Oldfield EH, Brawanski A, Merrill MJ. Expression of hypoxia-inducible carbonic anhydrases in brain tumors. Neuro Oncol 2005;7:465-475.

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome 1997;8:711-713.

Rogers DC, Jones DN, Nelson PR, Jones CM, Quilter CA, Robinson TL, Hagan JJ. Use of SHIRPA and discriminant analysis to characterise marked differences in the behavioural phenotype of six inbred mouse strains. Behav Brain Res 1999;105:207-217.

Rogers DC, Peters J, Martin JE, Ball S, Nicholson SJ, Witherden AS, Hafezparast M, Latcham J, Robinson TL, Quilter CA, Fisher EM. SHIRPA, a protocol for behavioral assessment: validation for longitudinal study of neurological dysfunction in mice. Neurosci Lett 2001;306:89-92.

Räisänen SR, Lehenkari P, Tasanen M, Rahkila P, Härkönen PL, Väänänen HK. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. FASEB J 1999;13:513-522.

Saarnio J, Parkkila S, Parkkila AK, Haukipuro K, Pastoreková S, Pastorek J, Kairaluoma MI, Karttunen TJ. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. Am J Pathol 1998a;153:279-285.

Saarnio J, Parkkila S, Parkkila AK, Waheed A, Casey MC, Zhou XY, Pastoreková S, Pastorek J, Karttunen T, Haukipuro K, Kairaluoma MI, Sly WS Immunohistochemistry of carbonic anhydrase

isozyme IX (MN/CA IX) in human gut reveals polarized expression in the epithelial cells with the highest proliferative capacity. J Histochem Cytochem 1998b;46:497-504.

Saarnio J, Parkkila S, Parkkila AK, Pastoreková S, Haukipuro K, Pastorek J, Juvonen T, Karttunen TJ. Transmembrane carbonic anhydrase, MN/CA IX, is a potential biomarker for biliary tumours. J Hepatol 2001;35:643-649.

Sender S, Gros G, Waheed A, Hageman GS, Sly WS. Immunohistochemical localization of carbonic anhydrase IV in capillaries of rat and human skeletal muscle. J Histochem Cytochem 1994;42:1229-1236.

Sender S, Decker B, Fenske CD, Sly WS, Carter ND, Gros G. Localization of carbonic anhydrase IV in rat and human heart muscle. J Histochem Cytochem 1998;46:855-861.

Shah GN, Hewett-Emmett D, Grubb JH, Migas MC, Fleming RE, Waheed A, Sly WS. Mitochondrial carbonic anhydrase CA VB: differences in tissue distribution and pattern of evolution from those of CA VA suggest distinct physiological roles. Proc Natl Acad Sci U S A 2000;97:1677-1682.

Shah GN, Ulmasov B, Waheed A, Becker T, Makani S, Svichar N, Chesler M, Sly WS. Carbonic anhydrase IV and XIV knockout mice: roles of the respective carbonic anhydrases in buffering the extracellular space in brain. Proc Natl Acad Sci U S A 2005;102:16771-16776.

Skaggs LA, Bergenhem NC, Venta PJ, Tashian RE. The deduced amino acid sequence of human carbonic anhydrase-related protein (CARP) is 98% identical to the mouse homologue. Gene 1993;126:291-292.

Sly WS, Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem 1995;64:375-401.

Sok J, Wang XZ, Batchvarova N, Kuroda M, Harding H, Ron D. CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI. Mol Cell Biol 1999;19:495-504.

Sowden J, Edwards M, Morrison K, Butterworth PH, Edwards YH. Erythroid expression and DNAaseI-hypersensitive sites of the carbonic anhydrase 1 gene. Biochem J 1992;288:545-551.

Supuran CT, Scozzafava A and Casini A. Carbonic anhydrase inhibitors. Med Res Rev 2003;23:146-189.

Svastová E, Zilka N, Zat'ovicová M, Gibadulinová A, Ciampor F, Pastorek J, Pastoreková S. Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with beta-catenin. Exp Cell Res 2003;290:332-345.

Taniuchi K, Nishimori I, Takeuchi T, Fujikawa-Adachi K, Ohtsuki Y, Onishi S. Developmental expression of carbonic anhydrase-related proteins VIII, X, and XI in the human brain. Neuroscience 2002;112:93-99.

Tashian RE, Hewett-Emmett D, Carter N, Bergenhem NC. Carbonic anhydrase (CA)-related proteins (CA-RPs), and transmembrane proteins with CA or CA-RP domains. EXS 2000;90:105-120.

Thatcher BJ, Doherty AE, Orvisky E, Martin BM, Henkin RI. Gustin from human parotid saliva is carbonic anhydrase VI. Biochem Biophys Res Commun 1998;250:635-641.

Turner JR, Odze RD, Crum CP, Resnick MB. MN antigen expression in normal, preneoplastic, and neoplastic esophagus: a clinicopathological study of a new cancer-associated biomarker. Hum Pathol 1997;28:740-744.

Türeci O, Sahin U, Vollmar E, Siemer S, Göttert E, Seitz G, Parkkila AK, Shah GN, Grubb JH, Pfreundschuh M, Sly WS. Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. Proc Natl Acad Sci U S A 1998;95:7608-7613.

Vermylen P, Roufosse C, Burny A, Verhest A, Bosschaerts T, Pastoreková S, Ninane V, Sculier JP. Carbonic anhydrase IX antigen differentiates between preneoplastic malignant lesions in non-small cell lung carcinoma. Eur Respir J 1999;14:806-811.

Whittington DA, Grubb JH, Waheed A, Shah GN, Sly WS, Christianson DW. Expression, assay, and structure of the extracellular domain of murine carbonic anhydrase XIV: implications for selective inhibition of membrane-associated isozymes. J Biol Chem 2004;279:7223-7228.

Wingo T, Tu C, Laipis PJ, Silverman DN. The catalytic properties of human carbonic anhydrase IX. Biochem Biophys Res Commun 2001;288:666-669.

Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 2000;60:7075-7083.

Závada J, Závadová Z, Pastorek J, Biesová Z, Jezek J, Velek J. Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion. Br J Cancer 2000;82:1808-1813.

Závada J, Závadová Z, Zat'ovicová M, Hyrsl L, Kawaciuk I. Soluble form of carbonic anhydrase IX (CA IX) in the serum and urine of renal carcinoma patients. Br J Cancer 2003;89:1067-1071.