Evaluation of the effects of inhaled nanoparticles on the central nervous system of mice

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„Ich liebe es, wenn ein Plan funktioniert!“
Colonel John “Hannibal” Smith (Das A-Team)

Meinen Eltern

Specific parts of this Thesis are derived from the following papers:

- Van Berlo D, Hullmann M, Schins RPF. Toxicology of ambient particulate matter. Experientia Supplementum Series *EXS* 2012; 101:165-217
- M. Hullmann, D. van Berlo, A. Wessels, F.R. Cassee, M.E. Gerlofs-Nijland, S. Weggen, C. Albrecht and R.P.F. Schins. Effects of short-term inhalation of carbon nanoparticles on brains and lungs of wildtype and p47phox$^{-/-}$ mice. Submitted

Abbreviations

°C	degrees Celsius
µl	microliter
8OHdG	8-hydroxydeoxyguanosine
A	alveolar
ABC	avidin-biotin-complex
AD	Alzheimer's Disease
APE/Ref-1	apurinic/apyrimidinic endonuclease/redox effector factor-1
APP	amyloid precursor protein
Aß	amyloid beta
BBB	blood-brain-barrier
BCA Assay	bicinchoninic acid assay
Bp	base pare
BSA	bovine serum albumin
CAPs	concentrated ambient particles
cDNA	complementary deoxyribonucleic acid
CGD	chronic granulomatous disease
cm^3	square centimetres
CNP	carbon nanoparticle
CNS	central nervous system
CO	carbon monoxide
COX-2	cyclooxygenase
CPC	condensation particle counter
Ct	cycle threshold
DAB	diaminobenzidine
DEE	diesel engine exhaust
DEP	diesel exhaust particles
DNA	deoxyribonucleic acid
DUOX	dual oxidase
ELISA	enzyme-linked-immunosorbent assay
EPA	environmental protection agency
EtOH	Ethanol

FAD	familiar Alzheimer disease
FCS	fetal calf serum
g	Gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GSD	geometric standard deviation
GTPase	guanosine triphosphate hydrolase
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HD	Huntington's disease
HE	haematoxylin-eosin
HEPA	High-Efficiency Particulate Air
HO	heme oxygenase
HPRT	hypoxanthine phosphoribosyltransferase
HRP	horse reddish peroxidise
IL-1β	interleukin-1β
KCl	potassium chloride
KH2PO4	Potassium phosphate
KO	Knockout
L	Liter
LTP	long term potentiation
M	Molar
m^3	cubic metre
MCI	mild cognitive impairments
mg	Milligrams
min	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	messenger ribonucleic acid
Na2PO4	sodium phosphate
NAAQS	National Ambient Air Quality Standard
NaCl	sodium chloride

NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NFT	neurofibrillary tangles
nm	Nanometer
NO	nitric oxide
NO_2	nitric dioxide
NO_x	nitrogen oxide
NOX	NADPH oxidase
NSAM	Nanoparticle Surface Area Monitor
NVI	national Vaccine Institute
OD	optical density
Ogg1	7,8- dihydro-8-oxoguanine-DNA glycosylase
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PCR	polymerase-chain-reaction
PD	Parkinson's disease
pg	Pictogram
Rcf	relative centrifugal force
RIVM	National Institute for Public Health and the Environment
RNA	ribonucleic acid
ROS	reactive oxygen species
sec	Seconds
SEM	standard error of mean
SN	substantia nigra
SPF	specific pathogen-free
TB	Tracheobronchial
TBE	Tris/Borate/EDTA
TNF	tumor necrosis factor
tris	tris(hydroxymethyl)aminomethane
TSP	total suspend particles
UFP	ultrafine particles
UV	Ultraviolet
V	Volt
VOC	volatile organic compounds

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1. Introduction

1.1. Aging - a biological companion of time

Life expectancy of mankind is continuously rising and people are getting older and older. Consequently, this results in an increasing number of people living in the developed countries that suffer from age-related diseases, including neurodegenerative diseases such as Alzheimer's disease (AD) (Podtelezhnikov et al., 2011), cardiovascular diseases (Dai et al., 2012), osteoporosis (Casuccio, 1962) and most types of cancer (Caruso et al., 2004). The central question for scientists working in the field of aging is: how and why does aging occur? However, this question cannot be answered very easy, as aging is a very multifaceted process and many different theories of the biology of aging are already proposed during the last centuries. In the 1990s, more than 300 different theories of aging have been suggested (Medvedev, 1990; Vina et al., 2007). In many cases, the authors try to explain the whole process of aging with one single theory even though it has become apparent that aging is the result of multiple sources and events (Holliday, 1997). The four main characteristics of aging include its progressive nature, endogenous origin, irreversible character and deleterious effects for the organism (Sanz et al., 2006).

Aging can be seen as the accumulation of changes in an individual over time (Bowen and Atwood, 2004). Indeed, aging may lead to changes in molecular genetics, e.g. protein modifications (Orgel, 1963), accumulations of DNA damage over time (Lindahl, 1993), telomere shortening (Goyns, 2002) or somatic mutations (Vijg, 2000). Additionally, aging may induce biochemical changes (Cote and Kremzner, 1983) of which the telomere theory and the oxidative stress theory are the most popular.

<u>The telomere theory:</u>

Telomeres consist of non-coding, tandem DNA repeats and telomere binding proteins (de Lange et al., 1990). They are known to form the end structures of human chromosomes (Blackburn, 1991) where their main function is to maintain chromosomal stability. In general, telomeres shorten with each single cell division due to the insufficient nature of DNA polymerase during the replication S-phase

(Morin, 1989). After continuous repetitions of cell division, telomeres are shorten too often and consequently they will lose their capping ability which could lead to replicative senescence or apoptosis (Shay and Wright, 2004). Consequently, their length has been proposed as kind of biological counter, measuring the replications and thus life span of a cell (Shay and Wright, 2007). Different data demonstrate that telomere shortening in different tissues during human aging correlates with diverse diseases often found in the elderly people, including Alzheimer's disease and arteriosclerosis (Frenck et al., 1998; Panossian et al., 2003; Samani et al., 2001).

The oxidative stress theory:

In 1956, Denham Harman formulated the oxidative stress theory of aging (Harman, 1956): free radicals derived from oxygen are responsible for damage in cell systems associated with aging (Vina et al., 2007). Reactive oxygen species (ROS) include the oxygen centered radicals ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\cdot}$), as well as non-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen; reactive nitrogen species include nitric oxide (NO) and peroxynitrite ($ONOO^{\cdot}$) (Fruehauf and Meyskens, 2007). Free radicals are chemical species containing one or more unpaired electrons and are normally produced within the cell by various enzymatic systems such as lipoxygenase, cyclooxygenase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Fruehauf and Meyskens, 2007). Depending on the cell type, further dominant producers are the cytochrome P450 and the mitochondria (Inoue et al., 2003). Due to their unpaired electrons, ROS are very reactive and can cause damage to important endogenous macromolecules such as DNA, RNA, proteins and lipids.

In normal conditions, the organism contains an elaborate network of so-called antioxidants, i.e. compounds that are able to neutralize ROS before they can cause damage to important endogenous macro molecules. If the antioxidant system, for some reason, is unable to counterbalance the presence of highly reactive molecules (including ROS) within the cell, an imbalance of pro- and antioxidants in favour of the first will be the consequence. This state is defined as oxidative stress (Sies, 1991). A result of oxidative stress is the accumulation of oxidative damage which may lead to the development of diseases due to e.g. ROS-mediated damage and mutations in mitochondrial DNA (Singh, 2006).

Besides causing damage due to their reactivity, ROS also play an important role in cell signalling, phagocytic function, inflammation, and apoptosis (Johnson et al., 1996; Kamata and Hirata, 1999; Ueda et al., 2002). Moreover, free radical damage within cells has also been linked to a broad battery of disorders including different types of cancer (Harris, 2002; Senthil et al., 2004; Senthil and Manoharan, 2004), diabetes (Victor et al., 2011), Alzheimer's disease and Parkinson's disease (Giasson et al., 2002). In association to the theories of aging, it was shown that mitochondria from older animals produce more ROS compared to those from younger ones (Sohal et al., 1990). Recent studies support this theory by showing the impact of higher or lower oxidative stress levels in different organisms (Muller et al., 2007). Also positive effects on health and lifespan of humans due to consumption of a diet high in antioxidants have been reported (Harman, 2006; Wright et al., 2006).

1.1.1. The aging brain

Aging is a major risk factor for most common neurodegenerative diseases including Alzheimer's disease, Huntington's disease and Parkinson's disease. As described in the previous paragraph, over time numerous biological changes take place in the body which spares no organ or system. Therefore the brain is no exception to this phenomenon. Indeed, aging results in structural, molecular and genetic changes within the brain as well as a loss of plasticity.

In 1955, Brody was the first to suggest that a decrease in brain weight during aging is indicative for the loss of neurons due to neuronal cell death in all cortical layers (Brody, 1955). At present, there are also studies indicating that age-induced cognitive deficits may not be due to neuronal loss or cell death in the brain after all. In fact, these deficits might be the result of small region-specific changes to the morphology of neurons (Burke and Barnes, 2006). Indeed, it has been shown that little or even no neuronal cell death develops during brain-aging (Gomez-Isla et al., 1997; Haug et al., 1984). Instead, morphological changes including progressive loss of grey matter density (Resnick et al., 2003), changes in thickness of the cortical zone (McGinnis et al., 2011), disruption of myelination and increasing reactive gliosis occur. These morphological changes may reflect dendritic shrinkage, synaptic loss (Morrison and Hof, 1997; Yankner et al., 2008) and glial dystrophy (Conde and Streit, 2006). As the brain is a very complex structure composed of many different compartments, tissues and cell types, it can be anticipated that different parts of the

brain are affected in a different manner during the aging process. Indeed, it could be shown that volume reduction in different brain regions is not uniform as some brain regions shrink up to 1 % per year whereas other parts of the brain do not show any changes in volume during the whole life span (Raz and Rodrigue, 2006).

In addition to structural, morphological and genetic changes in the aging brain, the aging process is also involved in a broad battery of molecular changes. Brain-derived neurotrophic factors (BNDF) (Erraji-Benchekroun et al., 2005) and neurotransmitters including serotonin (Wong et al., 1984), glutamate (Chang et al., 2009) and dopamine (Kaasinen et al., 2000) are shown to play an essential role in normal brain aging (Backman et al., 2006; Mattson, 2008).

The study of Rex *et al* in 2006 underlined the biological relevance of BDNF in the aging brain. Treating middle-aged rats with BNDF via infusion could restore age-related impairments in long term potentiation (LTP) (Rex et al., 2006), a phenomenon that is generally believed to be one of the main cellular mechanisms underlying memory and learning (Bliss and Collingridge, 1993).

In post-mortem analyses of aged human brains, serotonin was shown to have a diminished binding capacity to its receptors. Also the number of human brain serotonin (S-1) binding sites in the frontal cortex and hippocampus was decreased (Marcusson et al., 1984). Moreover, the neurotransmitter glutamate levels are affected with age as for example older humans display lower glutamate concentrations in regions of the cerebral cortex compared to younger subjects (Kaiser et al., 2005).

“Normal brain aging” can be described as aging in the absence of age-related clinically-diagnosed neurodegenerative diseases and without the detection of disease related pathologies (Glorioso and Sibille, 2011). However, molecular changes occurring during normal brain aging often overlap with those detected in neurodegenerative diseases. Consequently, age-related neuropathology found in various diseases such as Alzheimer’s disease, Parkinson's disease and arteriosclerosis make it difficult to recognize the occurrence of normal patterns of aging (Hedden and Gabrieli, 2004).

1.2. The NADPH oxidase and ROS formation: a double-edged sword

ROS are often pictured to be “the bad guys” within a cell as their production may lead to oxidative stress, which was shown to be a major contributor to different diseases in general and aging in the central nervous system (CNS) in particular. However, next to the induction of oxidative damage and inflammation, ROS formation also induces positive effects such as the generation of a host defence (Rada and Leto, 2008) and neuronal differentiation during development (Tsatmali et al., 2006). For a long time, mitochondria were thought to be the main source of ROS production in the CNS. However, growing evidence proposes the NADPH oxidase (NOX) enzymes as a main contributor to ROS production.

The NADPH oxidase enzyme family consists of 7 members: NOX1 to 5 and DUOX1 and 2. Among the various members of the NADPH oxidase family, NOX2 is the best investigated. Apart from its abundant expression in phagocytic cells, it is present in endothelial cells, neurons, astrocytes and microglia (Quinn et al., 2006). This ROS-generating NOX enzyme is a multi-subunit enzyme that catalyses the reduction of molecular oxygen and oxidation of NADPH to generate superoxide anion radicals ($O_2^{\bullet-}$) (Babior, 2004). NOX2 consists of two transmembrane proteins ($p22^{phox}$ and $gp91^{phox}$), forming the heterodynamic cytochrome b_{558}, and three cytosolic proteins ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) (Lambeth, 2004). Additionally, a GTPase (Rac1 and Rac2) is needed to form a functional, membrane–bound enzyme. Activation by a trigger will lead to the stimulation of the G protein and a phosphorylation of the $p47^{phox}$ subunit (Bedard and Krause, 2007; Clark et al., 1990). Next, the cytosolic elements $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ will translocate to the plasma membrane, bind to the cytochrome b_{558} complex, and form a functional complex that generates $O_2^{\bullet-}$. A schematic illustration of the NADPH oxidase activation is shown in Figure 1.

All these subunits are essential for the functioning of the enzyme and the absence of any of these proteins results in a dysfunctional NADPH oxidase (Quinn et al., 2006). Phagocytic cells lacking a functional NADPH oxidase are unable to eliminate pathogens and show impaired apoptosis and turnover. This can lead to severe recurrent bacterial and fungal infections and development of granuloma, i.e. a clustering of inflammatory cells including various phagocytic cells (Quinn et al., 2006).

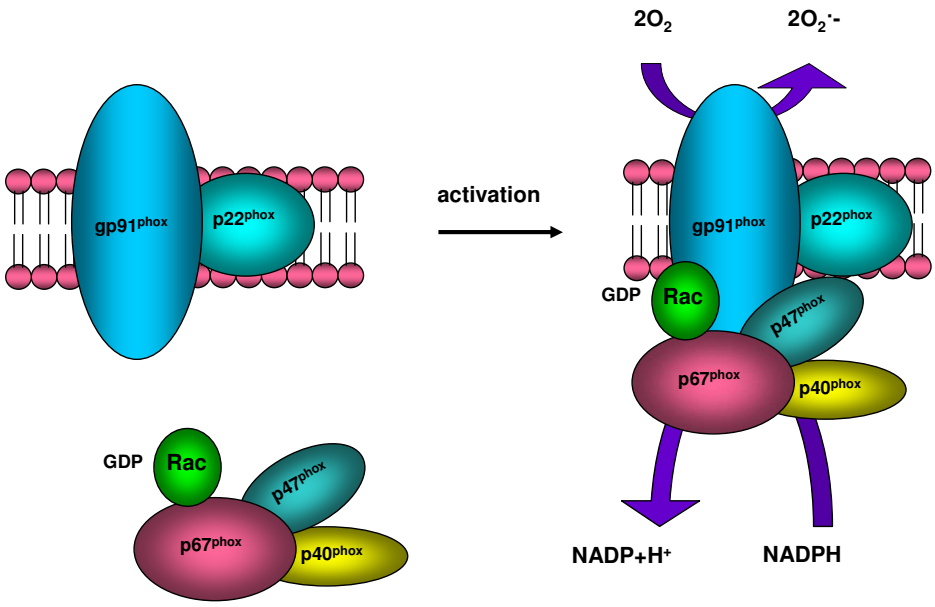

Figure 1: NADPH oxidase complex

After activation of the NAHDH oxidase it forms an active complex. This active complex then catalysed NADPH and oxygen which results in the production of superoxide.

Phagocytic cells like macrophages, neutrophils and monocytes are specialised in producing ROS to eliminate pathogens or foreign particles after activation of the NADPH enzyme complex. $O_2^{\cdot -}$ acts as a precursor for synthesis of numerous bactericidal compounds (Shatwell and Segal, 1996). The catalysis of superoxide from oxygen and NADPH occurs via the following reaction (Ago et al., 1999):

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$$

The important role of $O_2^{\cdot -}$ in eliminating microorganisms and pathogens becomes clear from the massive consequences that its absence has on individuals suffering from chronic granulomatous disease (CGD). The neutrophils of patients suffering from CGD are deficient in a functional $p47^{phox}$ subunit, resulting in an inoperable NADPH oxidase. Consequently, these patients lack a good phagocytic defence against intruders and thus suffer from strong bacterial and fungal infections (Shatwell and Segal, 1996).

1.3. Free radicals and the brain

The fact that NOX enzymes play an essential role in the CNS is becoming increasingly clear by the observations that different neurodegenerative diseases arise from a general increase in free radicals, in particular parts of the brain (Butterfield and Kanski, 2001). NADPH oxidase expression is up regulated in a vulnerable brain region of patients with mild cognitive impairments, suggesting NOX to play a role in the early pathogenesis of AD (Bruce-Keller et al., 2010). Additionally NOX activity is increased in the so-called amyloid beta (Aβ) protein plaques which are a typical hallmark in patients with AD (Bennett et al., 2009). Oxidative stress is also possibly involved in Aβ -mediated cell death in AD patients (Bruce-Keller et al., 2010). Also in microglia-mediated amyloid neurotoxicity, NOX seems to play a fundamental role (Qin et al., 2004). Microglia are a type of glial cells that are ubiquitously distributed in large non-overlapping regions throughout the brain (Kreutzberg, 1995). They function as resident macrophages of the brain and, as such, they can act as scavengers to protect the CNS from neuronal damage, infectious agents and the formation of protein plaques (Gehrmann et al., 1995). The fact that microglia are found around amyloid plaque formations in the brains of AD patients also suggests a possible role in different CNS diseases, but if this is a beneficial or harmful one still has to be elucidated (Choi et al., 2012).

Normal neuronal function depends on an accurate modulated redox ambience and can be swayed by a lack as well as by an overproduction of reactive species.

1.4. Neurodegenerative diseases

Everyone ages, but not all persons will develop a neurodegenerative disease during aging. Neurodegeneration is an umbrella term for a battery of morphological and structural changes in the brain or spinal cord that are distinctly different from the changes developing during “normal brain aging”. Neurodegenerative diseases are characterized by progressive loss and death of cells affecting specific neural systems. Core features and hallmarks of neurodegenerative diseases include misfolding and aggregation of proteins resulting in accumulation of atypical extra/ and intracellular filamentous deposition in specific cell types (Rubinsztein, 2006) and cell death in the nervous system (Bredesen et al., 2006). An important aspect of these hallmarks is the factor of time: a sufficient protein accumulation will occur first,

followed by a cascade of symptoms developing over years which will inevitably lead to increasing disability and, at worst, to death (Mattson and Magnus, 2006). Neuronal cell death is induced by necrosis or apoptosis (Kanduc et al., 2002; Yuan and Yankner, 2000). There are many different triggers of neuronal apoptosis including overactivation of glutamate receptors, DNA damage, accumulation of damaged proteins and oxidative stress (Mattson, 2008; Mattson and Magnus, 2006). Moreover, oxidative stress also represents a central feature of all neurodegenerative disorders and might also be induced by genetic mutations (Thompson, 2008) or mitochondrial disorders (DiMauro and Schon, 2008).

1.4.1. Neurodegenerative diseases and oxidative stress

NADPH oxidases, including NOX2, play an important role in the central nervous system (Infanger et al., 2006). The regulation and expression structure of these enzymes within the brain propose the superoxide to manage different functions in the brain (Serrano et al., 2003) as it is expressed in neurons (Vallet et al., 2005) and localized in synapses (Tejada-Simon et al., 2005).

Numerous of the neurodegenerative diseases of the CNS occur from augmented ROS production and/or a reduced level of antioxidants available in the cell to detoxify the free radicals. Uncontrolled ROS overproduction may result in chronic oxidative stress which has been associated with neuronal cell death, protein aggregation, and the pathogenesis of different neurodegenerative diseases and Aβ formation (Leuner et al., 2012). On the other hand, an inappropriate ROS production due to a dysfunctional NADPH oxidase may lead to synaptic plasticity deficits and mild memory impairments as has been shown using a mouse model lacking the transmembrane protein $gp91^{phox}$ or the cytosolic element $p47^{phox}$ (Kishida et al., 2006).

ROS formation can lead to significant biological changes and is discussed as a key mechanism in most common forms of neurodegenerative diseases like Parkinson's disease (PD), Huntington's disease (HD) and Alzheimer's disease (AD).
PD is a genetically complex and heterogeneous disorder which occurs with different symptoms like rigidity, slowness of movement, postural instability and tremor, its most well known indicator (Jankovic, 2008). The main causes of PD development are loss of dopaminergic neurons in the substantia nigra (SN), a region of the midbrain, and the accumulation of intraneuronal inclusions, consisting of the insoluble protein

alpha-synuclein, into so-called Lewy bodies within neurons (Jankovic, 2008). The suggestion that oxidative stress plays a major role in developing PD rises from the observation that mitochondrial dysfunction and oxidative damage to lipids, proteins and nucleic acids occur in PD brains. Moreover, oxidative damage has also been shown in the peripheral tissues of PD patients (Mancuso et al., 2006). The accumulation of iron, in combination with the protein inclusions in the SN, is also a characteristic hallmark of PD that may be related to the development of oxidative stress due to Fenton-like reactions (Hirsch, 2009; Sayre et al., 2005).

Huntington's disease is a progressive neurodegenerative disorder caused by a CAG repeat expansion within exon 1 of the gene encoding for the huntingtin-protein (Gardian and Vecsei, 2004). Early onset of the disease can be described with changes in personality, problems with muscle coordination, cognitive decline and physical skills (Walker, 2007). Although CAG expansions in the huntingtin-gene and errors in DNA replication are thought to be one of the main reason for this neurodegenerative disorder (Sinden, 2001), oxidative stress also seems to play an important role in the pathogenesis of HD. For example, the oxidative DNA lesion 8-hydroxydeoxyguanosine (8OHdG), which is also a sensitive indicator of oxidative stress, impaired metabolism and mitochondrial dysfunction, is increased in HD patients (Long et al., 2012). Increased 8OHdG can normally be found in DNA following oxidative attack (Kasai and Nishimura, 1984), whereas such oxidized base lesions can be removed by the enzyme 7,8- dihydro-8-oxoguanine-DNA glycosylase (OGG1) Interestingly, increased levels of OGG1 could be found in mitochondrial DNA from HD patients, suggesting upregulation of this enzyme as a result of increased oxidative damage (Polidori et al., 1999). As 8OHdG levels are increased in HD patients it might represent a marker for HD progression (Long et al., 2012).

For both PD and HD, it can be concluded that a normal CNS function depends on a concerted oxidant setting within the brain.

As a main part of my thesis will focus on Alzheimer's disease, this neurodegenerative disorder will be introduced in more detail in the next paragraph.

1.5. Alzheimer's disease

In 1906 the German psychiatrist and neuropathologist Dr. Alois Alzheimer was the first describing symptoms of one of the most common forms of dementia, later

named after him, i.e. Alzheimer's disease (Berchtold and Cotman, 1998). In 2006, 26.6 million people worldwide were affected by this neurodegenerative disease. In 2050, 1 in 85 persons is expected to suffer from this most disabling variant of neurodegenerative disorders (Brookmeyer et al., 2007). Additionally, 50 percent of those aged 85 or older will suffer from this neurological disorder (Suh and Checler, 2002), pointing out the growing socioeconomic and medical problems associated with AD.

In most cases, AD develops sporadic with increasing age and genetic risk factors are trivial or vague. Only very small numbers of patients suffer from the non-sporadic, familial form of AD (approximately 0.1% of total AD patients) (Harvey et al., 2003) which has an onset age below 65 years (Blennow et al., 2006). This form of AD, also known as the familial Alzheimer's disease (FAD), develops due to a mutation in one of at least 3 genes, namely presenilin 1, presenilin 2 and amyloid precursor protein (APP) (Ertekin-Taner, 2007). Until now, most of the animal models used to study AD are based on these mutations. Examples of such mouse models which are used in Alzheimer research are listed in Table 1.

Both forms of AD, i.e. sporadic AD as well as FAD, are characterized by behavioural changes as well as cognitive and memory impairments that result from brain region-specific neuronal loss (Querfurth and LaFerla, 2010). They are histopathologically indistinguishable. Behavioural and cognitive test in rodents, as developed for neuropharmacological and neurotoxicological research, are often included in AD mouse models (see Table 1).

Although the mechanisms underlying the pathogenesis of AD are still not completely understood, it is well known that AD is characterized by the development of two neuropathological hallmarks: the extracellular senile Aß plaques (Selkoe, 2001) and the intracellular neurofibrillary tangles (NFT) formed by abnormal hyperphosphorylation of a protein named tau (Terry, 1963).

Table 1: Examples of AD mouse models (modified from Elder et al, 2010 (Elder et al., 2010))

Mouse line	Mutation	Pathology Amyloid deposition/ Plaque formation	Other observations	Ref.
PDAPP	V717F (APP-Indiana)	6-9 month	synaptic loss, astrocytosis and microgliosis	(Games et al., 1995)
Tg2576	KM670/671NL (APPswe)	9-12 month	Impairment in learning and memory begin with 9 months of age	(Hsiao et al., 1996)
APP/PS1	APPswe/PS1dE9	3 month	numbers of cortical and hippocampal neurons are not diminished, neuritic abnormalities and reactive astrogliosis are not observed	(Borchelt et al., 1997)
TgCRND8	APPswe/APP-Indiana	3 month	Cognitive impairments, neuronal cytoarchitecture appeared normal	(Chishti et al., 2001)
PS1M146V	PS1M146V	no plaque formation	elevated aß42 level	(Duff et al., 1996)
3xTg	APPswe/PS1M146V/ tauP301L	6 month	LTP was severely impaired, synaptic dysfunction in an age-related manner	(Oddo et al., 2003)
5xFAD	APPswe/Ind/fl and a PS1 transgene carrying double FAD mutations (M146L and L286V)	2 month	Intraneuronal Aβ42-induced neurodegeneration, Reduced synaptic marker protein levels, Memory impairments	(Oakley et al., 2006)

Until now, the most accepted explanation for the pathogenesis of AD is the amyloid cascade hypothesis (Hardy and Higgins, 1992). This hypothesis describes that Aß formation derives from cleavage of the amyloid precursor protein (APP), which can occur via two different pathways. The first adverse cleavage pathway is the so called amyloidogenic pathway. Within this pathway, the first cleavage performed by β-secretase, results in the release of large soluble N-terminal fragments (sAPPβ) into the extracellular/luminal room and a membrane 99 bound

amino acid C-terminal remnants. This cleavage is then followed by a second proteolytic one which occurs in the intramembrane region by γ-secretase (Glenner et al., 1984) (see Figure 2). Due to different cutting sides of APP, Aß cleaving products can differ in length at the C-terminus. The most common isoforms are Aß 1-40 and Aß 1-42 with a total of 40 or 42 amino acids respectively. The tendency to form aggregates due to its hydrophobic properties is more pronounced for the Aß 1-42 isoform compared to the Aß 1-40. Aggregation of monomers of Aß 1-40 or 1-42 may lead to formation of dimers, trimers and finally this will lead to the formation of so called senile Aß plaques. According to the amyloid cascade hypothesis, the deposition of senile plaques is the original pathological beginning of AD. Subsequently, it will lead to the formation of neuronal cell death and dementia.

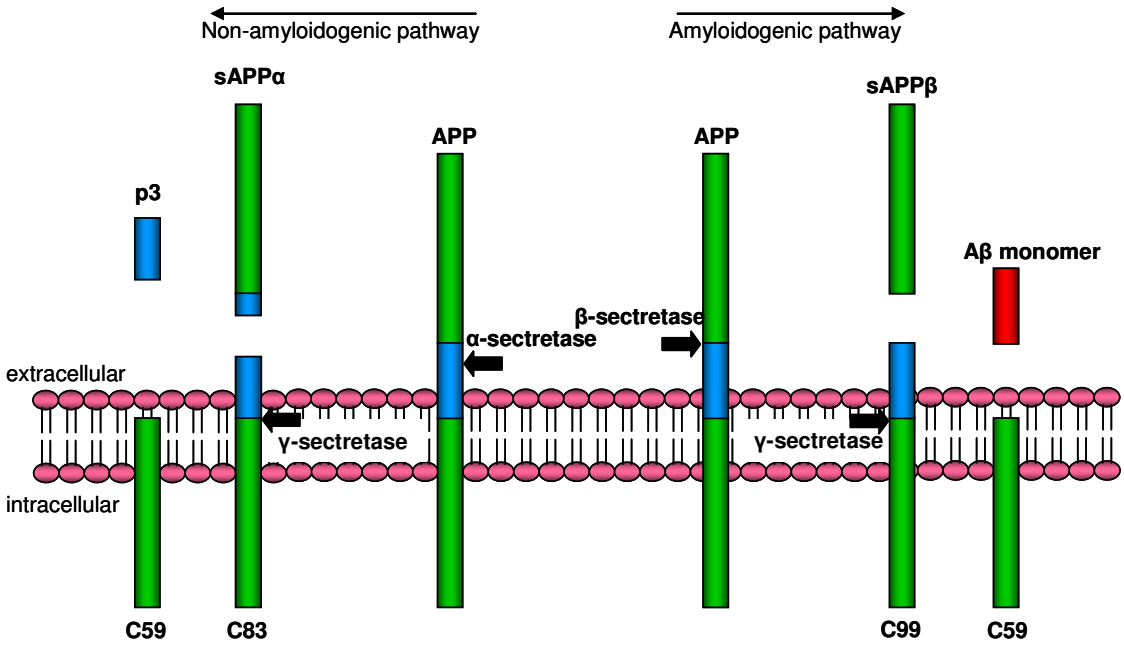

Figure 2: The amyloid cascade hypothesis

APP is cleaved by α-secretase and γ-secretase, resulting in the production of non-plaque forming p3, while β-secretase and γ-secretase produce the initial pathological trigger, the amyloid plaque-forming Aß monomer. (Modified from Cole and Vassar (Cole and Vassar, 2007))

Another possible pathway during APP cleavage is the non-amyloidogenic pathway. Within this pathway, the first cleavage is performed by α-secretase that releases the soluble N-terminal fragments sAPPα into the extracellular/luminal space. Concomitantly, a C-terminal membrane bound fragment of 83 amino acids is released. Additional cleavage by γ-secretase results in a secreted APP ectodomain

called p3, thus preventing Aβ generation. Even though Aβ plaque and NFT formation are considered to be the most prominent candidates to play a key role in AD, there are also other mechanisms postulated to have an impact on AD development. These mechanisms include pro-inflammatory responses (Wyss-Coray, 2006), mitochondrial dysfunction (Reddy, 2011), oxidative stress (Verri et al., 2012) or genetic and environmental factors (Nelson et al., 2011).

Current treatment options for neurodegenerative diseases such as AD only medicate the symptoms of the diseases. Until now, there are no medical healings available that can stop or even reverse the development of the pathological hallmarks of this progressive neurodegenerative disorder.

1.6. Particles in our environment: A key player in aging processes and diseases?

Particulate air pollution comprises a heterogeneous mixture of substances including carbon, metals, nitrates, sulphates and particulate organic matter. Its composition varies according to the predominant source of particles, season, and prevailing weather conditions (Schins et al., 2004; Turnbull and Harrison, 2000). In present-day Western urban environments, a major contributor to PM is engine exhaust released by diesel and petrol vehicles. Exposure to particulate matter (PM) already took place in the early stages of humanity. Forest and grassland fires, dust storms, volcanic activity and sea spray present different natural sources of aerosols which can get in contact with every individual. Already 400.000 years ago, the capability to use fire in a controlled way, increased the exposure to combustion-derived particles due to the application of fire for cave warming and indoor cooking (Bowman et al., 2009). Later in human history, a drastic increase in the concentration of ambient PM was caused by industrialisation. Also the invention and popularisation of the internal combustion engine commonly used to power the automobile lead to an increase in PM.

It is becoming increasingly clear that inhalation exposure to PM can induce or exacerbate various diseases. These diseases are not limited to the lung but extend to the cardiovascular system and possibly other organs and tissues like the brain. (Calderon-Garciduenas et al., 2004; Kunzli et al., 2005; Pope et al., 2002). In a cohort study of elderly women, Ranft and co-workers could show that mild cognitive impairments were consistently found to be affected in relation to traffic exposure. This

finding suggests that long-term exposure to PM might contribute to the development of AD (Ranft et al., 2009). Meanwhile, a number of independent studies in humans provided support for the hypothesis that PM adversely affects the CNS and hence may contribute to age-related diseases such as AD, PD and multiple sclerosis (Calderon-Garciduenas et al., 2008b; Gerlofs-Nijland et al., 2010; Oikonen et al., 2003). Further studies also provided additional clues that the environment appears to have an effect on aging. This is of special importance since our life expectancy will increase continuously, leading to an elongated exposure time to particulate matter, due to highly developed techniques in medicine and advances in medical care that result in successful aging (Rowe and Kahn, 1987). By reason of a reduced physiological capacity and the occurrence of biological changes associated with aging, elderly people get more vulnerable to PM-related damage and this may further increase the risk of developing age-associated diseases.

1.6.1. Particulate matter (PM): chemical and toxicological characteristics

Until several decades ago, PM concentrations in ambient air were typically measured as total suspended particles (TSP) (Lippmann, 2000). In 1987, the US environmental protection agency (EPA) revised the National Ambient Air Quality Standard (NAAQS) for PM, forwarding PM_{10} as a superior index since this fraction is more representative of the particles that can actually be inhaled and enter the human lung (Lippmann, 2000). PM_{10} can be defined as PM with a mean aerodynamic diameter of 10µm. The aerodynamic diameter is defined as the diameter of a spherical particle having a density of 1 g/cm^3 that has the same inertial properties in the gas as the particle of interest. PM_{10} is a complex mixture originating from both natural and anthropogenic sources. It consists of a broad range of particulate components as well as a plethora of substances bound to the core of the particles (Turnbull and Harrison, 2000). PM_{10} can be further divided in several size fractions, based on the aerodynamic diameter resulting from collection using specific particle matter samplers: coarse (2.5-10 µm), fine (0.1-2.5 µm) and ultrafine (< 0.1 µm). The latter size fraction has been referred to by toxicologists as ultrafine particles (UFP). However, on the basis of their size they are also being categorized as nanoparticles as will be discussed in the next paragraph.

Diesel exhaust particles (DEP) form a special group among the various types of particles that are used to address mechanisms of toxicity of PM. They contain

considerable amounts of metals and organics onto their carbonaceous core and can be generated and subsequently sampled in a highly controlled and reproducible manner for toxicology studies. Consequently, while representing a dominant component of PM in urban environments, DEP are typically used as model particles representing UFP or nanoparticles. An overview of the most common experimental approaches used to address PM toxicity is provided in Table 2.

Besides pure particle exposure, a lot of studies have been performed investigating the contribution of diesel engine exhaust to the adverse health effects that are attributed to air pollution. Of course, this is not a “pure” particle exposure, incorporating a large amount of non-particulate compounds such as carbon monoxide (CO), nitric oxides (NO, NO_2), sulphur dioxide (SO_2), hydrocarbons, formaldehyde and transition metals (Scheepers and Bos, 1992). Interactions may occur between solid and gaseous constituents and gaseous components, which can form further particulates. Also, synergistic effects can be elicited, like those reported for PM-bound metals and organics (Saldiva et al., 2002) which are both present in varying amounts in diesel engine exhaust (DEE) as particulates and gases. Therefore, these findings could be of relevance for adverse health effects related to PM exposure. An advantage of using DEE exposure in an experimental setting is that such (animal) studies feature inhalation. Inhalation results in a relatively homogeneous distribution of inhaled particles and is of course closer to the real-life situation than a bolus application of DEP into the lung as it can be achieved by pharyngeal aspiration or intratracheal instillation.

PM varies widely in composition and physicochemical properties. Coarse particles are often derived from natural sources and contain mostly crustal elements, while the fine fraction of PM is typically dominated by particles from anthropogenic sources including combustion processes. Hence, fine PM, and in particularly the ultrafine particle component herein, may contain large amounts of organic carbon compounds such as polycyclic aromatic hydrocarbons (PAH) and quinones (Li et al., 2003). For the investigation of possible mechanisms of PM-induced adverse health effects, many toxicological studies have used model particles representing different components of the complicated multiple entity that is PM. Commercially available particulate materials such as carbon black and titanium dioxide represent a model for the carbon core of combustion-derived particles and poorly soluble (nano)particles in general.

Table 2: Particle types used to investigate PM properties relevant for human health

Particle type	Category	Tested in	Reference
Concentrated ambient particles (CAPs)	Real life (direct/on-site exposure)	Humans volunteers, rats, mice, cell culture	*(Campbell et al., 2009; Harkema et al., 2009; Kleinman et al., 2008; Mills et al., 2008; Stringer et al., 1996)*
PM sampled on filters or in suspensions ("biosamples")	Real life (tested after storage and/or extraction)	Human volunteers, rats, mice, cell culture	*(Barlow et al., 2008; Gilmour et al., 2007; Knaapen et al., 2000; Monn and Becker, 1999; Schaumann et al., 2004),*
Diesel engine exhaust particles (DEP)	Model particle	Rats, mice, cell culture	*(Marano et al., 2002; Nemmar et al., 2009; Rao et al., 2005)*
Residual oil fly ash (ROFA); coal fly ash	Model particle	Rats, mice, cell culture	*(Salnikow et al., 2004; Smith et al., 2006; Steerenberg et al., 2003; Zanchi et al., 2011)*
Carbon black (fine, ultrafine)	Model particle	Rats, mice, cell culture	*(Donaldson et al., 2005a; Stone et al., 2000; Tong et al., 2009; Wilson et al., 2002)*
Titanium dioxide (fine, ultrafine)	Model particle	Rats, mice, cell culture	*(Hussain et al., ; Oberdorster et al., 2000; Singh et al., 2007)*

1.6.2. Nanoparticles (NP): Small in size, but big in character

In recent decades, extensive research has been performed to unravel the mechanisms whereby relatively low levels of particulate air pollution can produce adverse health effects and to identify crucial responsible components within this complex mixture. These studies have lead to the hypothesis that nanoparticles (NP, i.e. particles less than 100nm in three dimensions) play an essential role in inducing or worsening effects associated with ambient particulate matter exposure (Donaldson

et al., 2003; Ibald-Mulli et al., 2002; Oberdorster et al., 2005). Especially combustion derived NP, like diesel engine exhaust particles, are considered to be responsible for many of the adverse health effects triggered by PM; it has been suggested that the particle surface area is a main responsible factor (Donaldson et al., 2005b). The proportion of molecules bound to the surface increases exponentially when particle size decreases below 100 nm in diameter. This mirrors the importance of the surface area in terms of increased biological and chemical activity of NP (see Figure 3).

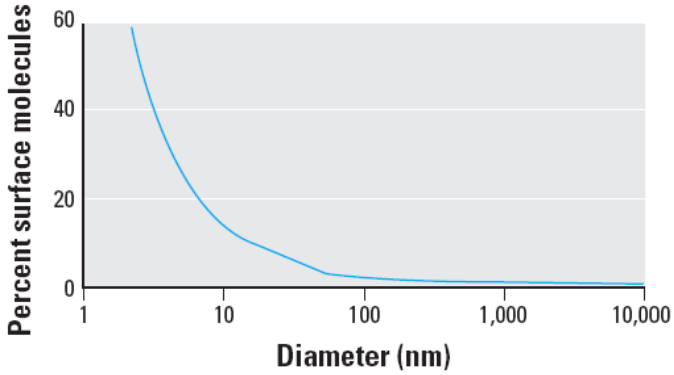

Figure 3: Surface - Volume - Ratio
The relationship of surface molecules and particles size (Taken from Oberdörster (Oberdorster et al., 2005)).

The increased biological activity of nanoparticles compared to their larger size counterparts can lead to positive and attractive effects such as increased antioxidant activity and carrier capacity for therapeutics. It can, however, also display negative and uninvited effects including toxicity, induction of oxidative stress and/or cellular dysfunction. Also a mixture of both effects is feasible (Oberdorster et al., 2005).

Apart from the size of NP and their potential to bind molecules on their surface, the composition of bound molecules also plays a significant role. A contamination of NP due to the binding of organics and metals is believed to be responsible for the enhanced induction of oxidative stress in cell systems. In addition, these organics and metals may lead to the activation of pro-inflammatory mediators (Borm and Muller-Schulte, 2006). If organic matter (like polycyclic aromatic carbohydrates), bound to the surface of NP, is released into biological environments, it may undergo redox cycling or phase I biotransformation reactions. These processes can result in formation of redox-active compounds and trigger oxidative

stress (Knaapen et al., 2004; Unfried et al., 2007). Additionally, if metal ions like iron, nickel or copper are released from the NP surface into biological environments, they can catalyse Fenton-like reactions which are also implicated in increased ROS formation (Knaapen et al., 2004; Schins and Knaapen, 2007).

1.7. Nanoparticle exposure routes: The nose-brain connection

Recent evidence suggests that the CNS represents a critical target of ambient air particles and NP. Early clues for potential targeting of the brain by NP were provided by the groups of Oberdorster and Kreyling who investigated the kinetics of translocation of inhaled NP to various organs in rats (Elder and Oberdorster, 2006; Kreyling et al., 2002; Oberdorster et al., 2002). They were able to demonstrate that carbon NP, generated by spark discharge, accumulate in the CNS upon inhalation. These particles were persistent for at least 7 days post-exposure in different regions of the brain, whereas the particles deposited in the lung were rapidly cleared (Oberdorster et al., 2004). In a follow-up study, rats were exposed to manganese particles whereby occlusion of one nostril resulted in a strong asymmetrical particle deposition in the olfactory bulb (Elder et al., 2006). This provided further support to earlier observations with intranasal instilled gold particles, showing that inhaled NP, upon deposition on the nasal olfactory epithelium, may reach the brain via translocation along the olfactory route (Elder et al., 2006). A second principal mechanism whereby inhaled NP may reach the brain is via successive translocation from the alveoli into the blood over the air-blood barrier and subsequently from the blood into the brain over the blood-brain barrier (BBB) (Elder and Oberdorster, 2006; Oberdorster et al., 2009). This translocation route might even be facilitated by the fact that the BBB can become damaged due to long term air pollution exposure (Calderon-Garciduenas et al., 2008b). Both possible translocation routes are summarised in Figure 4.

It is not yet fully established however, whether particles that translocate to the human brain can induce health effects. Additionally, the relative contribution of the two possible translocation routes (lung or olfactory route) still needs to be clarified. Finally, it is also still unknown whether potential health effects can be attributed to a direct effect of the translocated particles or whether, similarly to the cardiovascular effects of particles (see Figure 4), indirect effects driven by pulmonary inflammation

may be responsible. Indeed, systemic inflammation alone has already been known to elicit adverse effects in the brain (Qin et al., 2007; Rivest, 2001).

In conclusion, based on the currently available literature it can be stated that inhalation of particles may lead to adverse health effects in the CNS. Possible mechanisms behind these effects include the induction of oxidative stress and inflammation in the brain, two processes that are reported to be significantly involved in the pathology of various neurodegenerative diseases.

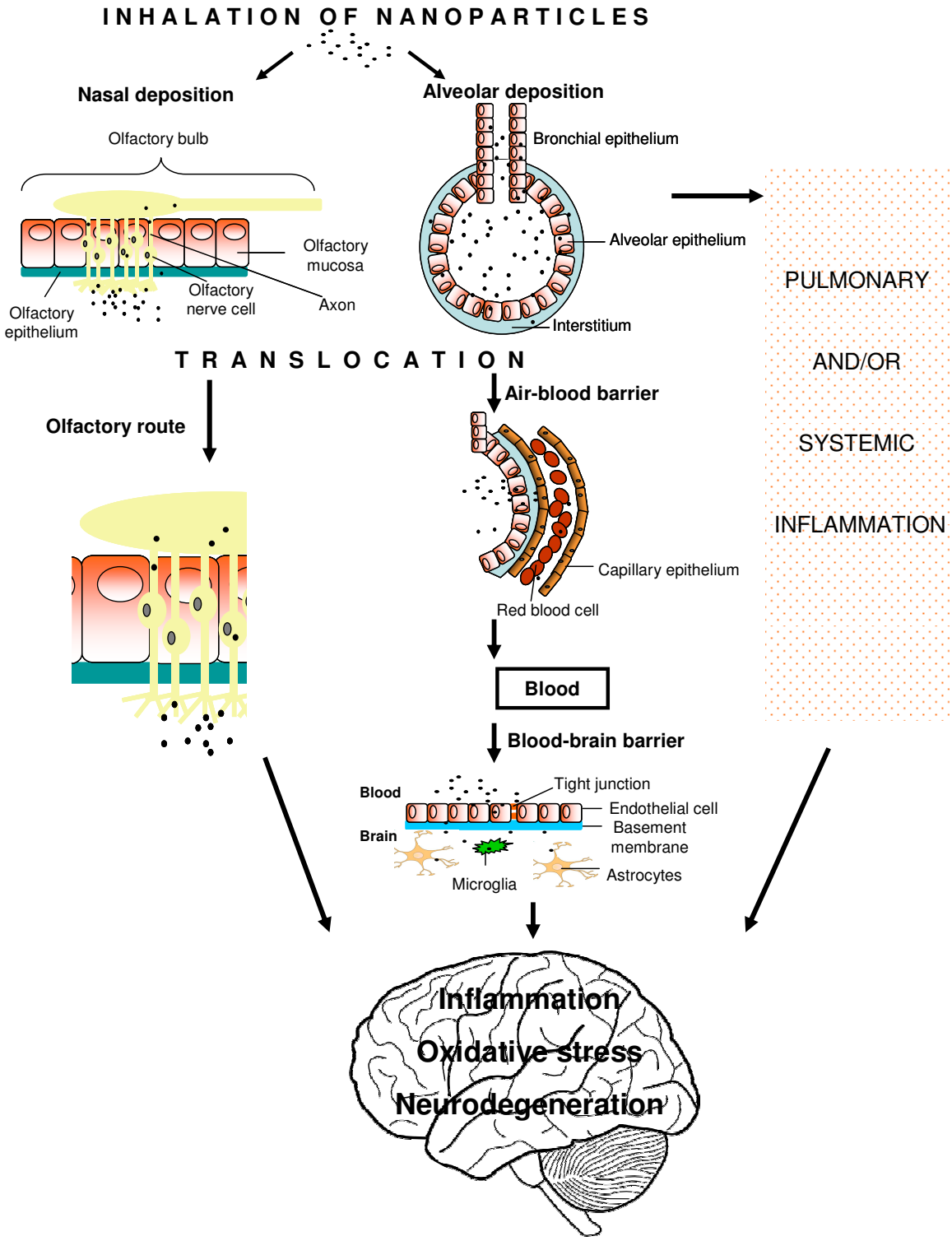

Figure 4: Nanoparticle exposure routes

Possible exposure routes after inhalation of NP. After nasal or alveolar deposition, NP may cause inflammation, oxidative stress or neurodegeneration in the brain.

1.8. Beyond the lung: The brain as a new target

In recent years, the possible effects of inhaled particles on the CNS have generated increasing interest. CNS pathology in humans has been connected with higher particulate air pollution levels in several studies. Further evidence has been emerging from *in vitro* as well as *in vivo* studies as will be discussed in the next paragraph.

1.8.1. Particulate air pollution-induced neurotoxicity

Several *in vitro* studies have demonstrated the ability of particles to induce toxicity in neuronal cells such as neurons and microglia. For instance, it has been shown by Block and co-workers, that DEP display a selective toxicity for dopaminergic neurons in a mesencephalic neuron-glia co-culture (Block et al., 2004). In subsequent experiments they demonstrated that neuron-glia cultures from NADPH oxidase deficient (phox–/–) mice (see also paragraph 2) were non-sensitive to DEP induced neurotoxicity when compared with control mice (phox+/+) (Block et al., 2004). In another study, concentrated ambient particles (CAPs) were found to trigger neurotoxic effects in microglia cells (Sama et al., 2007).

Important clues for the potential neuropathological effects of ambient air particles came from studies by Calderon-Garciduenas and co-workers (Calderon-Garciduenas et al., 2002; Calderon-Garciduenas et al., 2003). They have compared the brain histopathology from mongrel dogs that resided in highly polluted areas of Mexico City with those from dogs lived in a less polluted Mexican city (Tlaxcala). Dogs from polluted areas showed brain abnormalities like neuroinflammation and gliosis. In subsequent studies they also investigated the brains from inhabitants of highly polluted cities compared to those residing in cities with low levels of air pollution. Enlarged expression of cyclooxygenase-2 (COX-2) and Aβ42 were found in different areas of the brain, including the cortex and the olfactory bulb, from citizens living in polluted cities (Calderon-Garciduenas et al., 2004). COX-2 plays an important role in the inflammatory response induced by physiologic and stress stimuli and has been considered to play a role in the progression of neurodegenerative disorders including PD and AD (Heneka and O'Banion, 2007; Teismann et al., 2003).The investigations by Calderon-Garciduenas and co-workers have sparked the toxicological interest in the possible contribution of PM on adverse CNS effects.

Around the same time, Oikonen et al. reported a correlation between ambient PM levels and multiple sclerosis relapse. In their highest exposure group, the risk for the onset of relapse increased more than four times (Oikonen et al., 2003). In a further study by Calderon-Garciduenas and co-workers performed in children and young adults, it was shown that long-term air pollution exposure was associated also with neuroinflammation and an accumulation of Aß42 protein (Calderon-Garciduenas et al., 2008b). These studies also revealed indications of persistent inflammation in the brain characterised for instance by the increased expression of the transcription factor nuclear factor (NF)-κB and COX-2 (Peters et al., 2006).

Interesting observations were also made in a human volunteer study in which short-term controlled inhalation of DEE caused a rapid induction of brain activity in the frontal cortex, suggesting the presence of a cortical stress response (Cruts et al., 2008). In another study, traffic related exposure also tended to be related to cognitive score parameters in children: the observed effect was statistically significant for gross motor function (Freire et al., 2010). In a cohort study of elderly women, various neuropsychological tests to quantify mild cognitive impairments were also consistently found to be affected in relation to traffic exposure, suggesting that long-term exposure to fine PM might contribute to the development of AD (Ranft et al., 2009).

Apart from studies in humans, also an increasing number of animal experimental studies has been performed in recent years to address potential CNS effects by PM. Campbell and co-workers could demonstrate an increased expression of NFκB and interleukin-1α (IL-1α) in the brains of mice exposed to concentrated ambient particles (CAP) (Campbell et al., 2005). Elder et al. observed enhanced mRNA expression of the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNFα) in the olfactory bulb as well as other brain regions of rats exposed to MnO nanoparticles (Elder et al., 2006). Zanchi and colleagues investigated acute behavioural changes in Wistar rats after intranasal instillation of oil fly ash (ROFA) particles, representing a model of ambient particles rich in transition metals (Zanchi et al., 2008). Using an open field test to evaluate the motor/exploratory activities and emotionality resulted in a decrease of the frequency of peripheral walking and exploration in response to the ROFA treatment (Zanchi et al., 2008). Finally, in the framework of ongoing research collaborations between our institute and the National Institute for Public Health and the Environment (RIVM) in the Netherlands it could

also been shown that exposure to DEE can trigger oxidative stress and inflammation, characterised by enhanced mRNA expression of e.g. HO-1 and COX-2 (van Berlo et al., 2010a) and protein expression of IL-1α and TNF-α (Gerlofs-Nijland et al., 2010). Taken together, a number of *in vitro*, animal and human studies have provided further clues that the CNS may be indeed a highly relevant toxicological target for particulate air pollutants. The findings suggest that particle exposure might lead to the induction of oxidative stress and inflammation in the CNS, processes that are reported to be significantly involved in the pathology of various neurodegenerative diseases including Alzheimer's disease.

2. Aim of the Thesis

It is not yet fully established whether nano-size particles within an ambient PM mixture can translocate to the human brain in significant amounts that are then able to elicit toxic effects. Additionally, the relative contribution of two possible translocation routes (pulmonary or olfactory route) still needs to be clarified. Finally, it is also still unknown whether the potential adverse effects of PM can be attributed to a direct effect of the translocated particles or whether, similarly as discussed for cardiovascular effects of PM, indirect effects driven by pulmonary inflammation may be responsible. Irrespective of elucidating the mechanism whereby NP may reach and affect the brain directly, or alternatively, triggers brain effects "at a distance", concerns about the potential toxicity of ambient air particles and NP to the human CNS have increased considerably in recent years (Oberdorster et al., 2009; Peters et al., 2006). Consequently, there is an urgent need for further analyses displaying the potential adverse effects of inhaled NP for the brain and, more importantly, to elaborate on specific mechanisms implicated herein.

Increased oxidant levels and mild inflammation in the brain, both commonly observed in numerous experimental animal studies, are characteristic for normal brain aging processes (Beckman and Ames, 1998). However, they are also featured in various diseases of the central nervous system, including the common neurodegenerative disorders AD and PD (Quinn et al., 2006). Oxidative stress, inflammation and AD pathology represent main aspects of investigation in this thesis.

The aims of the thesis were:

a) To determine the effects of an acute exposure to carbon nanoparticles on oxidative stress responses and inflammation in the mouse brains of wildtype and $p47^{phox}$ knockout (KO) mice.

b) To investigate the effects of long-term repeated exposure to diesel engine exhaust particles on Alzheimer's disease (AD)-like pathology in an AD mouse model.

The $p47^{phox-/-}$ mice that were used for this thesis are deficient in their ability to generate superoxide radicals from phagocytic cells (Jackson et al., 1995) due to the impairment of a functional $p47^{phox}$ protein, which is a crucial component of the phagocytic NADPH oxidase enzyme complex NOX2. The $p47^{phox}$ KO mouse model has been shown to be of particular relevance in investigating the role of superoxide formation in various diseases. For the present study, the $p47^{phox}$ KO model has been used alongside their wildtype counterparts to address the role of NOX2 in nanoparticle-induced acute brain damage. In this study the mRNA expression levels of HO-1, TNFα, COX-2, Ogg1 and APE/Ref-1 were chosen as sensitive markers of oxidative stress, inflammation and/or oxidative DNA damage response(Albrecht et al., 2005; Gerlofs-Nijland et al., 2010; Klungland and Bjelland, 2007; Unnikrishnan et al., 2009; van Berlo et al., 2010a; van Berlo et al., 2010b). The selection was also made on the basis that those genes are expressed in lungs as well as in brains and are implicated in oxidative stress/inflammation associated brain diseases (Colombrita et al., 2003; Fillit et al., 1991; Heneka and O'Banion, 2007; Maeda et al., 2008; Singh and Englander, 2012; Teismann et al., 2003). In addition, the protein expression level of IL-1β was evaluated in the brain as a marker of inflammation (Yan et al., 1992).

The second animal study focused on the investigation of effects of repeated DEE particle inhalation over a 3 and 13 weeks interval in the 5xFAD mouse model. The 5xFAD mice mimic the development and progression of AD in humans by expressing human APP with the Swedish, Florida (I716V) and London mutations as well as the mutant PS1 (M146L, L286V) (Oakley et al., 2006). This animal model is characterised by a progressive, age-related cortical and hippocampal deposition of Aβ plaques, microglia activation and oxidative stress, starting around 3 months of age. Consequently, this animal study can be used to determine the effects of inhaled nanoparticles on mechanisms associated with both the development and progression of AD. The exposure duration of 13 weeks was selected as this reflects the generally accepted exposure duration of a so called subchronic rodent toxicology study. The key endpoints measured in this study were behaviour tests, Alzheimer-like histopathology and biochemical evaluation of brain tissue homogenate levels of Aβ42.

The anticipated outcome of the designated studies was to obtain novel insights on the role of environmental factors in the development and progression of neurodegenerative diseases. Specific emphasis was put on the elaboration of the contribution of the ultrafine component of ambient particulate matter herein. The results of the presented studies will contribute to our understanding of the molecular mechanisms involved in adverse health effects of nanoparticles and hence aid risk assessment strategies in relation to environmental as well as occupational exposure to both naturally occurring and engineered nanoparticles. The latter may be of special importance in relation to the application of novel nanoparticles that are designed for applications in nanomedicine and diagnostics.

3. Materials and Methods

3.1. Materials

3.1.1. Experimental animals: transgenic and knockout mice

$p47^{phox-/-}$ mice

The $p47^{phox-/-}$ mice (Taconic, Denmark) are deficient in their ability to generate superoxide from phagocytic cells (Jackson et al., 1995) resulting in an impaired phagocyte ROS generation. This is due to impairment of a functional $p47^{phox-/-}$ protein, which is a crucial component of the phagocytic NADPH oxidase system NOX2. The $p47^{phox-/-}$ KO mouse model has been shown to be of particular relevance in investigating the role of superoxide formation in various diseases.

All animals were housed in the IUF's accredited on-site testing facility under SPF conditions, according to the guidelines of the Society for Laboratory Animals Science (GV-SOLAS). Water and food were available ad libitum. Water for $p47^{phox-/-}$ mice contained antibiotics (Cotrimoxazol: 400mg sulfamethoxazol, 80 mg trimethoprim, 5ml per 1L drinking water). C57BL/6J wild-type mice served as control animals and were housed on hardwood whereas $p47^{phox-/-}$ mice on Alpha-Dri (Lillico, UK) bedding in plastic cages in an air-conditioned animal room (23 ± 2 °C) with a regular 12-h light/dark cycle.

$p47^{phox-/-}$ and C57BL/6J wild-type mice were used in the carbon nanoparticle (CNP) inhalation exposure study.

5xFAD mice

5XFAD transgenic mice (Tg6799) were used as a model for AD. These mice overexpress the 695 amino acid isoform of the human amyloid precursor protein (APP695) carrying Swedish (K670N), London (V717I) and Florida (I716V) mutations as well as the human Presenilin-1 (PS1) (M146L; L286V) mutations under transcriptional control of the neuron-specific mouse Thy-1 promoter (Oakley et al.,

2006; Ohno et al., 2006) that has been demonstrated to drive transgene expression predominantly in the CNS (Caroni, 1997).

These mice develop a specific phenotype showing high APP expression levels, amyloid deposition (and gliosis) beginning with two month of age, intraneuronal Aβ42-induced neurodegeneration, reduced synaptic marker protein levels and memory impairments (Oakley et al., 2006).

In the first year of the project, the mice were successfully introduced in the animal facility of the Leibniz Research Institute for Environmental Medicine via embryo-transfer and breeding was initiated to generate the required number of female 5xFAD mice.

Mice were backcrossed for 4 generations to C57Bl/6J wildtype (WT) mice before used in experiments. All experimental animals were born within a time range of 4 days, and age was approximately 10 weeks when inhalation exposure started.
All animals were handled according to guidelines of the Society for Laboratory Animals Science (GV-SOLAS). Temperature and the relative humidity in the inhalation units have been controlled at 22 ± 2°C and at 40-70%, respectively. Lighting was artificial with a sequence of 12 hours light (06:00-18:00) and 12 hours dark. Commercially available rodent food pellets and water were provided ad labium. As the inhalation itself took place in the Netherlands, Bilthoven, mice were transported to the National Institute for Public Health and the Environment (RIVM). They were allowed to acclimatize for 7 days. Experiments were approved by the Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI, Bilthoven, Netherlands). All 5xFAD mice were heterozygote with respect to the transgenic background. Non-transgenic wild-type littermates were used as a control.
5xFAD mice were used in the diesel engine exhaust (DEE) particle exposure study.

3.1.2. Chemicals and Antibodies

Name	Company, Head office
10x Reaction buffer	Peqlab, Erlangen
5xiScipt reaction Mix	BioRad, Hercules, USA
ABC solution	Sigma-Aldrich, St. Louis, USA
Acetic acid	Merck Millipore, Darmstadt
Anti-Amyloid ß42, primary antibody (mouse)	Merck Millipore, Darmstadt
Anti-Mouse, secondary antibody (horse)	Vectorlabs, Burlingame, USA
Assay Diluent RD1N	R&D systems, Minneapolis, USA
Bicinchoninic solution	Sigma-Aldrich, St. Louis, USA
Boric acid	Merck Millipore, Darmstadt
BSA	Carl Roth, Karlsruhe
CH_5N_3	Carl Roth, Karlsruhe
Chloroform	Sigma-Aldrich, St. Louis, USA
Citrate buffer	Carl Roth, Karlsruhe
Copper (II) tablets solution	Sigma-Aldrich, St. Louis, USA
Cotrimoxazol	Ratiopharm, Ulm
DAB	Vector, Berlingame, USA
DirectPCR-tail	Peqlab, Erlangen
EDTA	Carl Roth, Karlsruhe
Eosin	Merck Millipore, Darmstadt
Ethanol	Carl Roth, Karlsruhe
Ethidium bromide	Carl Roth, Karlsruhe
FCS	Sigma-Aldrich, St. Louis, USA
Formic acid	Carl Roth, Karlsruhe
Gel Loading dye Orange (6x)	New England Biolabs, Frankfurt
H_2O_2	Sigma-Aldrich, St. Louis, USA
HCl	Carl Roth, Karlsruhe
Hematoxylin-Solution after Gill III	Merck Millipore, Darmstadt
iQ SYBR Green Supermix	BioRad, Hercules, USA
iScript reverse transcriptase	BioRad, Hercules, USA
Isopropanol	Sigma-Aldrich, St. Louis, USA

KCl	Carl Roth, Karlsruhe
KH_2PO_4	Merck Millipore, Darmstadt
Milk powder	Carl Roth, Karlsruhe
Mouse IL-1ß Conjugate	R&D systems, Minneapolis, USA
Na_2HPO_4	Merck Millipore, Darmstadt
$NaCHO_3$	Carl Roth, Karlsruhe
NaCl	Carl Roth, Karlsruhe
Paraformaldehyde	Carl Roth, Karlsruhe
PBS	Sigma-Aldrich, St. Louis, USA
peqGOLD 100bp DNA-ladder	Peqlab, Erlangen
PeqGOLD dNTP-Mix	Peqlab, Erlangen
Primer	Operon, Köln
Protease inhibitor cocktail tablets	Roche Basel, Schweiz
Protein kinase K (20 mg/ml)	Peqlab, Erlangen
RNAfree water	Qiagen, Hilden
Rothi Histokitt (embedding medium)	Carl Roth, Karlsruhe
Taq-DNA Polymerase 5 u/ul	Peqlab, Erlangen
Tris	Carl Roth, Karlsruhe
Tris	Carl Roth, Karlsruhe
Triton-X 100	Sigma-Aldrich, St. Louis, USA
Trizol	Invitrogen, Carlsbad, USA
Tween 20	Serva, Heidelberg
Xylene	Carl Roth, Karlsruhe

3.1.3. Technical equipment

Name	Company, Head office
Camera AxioCam MRc	Carl Zeiss, Jena
Centrifuge 5417R	Eppendorf, Hamburg
Condensation Nucleus Counter	St Paul, USA
condensation particle counter model 3022A	St Paul, USA
Electrophoresis chamber	BioRad, Hercules, USA
FluorChem 8900	Biozym, Hessisch Oldendorf
Freezer -20°C	Liebherr, Bulle, Schweiz

Freezer -80°C	Hera Freeze Thermo Fischer Scientific, Schwerte
Fridge +4°C	Liebherr, Bulle, Schweiz
Gelelectroporese chamber	BioRad, Hercules, USA
Microscope Axiophot	Carl Zeiss, Jena
Microwave Micro-Chef MO505	Moulinex, Offenbach
Multiscan Ascent plate reader	Labsystems Oy Helsinki, Finland
Photometer Bio	Eppendorf, Hamburg
Power pac 3000	BioRad, Hercules, USA
Realtime-PCR System iQ5	BioRad, Hercules, USA
Sliding microtom HM 400	Thermo Fischer Scientific, Schwerte
Spark discharge generator	Palas GFG 1000 Soot Generator, Karlsruhe
Thermocycler Tprofessional	Biometra, Göttingen
Vacuum infiltration processor	
Wheaton Potter-Elvehjem tissue grinder	Thermo Fischer Scientific, Schwerte

3.1.4. Software

Program	Application
BioRad iQ5	Realtime-PCR-Data analysis
Image J	Image editing
Zeiss ZEN 2011	microscopical image analyses
SPSS version 15.0 for Windows	Statistical analysis

3.1.5. Buffers and Solutions

10x TBE pH 8.0

121.1 g/L	Tris
3.72 g/L	EDTA
51.53 g/L	Boric Acid

Aß42 ELISA brain homogenisation Buffer A

5M guanidine HCl
50mM Tris HCl, pH 8.0

Aß42 ELISA reaction buffer B pH 7.4

0.2 g/L KCl
0.2 g/L KH2PO4
8.0 g/L NaCl
1.150 g/L Na2PO4
5% BSA
0.03% Tween-20
1x protease inhibitor

IL-1ß brain homogenisation Buffer

0.2 g/L KCl
0.2 g/L KH2PO4
8.0 g/L NaCl
1.150 g/L Na2PO4
0.1 % Tween-20
1x protease inhibitor

DirectPCR-tail lyses reagent

500 µl DirectPCR-tail
470 µl RNA free water
15 µl Proteinkinase K (20mg/ml)

3.1.6. Primer sequences

HO-1 Forward	5´-CCTCACTGGCAGGAAATCATC-3´
HO-1 Reverse	5´-CCTCGTGGAGACGCTTTACATA-3´
COX-2 Forward	5´-AGGGTTAAACTTCCAAAGGAGACA-3´
COX-2 Reverse	5´-CAGCCTGGCAAGTCTTTAACCT-3´
TNFα Forward	5´-AGGCTGCCCCGACTACGT-3´
TNFα Reverse	5´-ACTTTCTCCTGGTATGAGATAGCAAAT-3´
Ogg1 Forward	5´-CCAAGGTGTGAGACTGCTGAGA-3´
Ogg1 Reverse	5´-AGCAATGTTGTTGTTGGAGGAA-3´
APE/Ref-1 Forward	5´-TCAAGAAGGCCGGGTGATT-3´
APE/Ref-1 Reverse	5´-TTGGGAACATAGGCTGTTACCA-3´
HPRT Forward	5´-AAGACTTGCTCGAGATGTCATGAA-3´
HPRT Reverse	5´-AAAGAACTTATAGCCCCCCTTGA-3´

3.1.7. Laboratory equipment / consumables

Name	Company, Head office
Falcon tubes	BD Bioscience, Canaan, USA
Mikroliterpipets Research (1-1000 µl)	Eppendorf, Hamburg
Pipettipps	Greiner bio-one, Freckenhausen
Reactiontubes (0.5-2.0 ml)	Eppendorf, Hamburg
Realtime-PCR 96 well Plate	Peqlab, Erlangen
Superfrost Ultra Plus Slides	Thermo Fischer Scientific, Schwerte
UV-cuvette	Brand, Wertheim

3.2. Methods

3.2.1. Isolation of DNA from Tailcuts

PCR amplification was used to genotype the offspring of 5xFAD animals. Therefore tail cuts of the animals (3-4 mm) were used to isolate DNA after lyses in 150 µl DirectPCR-tail lyses reagent (see 3.1.5. Buffers and Solutions) at 56°C over night while shaking the samples. Next day the proteinase K was inactivated for 45 minutes at 85°C. DNA solution was then directly used to prepare a genotyping PCR.

3.2.2. Genotyping of 5xFAD mice via PCR (polymerase chain reaction)

To amplify the DNA from mice tissue sequence-specific primers were used amplifying the transgenes of 5xFAD mice. Primer sequences were 5´- AGG ACT GAC CAC TCG ACC AG - 3´ as 5xFAD forward primer and 5´- CGG GGG TCT AGT TCT GCA T - 3´ as 5xFAD reverse primer. In heterozygote animals primers can bind to the DNA and amplify a fragment of 377 bp. Hence, these mice are transgenic and develop the Alzheimer phenotype.
Amplification of the DNA was done as followed:

5 µl	10x Reaction buffer
1 µl	PeqGOLD dNTP-Mix
2 µl	5xFAD forward primer, 0.5 µM
2 µl	5xFAD reverse primer, 0.5 µM
0.25 µl	Taq-DNA Polymerase
38.75 µl	RNA free water
1 µl	DNA template

Amplification of DNA occurred in three steps. The first is the heat-denaturation phase in which the two strands of DNA were separated into single strands. During the annealing phase the temperature is lowered so that the primers are able to bind to the single DNA stands and build up stable double-strain-parts between primer and DNA. During the last phase, the elongation, new DNA strains are synthesized via

Taq-DNA Polymerase. At 72°C the polymerase develop the highest activity, ensure an optimal primer elongation.

Reaction occurred under following condition:

	94°C	3 min	
Denaturation	94°C	30 sec	35 cycles
Annealing	52°C	1 min	
Elongation	72°C	1 min	
	72°C	2 min	

3.2.3. Gel electrophoresis

After DNA amplification via PCR DNA fragments were separated by length, to estimate the size of the fragments by applying an electric field. The negatively charged nucleic acid molecules migrate through an agarose gel. Shorter molecules move faster through the agarose matrix than longer once. Separation took place on a 1.5% agarose gel. Therefore 1.5 mg agarose was boiled up in 100 ml TBE buffer, 1 µl ethidium bromide (10 mg/ml) was added and poured in an electrophoresis chamber. After the agarose gel was sat it was covered with a layer of TBE buffer. The samples are dispensed into a well in the gel which is then connected to a power source. Samples and a DNA marker (100-1000 bp) were then separated at 70 V. Subsequently the DNA fragments were detected via UV-light and documented with the FluorChem 8900.

3.2.4. Diesel exposure and characterisation of test atmosphere

All animals were exposed in whole body inhalation chambers in separate inhalation units for 5 days/week, for 6 hours a day during either 3 or 13 weeks. Their food was changed on the Monday, Wednesday and Friday afternoon directly after each diesel exposure. Drinking water was provided through an automatic drinking water system. Animals were exposed to control (conditioned, purified and HEPA filtered) air or DEE nanoparticles diluted by mixing freshly generated exhaust from a stationary diesel engine (Common-rail motor, 35 KW load) with conditioned purified air. Particle number and mass concentrations were determined continuously using a

condensation particle counter (CPC model 3022A, TSI St. Paul, Minn., USA) and a nephelometer (DATARAM 2000, MIE, Billerica, Mass., USA). Final particle mass concentration was 1mg/m^3 with a geometric median diameter of 81 nm and geometric standard deviation of 1.75 nm. Time-integrated particle concentration was analyzed by gravimetric analysis. A carbon sampler tube was placed downstream of one of the filters at the outlet to collect the volatile organic compounds (VOCs), which were measured by means of GC-MS (RIVM, Bilthoven the Netherlands).

3.2.5. CNP generation and exposure characterisation

C57BL/6J and $p47^{phox-/-}$ mice were exposed by nose-only inhalation to CNP or HEPA filtered air for 4 hours on one day (10 animals per group). The CNP were generated on-site in an argon atmosphere from graphite electrodes in an electric spark discharge generator (Palas GFG 1000 Soot Generator, Karlsruhe, Germany). Throughout the inhalation exposures, test atmospheres were constantly monitored using a Condensation Nucleus Counter (3022A, TSI Inc. St Paul MN, USA) as well as a Nanoparticle Surface Area Monitor (NSAM 3550, TSI), and by a Scanning Mobility Particle Sizer (SMPS, TSI) at 30 min measurement intervals. Time-integrated mass concentrations were determined gravimetrically by means of particle collection on two parallel Teflon R2PJ047 filters (Pall corp., Ann Arbor MI, USA), with sampling at a flow rate of 2 l/min. Characteristics of the exposure concerning mass, particle and surface area concentration levels are listed in Table 3.

Table 3: Exposure characteristics of inhalation study

		4 h CNP exposure
Mass concentration (µg/m^3)		154
Particle number concentration (#/cm^3)		$7.51x10^6$
Mean size (nm), (GSD)		58.8 (1.65)
Surface area [#]	TB (µm^2/cm^3)	$2.35x10^3$
	A (µm^2/cm^3)	$8.7x10^3$

[#] Human lung-deposited surface area corresponding to tracheobronchial (TB) and alveolar (A) lung regions.

3.2.6. Behaviour tests

String suspension task

The motor coordination and the grip strength of the animals were tested using the string suspension task. Therefore a 2 mm broad and 35 cm long cotton string was stretched between two vertical poles above a cushioned bottom (see Figure 5A). The animals were carried by their tails and permitted to grasp the string by their forepaws before their release.

A **B**

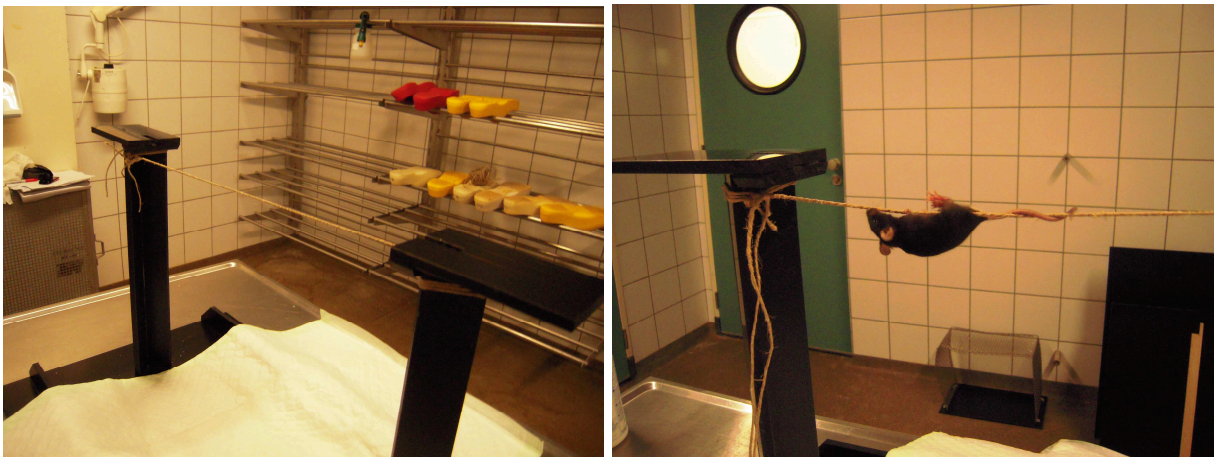

Figure 5: String suspension task

(**A**) Photo from the string suspension task equipment. (**B**) Tested WT littermate mouse on its way to escape to the platform.

A rating system from 0 to 5 was assigned to each animal during a single 60 seconds session (Moran et al., 1995).

Table 4: Rating system string suspension task

0	unable to hang on the string
1	hangs only by forepaws
2	attempting to climb the string
3	climbing the string with four paws successfully
4	moving laterally along the string
5	escaping to one of the ends of the string

Y-maze and Cross-Maze

The Y-maze and Cross-maze tasks were applied to reflect spatial working memory of mice by spontaneous alternation.

Spontaneous alternation procedure is based on the natural affinity of mice to explore a novel environment. When a mouse is placed in Y-or X-maze usual mice prefer to explore the least recently visited arm. Therefore mice tend to rotate in their visits between the arms. To explore the arms (three in the Y-maze and four in the X-maze) in a successful manner the mouse must perpetuate an ongoing record of most recently visited arms. These records must then also be updated continuously by the mice. A mouse with impaired working memory cannot memorize in which arm it just was. This results in a decreased spontaneous alternation (Holcomb et al., 1999).
For the Y-maze, the spontaneous alternation percentage could be measured by a triangular Y-shaped maze built up from a black plastic material. The three arms were arranged in 120° position extending from a central space. Arm sizes comprised 30 cm length, 8 cm width and 15 cm height (see Figure 6). Each animal was placed randomly in one arm and allowed to discover the maze freely for 10 minutes.

Figure 6: Y-maze task
Photo of the Y-maze task equipment. A 5xFAD mouse is sitting in the centre of the maze.

Alternation was considered as successful when entries into three different arms in overlapping triplet sets were made (for example 1, 2, 3 or 2, 3, 1 but not 1, 2, 1).

The X-maze differs from the Y-maze, as it consists of 4 arms, arranged in 90° position extending from a central space, which makes it more discriminative (arm

sizes: 30 cm length, 8 cm width and 15 cm height, build up from black plastic material). Alternation was considered as successful when entries into four different arms in overlapping quadruple sets were made (for example 1, 2, 3, 4 or 2, 3, 4, 1 but not 3, 2, 1, 3).

For both mazes, alternation percentages could be calculated as the percent of the actual alternations to the possible arm entries. To avoid influence on the behaviour of the mice due to odour of mice tested before, the maze was cleaned after every trial with 70% ethanol.

3.2.7. Necropsy of 5xFAD animals

WT littermates (n=10 per treatment and time point) and 5xFAD mice (n=16 per treatment and time point) were sacrificed by cervical dislocation one week after the last exposure to DEE nanoparticles or HEPA filtered air. Whole brain was carefully removed after open the scull and dissected into different parts (n=10 WT air; n=10 WT DEE; n=16 5xFAD air; n=16 5xDAS DEE). The left brain hemisphere was stored for immunohistochemical analyses after fixation with 4% paraformaldehyde at 4°C and subsequent embedding in paraffin. Right hemispheres were snapfrozen for later biochemical analyses (n=5 WT air; n=5 WT DEE; n=8 5xFAD air; n=8 5xDAS DEE). or dissected into three parts, i.e. the olfactory bulb, cerebellum and the remaining part (n=5 WT air; n=5 WT DEE; n=8 5xFAD air; n=8 5xDAS DEE). All parts were separately snapfrozen in liquid nitrogen in 1 ml Trizol for later mRNA expression analyses. Lungs were used for immunohistochemical analyses after perfusion and fixation in formaldehyde (n=5 WT air; n=5 WT DEE; n=8 5xFAD air; n=8 5xDAS DEE) or dissected, followed by snapfreezing (n=5 WT air; n=5 WT DEE; n=8 5xFAD air; n=8 5xDAS DEE). Therefore the left lungs were stored at -80°C for later protein analyses and the other lobes were also stored at -80°C for later mRNA expression analyses after addition of 1 ml Trizol before snapfreezing in liquid nitrogen. Also heart, liver and spleen were removed and either snapfrozen or fixated in paraformaldehyde for eventual further analyses.

3.2.8. Necropsy of p47$^{phox-/-}$ animals

After the exposure, 7 animals of each group were deeply anesthetized by a single intraperitoneal injection of Na-pentobarbital, followed by weighting and subsequently mice were exsanguinated via the abdominal aorta.

Tissues of the animals were removed in an average of 3 hours after last exposure. The post mortem period was similar for controls and exposed animals. For brain isolation the skull was opened and brains were removed. The Olfactory bulb was isolated and stored in Trizol (Invitrogen). The remaining part of the brain was divided into their hemispheres which were then separated in 2 different parts (cerebellum and remaining part, excepting for the brain stem). One half was stored in Trizol for later RNA isolation and the other without Trizol for protein isolation. All samples were immediately frozen in liquid nitrogen and kept at -80°C until further use.

3.2.9. RNA extraction from tissues

Tissue samples stored in 1 ml TRIzol were thawed on ice. Homogenisation with the TissueLyser took place for 2 min (30 x / sec) with the help of a steel ball in a 2 ml eppendorf cup. Upon homogenization and addition of 200 µl chloroform samples were incubated for 3 min followed by centrifugation for 15 min at 12,000 x g at 4°C. 450 µl of supernatants was transferred in a new cup and 500 µl isopropanol was added. After incubation at room temperature for 10 minutes, the samples were centrifuged for 15 min at 12,000 x g at 4°C. Supernatants were discarded, the pellets washed with 1 ml ethanol and then centrifuged for 5 minutes at 7,500 x g at 4°C. Again, supernatant was discarded and the pellet resuspended in 100 µl RNase free water. After incubation for 10 min at 60°C, RNA concentration was quantified by measuring the absorbance at 260 and 280 nm in a photometer (see 7.10.). Samples were stored at -80°C until further use.

3.2.10. Photometrical analysis of RNA concentration

Ultraviolet (UV) spectroscopy was used for assessing RNA concentration and purity. Nuclei acids absorb wavelength at the ultraviolet UV range due to their aromatic structure, and RNA has its absorption maximum at 260 nm. An optical density of 1.0 at 260 nm is equivalent to about 40 µg/ml of single-stranded RNA. The

ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. A value between 1.8 and 2.0 is deemed to be clean and show no Protein contamination. Lower values can suggest protein contamination within the nuclei acid preparation.

The nuclei acid concentration was calculated using the Beer-Lambert law. It calculates a linear change in absorbance with concentration:

Concentration of RNA sample = 40 x OD 260 x dilution factor

To determine the concentration and purity of the isolated RNA 2 µl nuclei acid solution were added to 98 µl RNase free water carried into a UV-cuvette and optical density was measured at 260 nm and 280nm.

3.2.11. Synthesis of complementary DNA (cDNA)

iScript reverse transcriptase is a modified MMLV (Moloney Murine Leukemia Virus) -derived reverse transcriptase that was used for the synthesis of complementary DNA (cDNA). Messenger RNA (mRNA) was incubated with reverse transcriptase, which uses the mRNA as a template for synthesis of a cDNA strand. Oligo(dT) were used as primers as they bind to the terminal poly-A tail of the mature mRNA template at its 3prime end.
Mixture of components:

4 µl	5x iScript reaction mix
1 µl	iScript reverse transcriptase
15 µl	RNA template (0.5 ug RNA in total)

To enable optimal working conditions for the elongation phase of the cDNA samples were then treated to different temperatures within the PCR cycler:

25°C	5 min	primer extension
42°C	30 min	synthesis step
85°C	5 min	inactivation of reverse transcriptase

cDNA was directly used for semi-quantitative Realtime-PCR or stored at -20°C.

3.2.12. Gene expression analyses via semi-quantitative Realtime-PCR

The semi-quantitative real time-polymerase chain reaction (RT-PCR) has become a standard tool in gene expression analysis studies which is used to amplify and simultaneously quantify a targeted nucleic acid. The use of a fluorescence dye (SYBR-green) that intercalated with double strain DNA enables the quantification of target genes as the fluorescence signal increases proportionately to the amount of amplificated DNA. Relative quantification is based on internal reference gene to conclude fold-differences in expression of the target gene.
In the last step of semi-quantitative Realtime-PCR a melt curve was run to ensure primer specify.

Mixture of components:

12.5 µl	iQ SYBR Green Supermix
2.5 µl	Primer (forward), 0.375 µM
2.5 µl	Primer (reverse), 0.375 µM
2.5 µl	RNase free water
5 µl	cDNA (1:15 diluted)

Reaction took place in the Realtime-PCR System iQ5 under following conditions:

95°C	3 min	
95°C	15 sec	X 40
60°C	45 sec	

To perform a melting curve analyses fluorescence data were collected slow but constantly over a temperature range of 56°C to 95°C raise the temperature all tenth seconds in 0.5°C increments.

The housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) served as an internal control. This gene should be expressed independent of treatment of the organism therefore expression level should be approximately the same in treated and untreated groups.
To analyse changes in gene expression levels in target gens first the C_t-values (*cycler threshold*) were determined. C_t-values describe the number of cycles at which

the fluorescence signal goes above a certain threshold of the background fluorescence. The sooner the threshold is exceeding the higher is the initial amount of a certain transcript before the amplification. To calculate the gene expression of a special gene of interest X following formula was used presented as $2^{-\Delta\Delta c(t)}$ values (Livak and Schmittgen, 2001).

Relative gene expression of gene X from sample A compared to sample B

$$= 2^{-\{[Ct(\text{gene X, A}) - Ct(\text{HKgene, A})] - [Ct(\text{gene X, B}) - Ct(\text{HKgene, B})]\}}$$

3.2.13. Tissue homogenisation

To prepare brains for later Enzyme Linked Immunosorbent Assay (ELISA) analyses brain tissues were homogenised in a potter tissue grinder. The buffer that was used for homogenisation was chosen dependently on subsequently performed ELISA (see 3.1.5. Buffers and Solutions). Consequently frozen tissues were put in the grinder, covered with corresponding homogenisation buffer (650 µl for IL-1ß ELISA samples and 1500 µl for aß42 ELISA samples) and manually homogenised. To get rid of cell debris that might disturb following ELISA analyses, all samples were centrifuged for 15 min at 14,000 x g. Supernatant was then transferred to a new eppendorf tube, aliquoted and stored at -20°C until further use or directly used to determine protein concentrations.

3.2.14. Bicinchoninic acid assay (BCA Assay)

The bicinchoninic acid assay provides an accurate determination of a total protein concentration in a solution. The principle of the BCA assay based on formation of a reduction Cu^{2+} complex to Cu^{1+} resulting in a colour change of the samples from green to purple as BCA is a chromogenic reagent chelating the reduced copper under production of a purple complex. The intensity of the purple colour gives information of the amount of protein available in the prepared samples as the amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution (Smith et al., 1985).

Concentrations of protein-levels in the samples were determined by evaluation of a BSA standard. BSA-protein serial dilutions were prepared (0 -15 µg / µl) and 10 µl of every dilution or sample was transferred to a 96-well plate (performed as duplicates). BCA solution A and copper (II) sulphate solution B were mixed in a 1:50 ratio and 200 µl were pipetted to each well. After 10 min at 37°C the absorption of the purple complex was measured in a plat reader at 540nm. The reactions of the standards were used to calculate a standard curve and a linear equation (y = m x + a). The absorbance values of the unknown samples were then interpolated onto the formula of the standard curve to identify their protein-concentrations.

3.2.15. Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA technique is used to detect the presence of a specific protein in a liquid sample. Therefore a monoclonal antibody against the antigen of interest is fixed to a microtiter plate followed by incubation with sample solutions containing the antigen. Antigens now bind to the immobilized antibodies; unbound antigens are washed away followed by addition of an enzyme-linked secondary antibody specific for the target antigen to the wells. After all unbound enzyme-linked antibody conjugates were removed a substrate solution is added to the wells. This substrate is converted by the enzyme into a colour whose intensity can be determined by measure the optical density in each well. Optical density unit of the sample is interpolated into a standard curve of e serial dilution of the target antigen. The intensity of the coloured product is directly proportional to the concentration of the analysed antigen present in the sample.

In this dissertation, two different ELISA were performed.

IL-1ß ELISA (R&D Systems)

IL-1ß ELISA was done referred to the provided manual (R&D Systems).
Each wells of the microtiter plates pre-coated with a monoclonal antibody specific for mouse IL-1ß was covered with 50 µl *Assay Diluent RD1N*. 50 µl of serial dilutions of the standard and 50 µl of samples (performed as duplicates) were then added to the wells, covered with an adhesion strip and incubated for 2 hours at room temperature, followed by a washing step with provided wash buffer for a total of 5 washes. 100 µl of *Mouse IL-1ß Conjugate* was pipetted to the wells which were then covered with an adhesion strip and again incubated for 2 hours at room temperature. Subsequently

wells were washed 5 x with wash buffer. 100 µl of provided *Substrate Solution* was added to the wells and incubated in the dark for 30 min at room temperature. After adding 100 µl provided *Stop Solution* optical density was measured at 450 nm at the plate reader.

Human Aß42 ELISA (Invitrogen)

Human Aß42 ELISA was done referred to the provided manual (Invitrogen). This assay recognizes both natural and synthetic forms of human Aß42. Microtiter plate was pre-coated with monoclonal antibody specific for the NH_2-terminus region of human Aß42.

Serial dilutions of the Aß peptide standard were prepared and samples were diluted with Aß42 ELISA reaction buffer B (see 3.1.5. Buffers and Solutions). Consequently all samples offer the same amount of total protein concentration. 50 µl of Aß peptide standards and samples were added to the wells (performed as duplicates) followed by addition of 50 µl *Hu Aß42 Detection antibody*. This rabbit antibody is specific for the COOH-terminus of the 1-42 Aß sequence. Plate was covered with an adhesion stripe and incubated for 3 hours at room temperature with shaking. Solutions were then decanted and plate was washed for four times with provided wash buffer. After pipetting 100 µl *Anti-Rabbit IgG HRP* solution to each well plate was covered and incubated for 30 min at room temperature. Wells were washed again four times followed by addition of 100 µl *Stabilized Chromogen* to each wells. Incubation took place for 15 minutes at room temperature in the dark. After adding 100 µl *Stop Solution* to every well absorbance was measured at 450 nm at the plate reader.

3.2.16. Immunohistochemical analyses of paraffin slices

After sacrificing the animals and careful dissection of their brains, the right half of the brain was fixated in 4% buffered formalin at 4°C for a minimum of 24 hours. Dehydration in a series of EtOH was followed by a transfer into xylene. Afterwards brains were embedded in liquid paraffin. 4 µm paraffin sections were cut at the sliding microtome and transferred on Superfrost Ultra Plus object slides and dried over night at 40°C. Subsequently sections were deparaffinised in xylene, followed by a rehydration in a series of ethanol (100%, 96%, 70%). To block endogenous peroxidise slices were treated with 0.3% H_2O_2 in PBS, followed by antigen retrieval

via boiling slices in 10mM citrate buffer, pH 6.0. After that sections were incubated in 88% formic acid for 3 minutes. Before application of the primary antibody unspecific binding sides were blocked for 1 hour via incubation in 0.01 M PBS plus 10 % fetal calf serum (FCS) and 4% skim milk. Incubation with primary antibodies, diluted 1:1000 in 0.01 M PBS and 10% FCS, was done over night in a humid chamber at room temperature. Slices were then incubated with biotinylated secondary antibody, diluted 1:200 in 0.01M PBS and 10% FCS. Signal detection occurred via avidin-biotin-complex-method (ABC) by Vectastain kit. Treatment with diaminobenzidine (DAB) as chromogen resulted in a reddish-brown colour. Hematoxylin was use to perform the counterstaining and an elevation of the pH due to tab water flushing led to a blue staining of the nucleus.

Lightmicroscopical pictures from cortex and hippocampus were taken with 100 x or 50 x magnification, respectively. Quantitative Aß42 plaque analyses were done via calculation the % of total plaque load in the analysed section. Plaque load was determined using image analysis software (ZEN2011, Zeiss) after a fixed adjustment of contrast threshold for stained Aß42 plaques. For determination of plaques in the cortex whole image section were evaluated while the hippocampus region was defined by hand (see Figure 7).

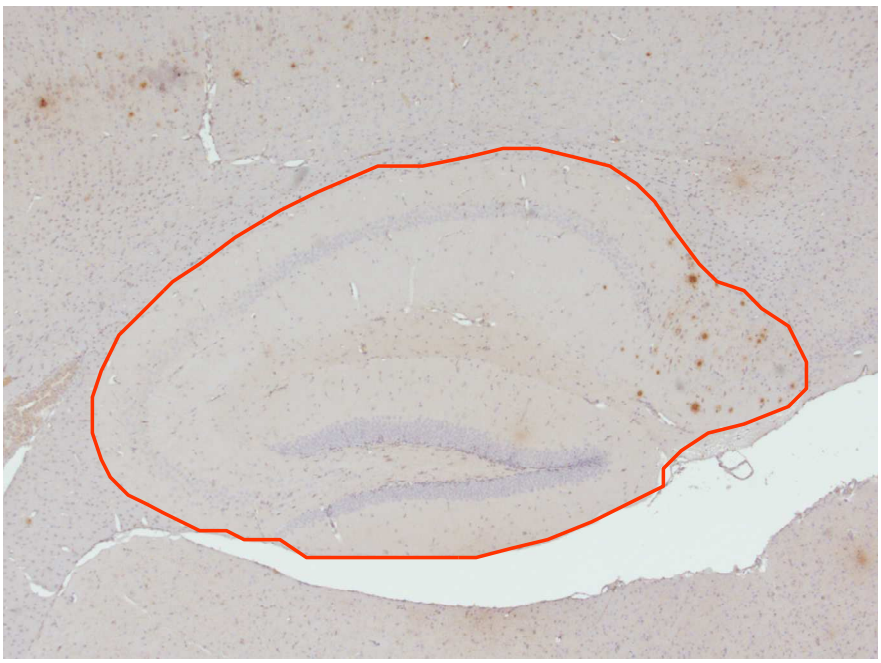

Figure 7: Quantitative analyses of the Hippocampus

Area for measurements in the hippocampus was selected manually (red line). Only plaques within these defined area or tangent the red line were counted and included for calculation.

3.2.17. Haematoxylin-Eosin staining

Haematoxylin-Eosin (HE) was used for immunopathological analyses of lung tissues. The oxidation product of haematoxylin is haematin which then represents the active ingredient in the staining solution. This staining method is used to visualise cells as it stains cell nuclei into a blue colour. Staining with haematoxylin was done in conjunction with the use of eosin which stains the cytoplasm and collagen of the cells in red.

The solution that was used was haematoxylin-solution modified after Gill III. Following sacrifice of the animals the lungs were carefully removed and fixed in 4 % Paraformaldehyde in PBS (pH 7.4) at 4°C for a minimum of 24 hours. The dehydration occurred in a series of EtOH, followed by a transfer into xylene. Lungs were then embedded in liquid paraffin. Sections were cut at the sliding microtome and shifted on auf Superfrost Ultra Plus object slides and dried over night at 40°C. Afterwards slices were deparaffinised in xylene, followed by a rehydration in a series of ethanol (100%, 96%, 70% EtOH). Sections were then incubated for 15 minutes in haematoxylin-solution, flushed with tab water and incubated for 1-2 minutes with eosin (0.1% in 70% EtOH). Dehydration took place in series of ethanol (70%, 96% and 100%) and afterward slices of tissues were mounted and pictures were taken using a light microscope.

3.2.18. Statistical evaluation

Data are expressed as mean ± SEM unless stated otherwise. For the evaluation of treatment-related differences as well as genetic background-related differences, data were analysed by students t test using SPSS version 15.0 for Windows. A difference was consider to be statistically significant when $p < 0.05$.

4. Results

4.1. Phox Study

This inhalation study evaluated the effects of a single short term (4 h) nose-only inhalation exposure to model carbon NP (generated by spark discharge) or filtered air in the lungs and brains of wildtype (WT) and $p47^{phox-/-}$ mice. The $p47^{phox-/-}$ mice are deficient in their ability to generate superoxide anion radicals from the NOX2 NADPH oxidase enzyme complex within phagocytic cells, like pulmonary macrophages and microglia cells within CNS. The $p47^{phox-/-}$ model was used to address the role of reactive oxygen species formation in NP-induced oxidative stress and inflammation in lung and brain tissues.

4.1.1. Quantitative RT-PCR in lung tissues of p47phox-/- and WT mice after CNP exposure

Pulmonary effects in the mice were evaluated by measurement of the mRNA expression of a set of marker genes by qRT-PCR after controlled exposure of the animals to CNP. Analyses of mRNA expression levels changes of HO-1, TNFα and COX-2 were chosen as well-established markers of oxidative stress and inflammation(Albrecht et al., 2005; van Berlo et al., 2010a; van Berlo et al., 2010b). Oxidative DNA damage responses were evaluated by evaluation of the mRNA changes in the DNA base excision repair genes Ogg1 and APE/Ref-1(Klungland and Bjelland, 2007; Unnikrishnan et al., 2009). The results of the pulmonary mRNA expression level changes after inhalation of CNP in WT or knockout mice are presented in Figure 8.

For none of the genes that represent changes in inflammation state or induction of oxidative stress a significant change in mRNA expression level could be detected. HO-1, TNFα and COX-2 expression levels were not significantly altered after inhalation, neither in the WT animals nor in the knockout mice. However, the mRNA expression levels of the genes coding for base excision repair genes were significantly increased in the WT mice in association with the CNP exposure. In contrast, no changes were observed in the $p47^{phox-/-}$ mice. The wildtype animals exposed to CNP showed on average a 2-fold increase in expression levels for both

Ogg1 and APE/Ref-1. As such, the lungs of p47phox knockout mice were found to be less responsive to inhalative exposure to CNP than the WT animals.

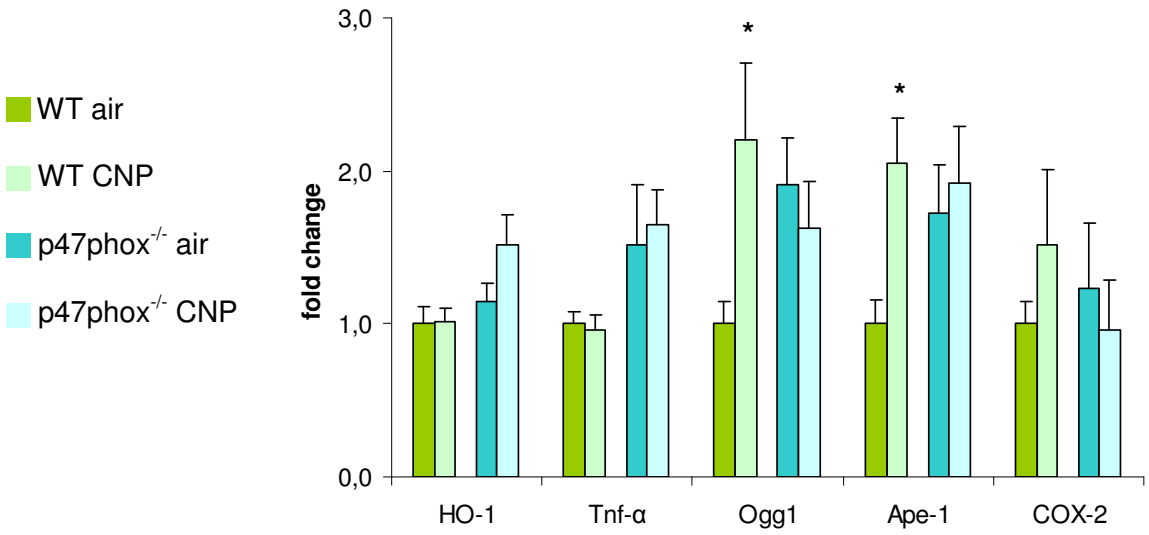

Figure 8: mRNA expression levels changes in lungs after CNP exposure

The mRNA expression levels of selected genes were determined in mouse lungs after short-term exposure to filtered air or CNP. Data represent mean values and standard errors of n=7 animals per treatment group (n=7) and are expressed as HPRT-corrected fold-change mRNA expression compared to WT filtered air-exposed animals. * $p< 0.05$ versus filtered air exposed WT animals.

4.1.2. qRT-PCR of brain tissues of p47$^{phox-/-}$ and WT mice after CNP exposure

The mRNA expression levels of HO-1, TNFα, COX-2, Ogg1 and APE/Ref-1 were analysed in three different sections of the mouse brains. After exposure to CNP or filtered air for 4 hours the olfactory bulb (OB), the cerebellum (CB) and the remaining part of each brain (MB) were analysed. Data were corrected for HPRT and expressed as fold expression change to the expression of the air-exposed animals and are shown in Table 5. No treatment related effects were detected for any of the investigated markers. Neither the WT animals, nor the p47$^{phox-/-}$ animals showed statistically significant changes in mRNA expression levels that could be attributed to CNP exposure. Overall also no clear consistent trends could be detected that would point towards unambiguous treatment related responses. Also no clues could be provided on the differences in responsiveness of the different brain regions. As the

expression data are calculated and expressed relatively to the air exposed WT animals, the expression differences in the air exposed animals from both backgrounds become visible. However, in this regard, no consistent data were observed that would point towards potential differences in constitutive mRNA expression patterns between the p47$^{phox-/-}$ and the WT mice.

Table 5: mRNA expression levels of selected genes in mouse brains measured in three different regions of the brain after short-term exposure to air or CNP. Results are represented as HPRT-corrected fold change compared to the air exposed WT mice. Data are shown as mean ± standard errors of mean [(2 $^{-\Delta\Delta c(t)}$)±SEM], and represent n=5 animals for the WT CNP group and n=6 per group for the other three study groups.

		WT		**p47$^{phox-/-}$**	
		Air	CNP	Air	CNP
HO-1	OB	1.0 (±0.2)	1.0(±0.4)	0.4(±0.1)	1.3(±0.8)
	MB	1.0 (±0.6)	2.1(±1.5)	1.7(±0.4)	2.1(±0.8)
	CB	1.0 (±0.5)	0.3(±0.1)	0.9(±0.4)	1.9(±0.6)
Tnf-α	OB	1.0 (±0.8)	0.1(±0.04)	0.3(±0.1)	0.6(±0.5)
	MB	1.0 (±0.9)	0.1(±0.05)	1.1(±0.9)	0.2(±0.1)
	CB	1.0 (±0.3)	4.5(±3.8)	4.3(±3.0)	4.7(±2.3)
Ogg1	OB	1.0 (±0.4)	1.0(±0.3)	0.7(±0.2)	1.1(±0.3)
	MB	1.0 (±0.5)	1.6(±1.2)	1.7(±0.5)	1.6(±0.5)
	CB	1.0 (±0.5)	0.5(±0.1)	0.8(±0.3)	1.5(±0.6)
APE/Ref-1	OB	1.0 (±0.2)	1.3(±0.4)	1.3(±0.3)	1.4(±0.6)
	MB	1.0 (±0.4)	0.8(±0.5)	1.2(±0.5)	1.2(±0.8)
	CB	1.0 (±0.2)	0.9(±0.2)	2.1(±1.0)	4.5(±2.1)
COX-2	OB	1.0 (±0.3)	0.9(±0.3)	1.0(±0.3)	1.3(±0.6)
	MB	1.0 (±0.3)	0.8(±0.4)	1.4(±0.4)	1.5(±0.8)
	CB	1.0 (±0.4)	0.7(±0.2)	2.0(±1.1)	5.3(±3.3)

4.1.3. IL-1ß ELISA with brain homogenates of p47$^{phox-/-}$ and WT mice after CNP exposure

To further evaluate the potential inflammatory effects of the short-term CNP exposure on the brains of the WT and p47phox knockout animals, the IL-1ß protein expression levels were evaluated in the brain tissue homogenates of the mice by ELISA. Results are shown in Figure 9. As can be seen in the graph, no differences in brain IL-1ß levels were observed between the air and CNP exposed mice. Neither

the WT mice nor the p47$^{phox-/-}$ mice revealed changes in protein expression levels of IL-1ß in response to the nanoparticles exposure. Remarkably, however, there were significant differences in the amount of IL-1ß between the p47$^{phox-/-}$ and the WT mice. Irrespective of the exposure, the brain homogenate levels of IL-1ß tended to be higher in the wildtype animals. For the air exposed groups this defence was statistically significant.

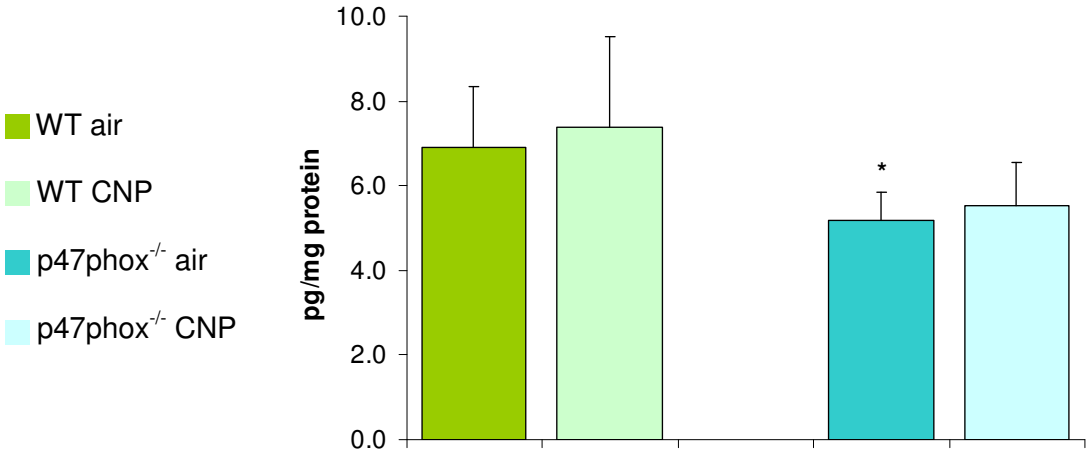

Figure 9: Interleukin-1ß protein expression

IL-1ß protein expression was measured by ELISA in mouse brain homogenates of after short term exposure to CNP or filtered air. * p< 0.05 versus filtered air exposed WT animals. n=7 for WT air, n=7 for WT CNP, n=7 for p47$^{phox-/-}$ air and n=7 for p47$^{phox-/-}$ CNP.

4.2. 5xFAD study

In recent years, it has been suggested that nanoparticles generated from combustion processes (e.g. diesel engine exhaust particles), may contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD). The study in the 5xFAD mice was performed to determine whether subchronic exposure to diesel engine exhaust (DEE) nanoparticles could affects AD pathology. The 5xFAD mouse model, is characterised by development of progressive behavioural deficits as well as amyloid plaque formation and neuron loss. Ten weeks old female 5xFAD mice and their non-transgenic littermates were exposed by whole body inhalation to diluted DEE (~1 mg particles/m^3) or clean air (controls) for 3 or 13 weeks (5 days/week and 6 hour/day). Subsequently, all animals were subjected to a series of

behavioural tests. At day ten post-exposure, mice were sacrificed to investigate lung and brain tissues for pathological, biochemical and molecular-biological changes.

4.2.1. Evaluation of body and organ weights

To evaluate whether the DEE exposures would affect the general physiological state of the transgenic 5xFAD and the wildtype mice, body and organ weights were determined after 3 and 13 weeks of exposure. This also allowed for the evaluation of the body and organ weight changes in relation to the genetic background of the animals.

The body weights of 13 weeks old 5xFAD mice and their transgenic littermates can be described as normal compared to female C57Bl/6J wildtype mice. WT mice should show a body weight about 21.21g ± 1.8g (information from Jackson laboratories: http://jaxmice.jax.org/support/weight/000664.html). In general one could observe that 5xFAD mice tended to have slightly lower weight compared to their littermates.

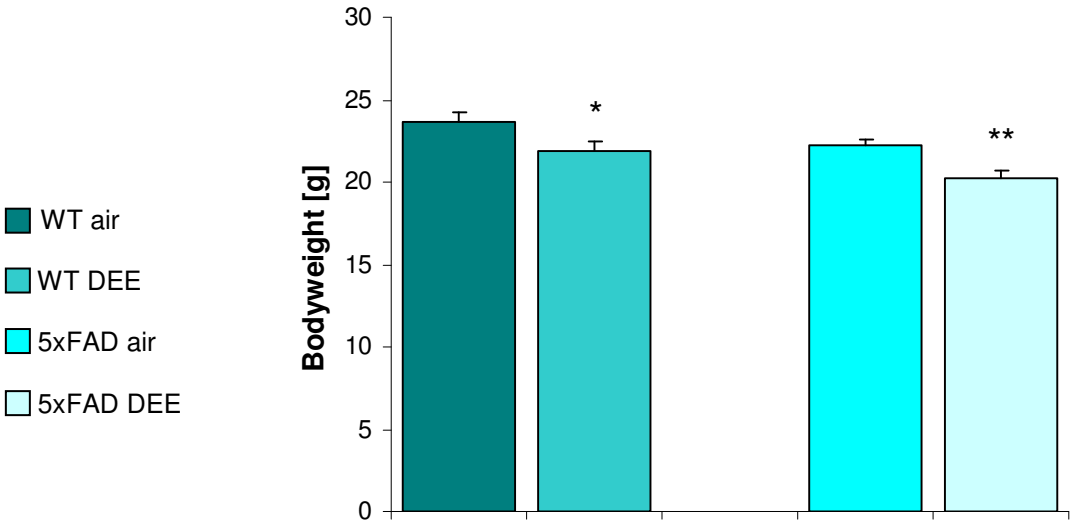

Figure 10: **Bodyweight of 3 month old mice**
Bodyweight measurements of transgenic 5xFAD and their littermates after 3 weeks of exposure with DEE or HEPA filtered air. Age of mice was 13 weeks. n=10 for WT air, n=10 for WT DEE, n=15 for 5xFAD air and n=16 for 5xFAD DEE. * $p < 0.05$, ** $p < 0.01$ versus air exposed animals (WT air vs. WT DEE and 5xFADair vs. 5xFAD DEE).

Exposure to DEE appeared to have an effect on the body weight of the mice. Figure 10 shows that after 3 weeks inhalation, the animals exposed to DEE showed a significant decrease in bodyweight in contrast to animals that inhaled clean air. This effect appeared to be more pronounced in 5xFAD transgenic mice. The body weight of mice exposed for 13 weeks was also evaluated before sacrificing when mice were 23 weeks old. Compared to the younger group body weight of DEE particle exposed animals moving closer to the control group. WT DEE animals are even slightly heavier than the air treated animals. Transgenic mice show more or less the same body weight after 13 weeks of DEE particle or air exposure (see Figure 11). With increasing age no differences between genetic background and treatment can be detected any longer.

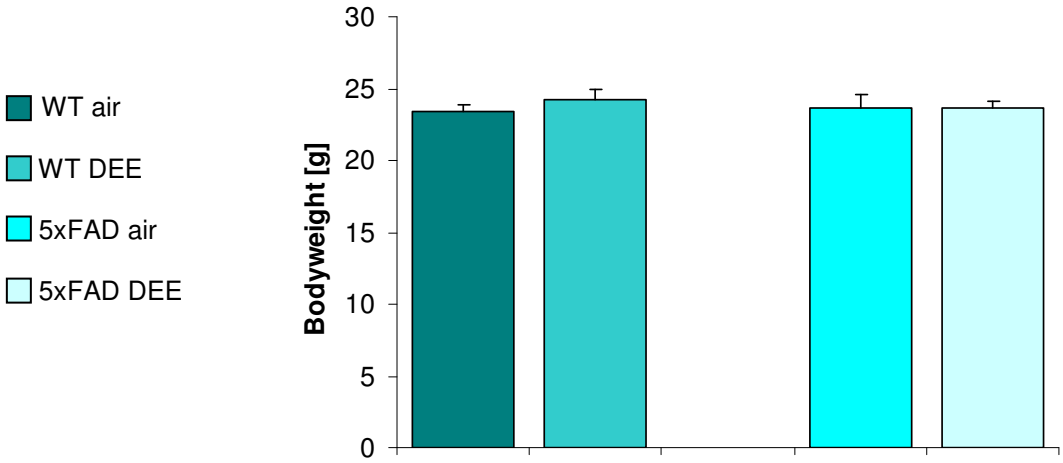

Figure 11: Bodyweight of 6 month old mice

Bodyweight measurements of transgenic 5xFAD and their WT littermates after 13 weeks of exposure with DEE or HEPA filtered air. Age of mice was 23 weeks. n=10 for WT air, n=8 for WT DEE, n=14 for 5xFAD air and n=18 for 5xFAD DEE.

After scarification of the animals, the weights of lung, heart, liver, kidney and spleen were also evaluated. After 3 weeks of exposure no differences in organ weights were detected. Neither between littermates and transgenic animals nor between the DEE and clean air exposed mice. The liver weights of both the WT and the transgenic 5xFAD mice showed some decreasing trends in association with the DEE exposure, but this did not reach a statistical significance (see Figure 12).

Taken together, there were no obvious differences in appearance of the mice at this time point of development and in relation to the 3 weeks DEE exposure. Both genetic background groups, treated or untreated, develop normally.

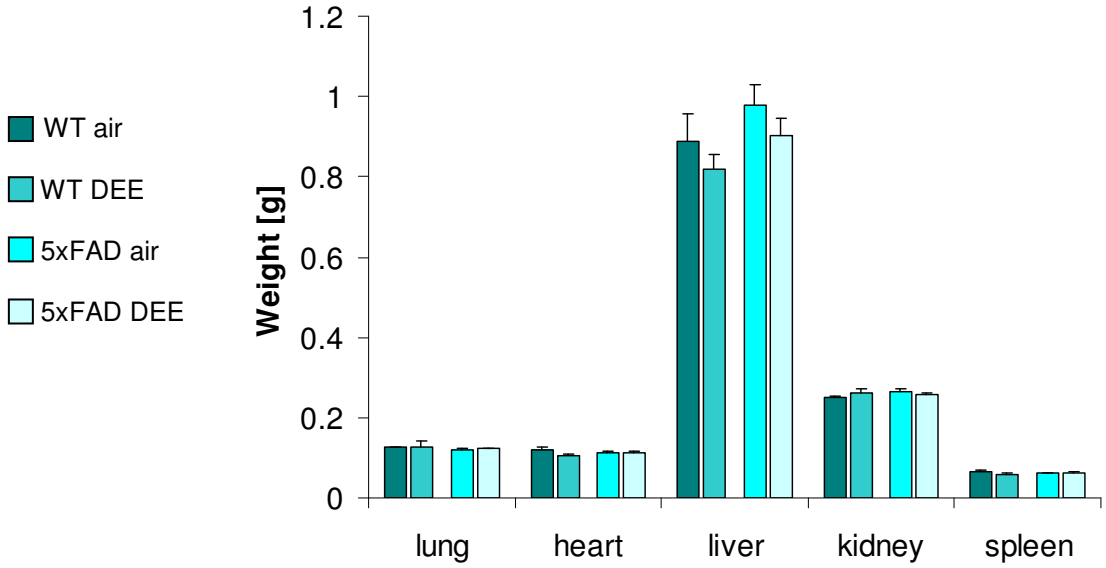

Figure 12: Organ weights of 3 month old animals

Weights of lung, heart, liver, kidney and spleen of transgenic 5xFAD mice and their WT littermates was evaluated after 3 weeks of exposure with DEE or filtered air. n=5 for WT air, n=5 for WT DEE, n=8 for 5xFAD air and n=8 for 5xFAD DEE

Also after the 13 weeks exposure time the weights of the organs of the mice were determined after sacrifice (see Figure 13). In concordance with the datas obtained for the younger animals (and after 3 weeks of exposure), no significant differences in organ weights could be detected. Also here some variations in liver weight could be noticed for the different genetic backgrounds, but again this did not reach statistical significance. Moreover, the pattern differed between the 3 and 13 weeks exposed animals, while in the younger animals liver weights tended to be higher for the transgenic animals (Figure 12), in the 10 week older animals the reverse was found.

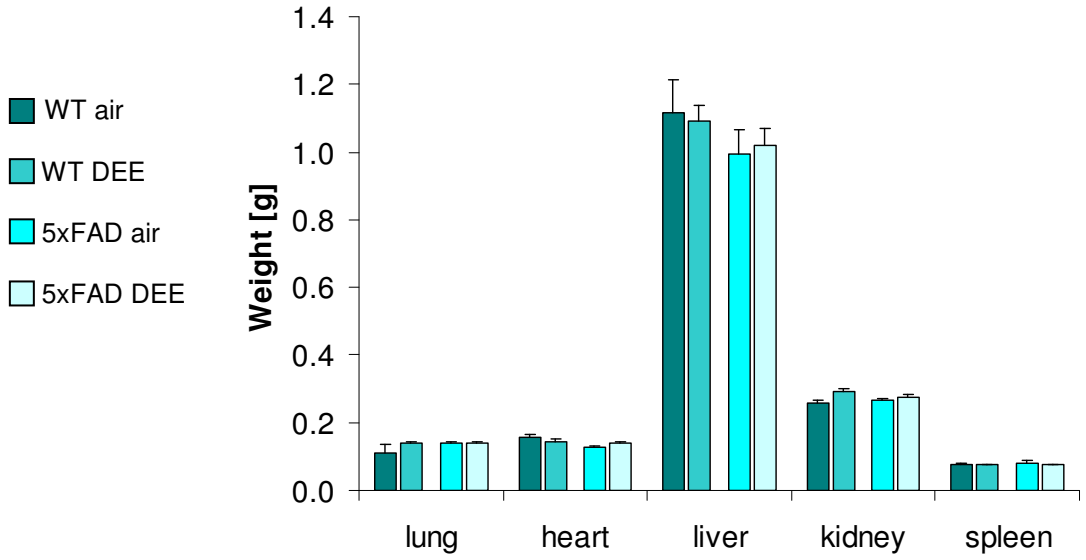

Figure 13: Organ weight of 6 month old animals

Weight of lung, heart, liver, kidney and spleen of transgenic 5xFAD mice and their littermates was evaluated after 13 weeks of exposure with DEE or HEPA filtered air. n=5 fir WT air, n=5 for WT DEE, n=8 for 5xFAD air and n=8 for 5xFAD DEE

4.2.2. Behaviour studies

Awareness and memory are principal items and requirement for certain behaviour. Due to such cognitive abilities and interpretation of circumstances and situations organisms are able to use this information to act target-oriented. Particular behaviour is the consequence. On this account diseases affecting cognitive functions have a strong impact. To understand underling processes from diseases like Alzheimer's the analysis of cognitive deficits in AD mouse models is vitally important. Learning and spatial working memory impairments can be detected with the help of numerous behaviour tests in diverse animal models. For the evaluation of the physical status of the mice in the present study a string suspension task was executed. Learning and spatial working memory in the mice was evaluated with the help of a Y-maze and X-maze task. These investigations were performed to obtain information about the development of neurodegenerative disease in the 5xFAD mouse model in general (i.e. comparison of transgenic and WT littermates) as well as in specific association with the DEE exposure (i.e. comparison of transgenic mice with WT littermates at both time points).

String suspension task

During this test the motor coordination and grip strength of the mice was tested. A normal developing mouse should not have any problems to hold itself on the string and escape to one of the platforms. With 3 month of age most of the WT mice were able to adhere at the string or even escape onto one of the platforms. Exposure to DEE particles or HEPA filtered air did not lead to differences in behaviour for this test, since for both backgrounds, the different exposure groups demonstrated similar abilities in motor coordination/grip strength (see Figure 14, panel A). The 5xFAD mice in general tended to have slightly more problems with hanging on the string and subsequent escaping. These differences were however not statistically significant. Therefore, it can be concluded that at this time point of development, there was no effects of the DEE exposure on the string suspension task detectable.

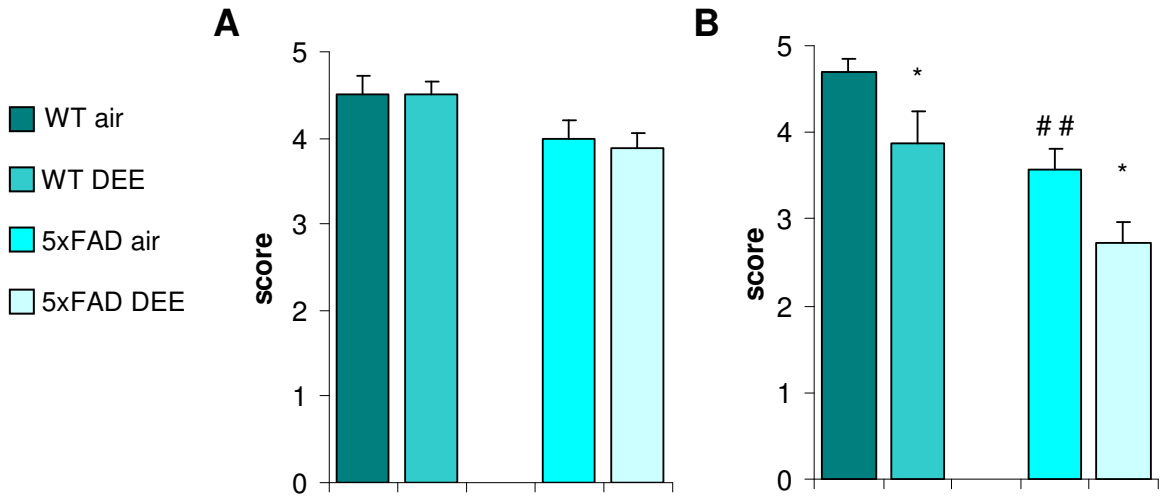

Figure 14: String suspension task

Two differently exposed groups of 5xFAD transgenic mice or their littermates were allowed to grasp a cotton string stretched between two vertical poles. Their ability to hang on the sting and escape to a platform within 60 seconds was measured with the help of a rating system from 0-5 (see 7.6.1. for details). (**A**) String suspension task after 3 weeks of inhalation exposure. n=12 for WT air, n=10 for WT DEE, n=14 for 5xFAD air and n=16 for 5xFAD DEE (**B**) String suspension task after 13 weeks of exposure. n=10 fir WT air, n=8 for WT DEE, n=16 for 5xFAD air and n=18 for 5xFAD DEE. * $p< 0.05$, versus filtered air exposed animals, background specific (WT air vs. WT DEE and 5xFADair vs. 5xFAD DEE), $^{\#\#}$ $p< 0.01$, 5xFAD versus WT air animals.

Motor coordination and grip strength was also determined via string suspension task for the 13 weeks exposed mice. The results are shown in Figure 14 (panel B). At this time point mice, the age of the animals was almost 6 months on average. The observed effects were clearly different from the effect seen after 3 weeks. The untreated WT littermates showed no impairments in grip strength with increasing age. In both stages of age, the mice completed the test without big efforts. However, the older aged groups of animals showed differences in motor coordination/grip strength concerning their genetic background and their exposure treatment. The 5xFAD mice had significantly larger problems to hold itself on the sting and to escape to one of the platforms. A clear and significant difference was observed in the test performance between the WT mice and the transgenic 5xFAD littermates at the age of 6 months (See figure 14 B). Moreover, the subchronic exposure to the DEE resulted in a significant diminishment in test performance in both groups. As a consequence of the 13 weeks exposure to the DEE, the WT littermates as well as 5xFAD transgenic mice appeared to have problems to hang on the string and escape to the platform.

Y-maze and X-maze task

The Y-maze and X-maze tasks were applied to reflect spatial working memory of mice by spontaneous alternation based on the natural affinity of mice to explore a novel environment.

The results of the Y-maze tasks are shown in Figure 15. After three weeks of exposure to DEE particles or clean air there was no difference in the test results (See panel A of the Figure). Air and DEE exposed WT animals showed similar alternation rates in both tests. Similarly, in the 5xFAD transgenic group exposure to the DEE particles did not lead to significant decreased or increased performance in the spatial working memory tests. Importantly, in the present study the 5xFAD Alzheimer mouse model also did not reveal a significant impairment in learning and memory compared to the non transgenic littermates. Consequently until this developmental stage there is no difference in spatial working memory by spontaneous alternation between the two groups of mice. As shown in panel B of Figure 15, the 6 months old mice showed slight differences in spatial working memory. The subchronic diesel exposure did not lead to adverse changes in working memory. The clean air exposed 6 month old 5xFAD mice behaved as expected, as these mice have more problems to explore

the Y-maze labyrinth in an efficient manner. The alternation rate was found to be significantly decreased when compared to the air treated WT littermates, underlining the developing Alzheimer phenotype.

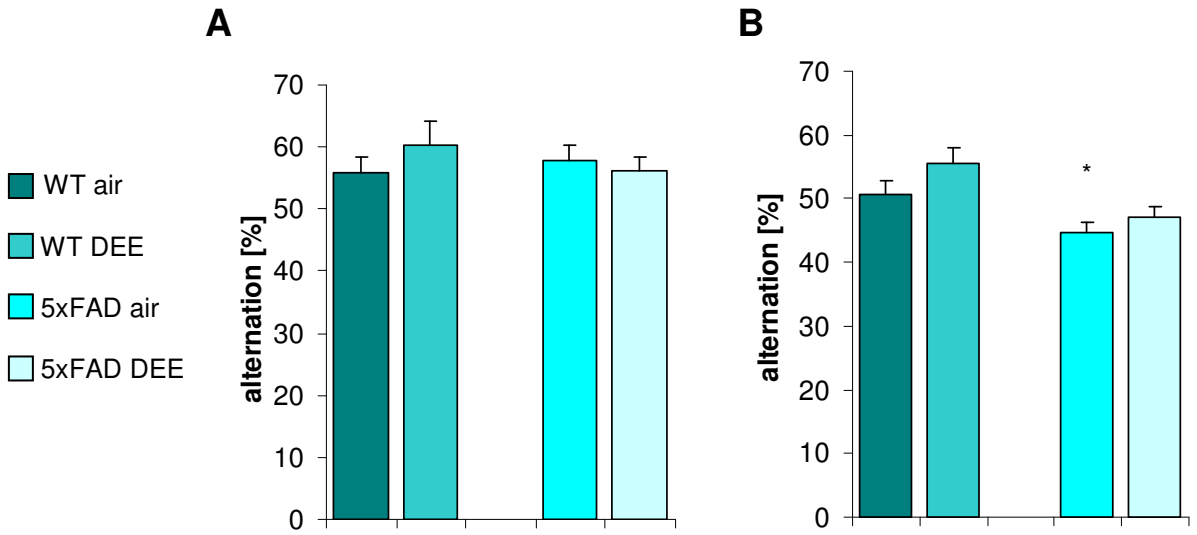

Figure 15: Y-maze task

5xFAD transgenic mice and their littermates were tested for spatial working memory by spontaneous alternation after exposure to DEE particles or HEPA filtered air.
(**A**) Alternation rate after 3 weeks exposure to DEE or filtered air. n=12 for WT air, n=10 for WT DEE, n=14 for 5xFAD air and n=16 for 5xFAD DEE. (**B**) Alternation rate after 13 weeks exposure to DEE or filtered air. n=10 for WT air, n=8 for WT DEE, n=16 for 5xFAD air and n=18 for 5xFAD DEE. * $p < 0.05$, versus WT air animals.

The X-maze task, which was also performed in present study, is based on the same principle as the Y-maze task. It can evaluate working and learning memory by calculating spontaneous alternation. Due to addition of a supplemental arm this method is more discriminative and smaller changes in behaviour due to the DEE inhalation would have been detectable. Results are shown in Figure 16. The tendencies of the results of the X-maze task after 3 and 13 weeks exposure did not differ from those that wee obtained with the Y-maze task. After 3 weeks, no significant differences between the diverse genetic background groups or the different exposure groups were detected (see Figure 16, panel A). The transgenic mice that were exposed to clean air do not show any differences in alternation rate when compared to their air treated WT littermates. Also the DEE particles exposure did not have any significant influence on working memory for both genetic

backgrounds. The results for the 13 week exposed animals are depicted in Figure 16, panel B. These data demonstrate that statistical significant problems with learning memory and spontaneous alternation in the transgenic 5xFAD mice can also be detected with the X-maze task. However, for the X-maze task also no effects of the DEE nanoparticles could be detected after 13 weeks of inhalation.

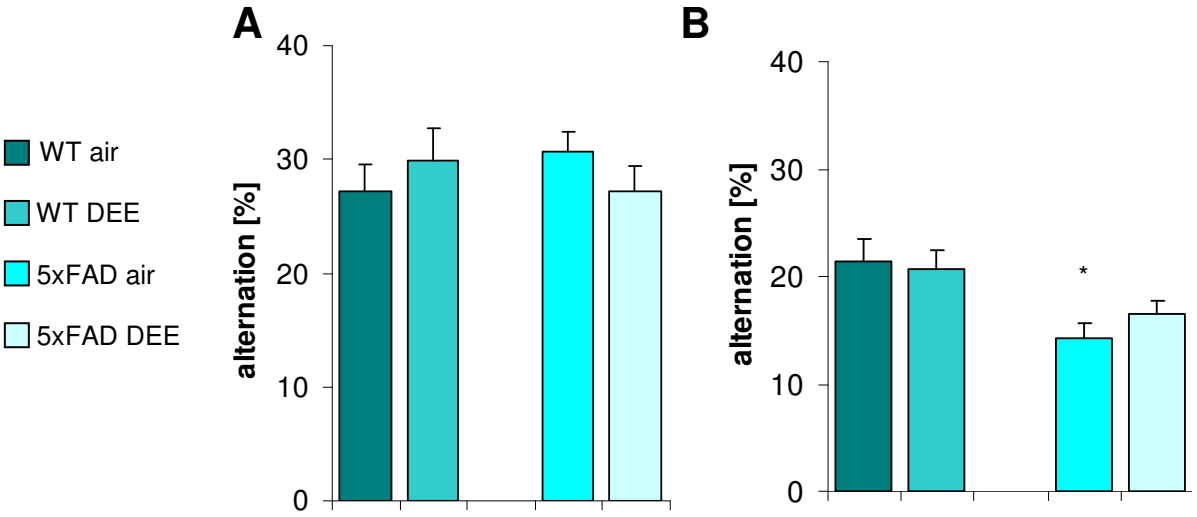

Figure 16: X-maze task

5xFAD mice and their littermates were tested for spatial working memory by spontaneous alternation in the X-maze task after exposure to DEE particles or HEPA filtered air.
(**A**) Alternation rate after 3 weeks exposure to DEE or filtered air n=12 for WT air, n=10 for WT DEE, n=14 for 5xFAD air and n=16 for 5xFAD DEE. (**B**) Alternation rate after 13 weeks exposure to DEE or filtered air. n=10 for WT air, n=8 for WT DEE, n=16 for 5xFAD air and n=18 for 5xFAD DEE. * $p < 0.05$, versus WT air animals.

Thus, the more sensitive X-maze task also failed to detect any significant effect of the exposure to DEE in the mice. However, it demonstrates the differences in spatial working memory between the transgenic mice and the WT littermates. The 5xFAD mice have significantly more problems with their working memory when growing older. Again, this effect was anticipated on the basis of the rapid developing Alzheimer phenotype in this transgenic mouse model.

The inhalation exposure to the DEE also appeared not to influence the general activity of the mice in the performed tests. As shown in Table 6, air and diesel treated

mice did not show significant differences in the number of arm entries after 10 min of exploration time, representing a similar activity state

When comparing the mice of the different genetic background, also no significant variations in total number of arm entries were observed. Thus, the observed differences in the alternation rate in the Y-maze and X-maze labyrinths appeared not to be due to a decreased activity of the animals. The general ratio of alternation rate decreased in non transgenic littermates with increasing age in the Y-maze as well as in the X-maze task. Therefore the younger group scored better, and an increase of age is associated with a loss of working memory.

Table 6: Number of total arm entries

	3 weeks exposure		13 weeks exposure	
	Y-maze	X-maze	Y-maze	X-maze
WT air	40.09 (±2.52)	52.92 (±3.85)	46.20 (±4.00)	61.10 (±4.43)
WT DEE	39.00 (±4.03)	53.10 (±5.83)	51.25 (±4.83)	63.63 (±5.63)
5xFAD air	37.75 (±3.17)	47.57 (±4.27)	56.50 (±3.53)	67.64 (±8.09)
5xFAD DEE	34.81 (±2.64)	46.69 (±3.55)	44.61 (±3.81)	56.11 (±3.92)

4.2.3. Increase of human Aß42 protein levels after DEE exposure

An augmented expression of Aβ42 protein level was measured in the frontal cortex, hippocampus and olfactory bulb from human individuals with residence in polluted cities (Mexico City) indicative of the progress of Alzheimer's disease (Calderon-Garciduenas et al., 2004). Based on the observations of Calderon-Garciduenas and co-workers, it was hypothesised that DEE exposure would cause an increase in Aß42 protein deposition in the brains of 5xFAD mice. Determination of total human Aß42 protein levels in the brain hemispheres of the 5xFAD mice was done with the use of an ELISA. With the applied method, both soluble and insoluble Aß42 fragments were detected. Figure 17, panel A, demonstrates that after 3 weeks of diesel particles exposure a massive increase in human Aß42 protein level (65.39 ng/mg Protein) can be detected in brain homogenates in comparison to the homogenates of the control group (13.55 ng/mg Protein). The human Aß42 level increased more than three fold in relation to the DEE exposure. This effect was highly significant. However, the effect of the DEE exposure could not longer be observed in

older animals (Figure 17, panel B). After exposure to the DEE particles for 13 weeks, the human Aß42 levels in the brains of the air-exposed transgenic animals aligned to those of the DEE-exposed animals. The clear difference in Aß42 levels in the air-exposed 5xFAD mice of different age (Figure 17, panel A versus B) are in concordance with the rapid disease development in this transgenic model.

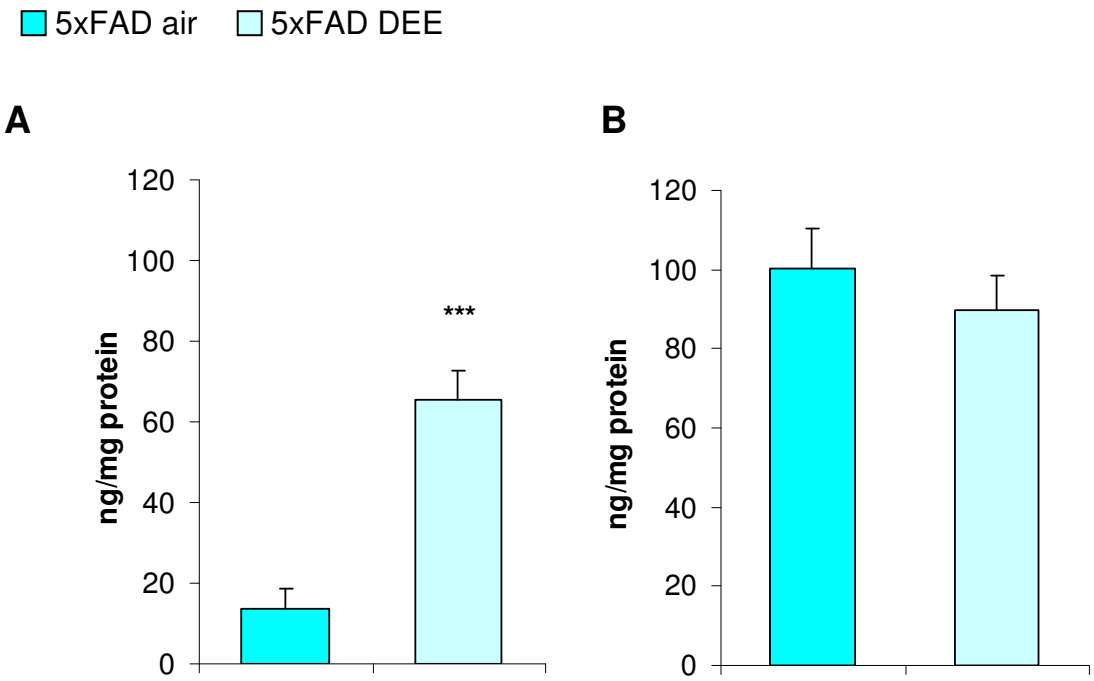

Figure 17: Quantitative analysis of human Aß42 protein levels
Human Aß42 protein levels were determined in brain homogenates via an ELISA according to the provided manual (Invitrogen) after 3 weeks of DEE or air exposure (**A**) or 13 weeks exposure (**B**). n=8 for 5xFAD air and n=8 for 5xFAD DEE. *** $p < 0.001$

4.2.4. Changes in plaque load of 5xFAD mice after DEE exposure

In order to visualize plaque accumulation in specific regions of the brains of 5xFAD mice, 4µm thick parasagittal brain slices were stained with an antibody against Aß42. Quantitative analyses was performed after the two different treatment time points and compared to control animals. Representative pictures of 5xFAD brain slices stained for Aß42 (reddish-brown colour) are shown in Figure 18. Differences in Aß42 plaque deposition in the younger 5xFAD mice were obvious in both analysed regions. The brains of WT animals were also stained, but no plaques could be detected in any sample.

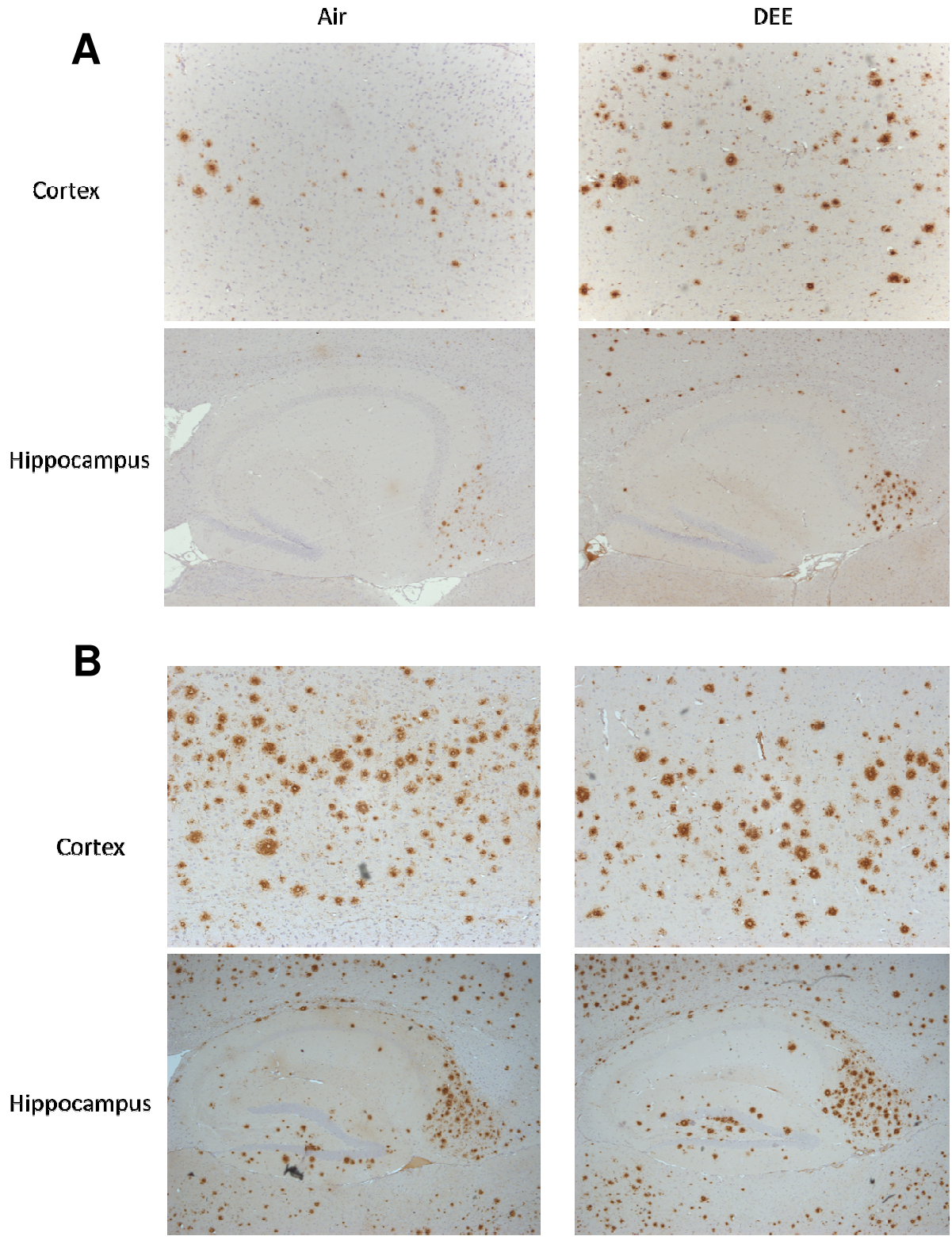

Figure 18: Representative pictures of Aß42 plaque staining in 5xFAD mice

Accumulation of Aß42 (reddish-brown colour) in cortex and hippocampus in 5xFAD mice exposed to diesel particles or air. Aß42 was localised by IHC in sections of paraffin-embedded brain hemispheres. Hippocampus (50 x magnification) and cortex (100 x magnification) from the same animal are shown for each time point. (**A**) 3 weeks exposed 5xFAD animals; (**B**) 13 weeks exposed animals.

After 3 weeks of nanoparticle exposure, plaque formation was found to differ between the different treatment groups (see figure 19A). Animals exposed to DEE particles tended to show increased plaque formation in the cortex as well as in the hippocampus in comparison to the air exposed animals. The plaque development in the cortex appeared to be much higher in comparison to control animals and was found to be statistically significant. However, also the hippocampus revealed a clear trend. Both areas showed higher plaque load levels after DEE particles exposure.

The observed trend could no longer be observed after 13 weeks of particle exposure (see Figure 19, panel B). The range of the areas which were covered with Aß42 protein plaques in the cortex and the hippocampus were similar in treated and untreated animals. In the hippocampus of the DEE particle exposed mice even show a slightly lower level of plaque deposition, but this was not significant. Plaque load in cortex is more or less the same in both groups, here, no clear difference can be detected.

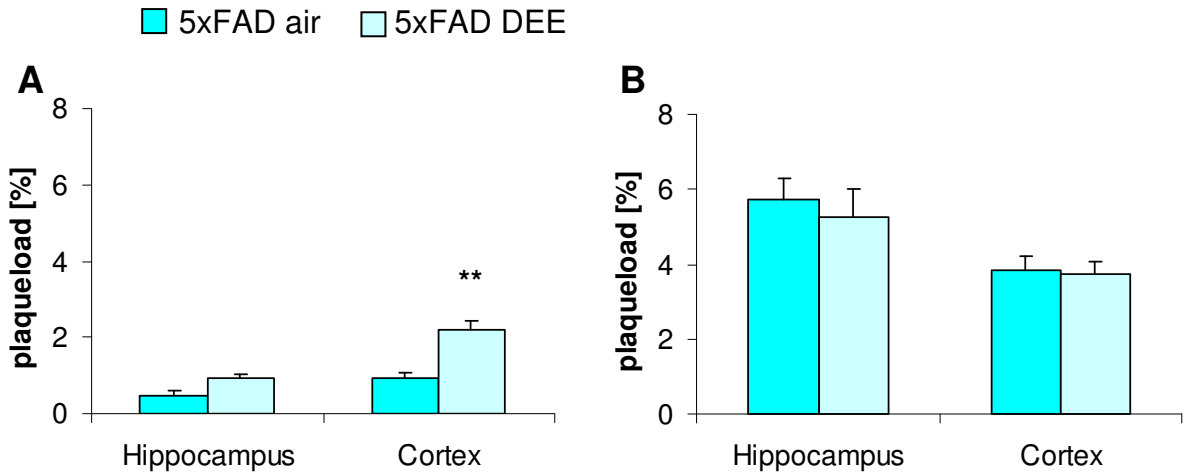

Figure 19: **Quantitative image analyse of Aß42 IHC**.
Parasagittal brain slices were stained with an antibody against Aß42. Areas of hippocampus and cortex were quantitative analysed. (**A**) Analysis after 3 weeks of diesel particle exposure. For hippocampus: n=10 for 5xFAD air and n=10 for 5xFAD DEE, for cortex n=10 for 5xFAD air and n=16 for 5xFAD DEE, ** $p < 0.01$. (**B**) Analysis after 13 weeks of diesel particle exposure. For hippocampus: n=12 for 5xFAD air and n=11 for 5xFAD DEE, for cortex n=15 for 5xFAD air and n=16 for 5xFAD DEE.

After 3 weeks, plaque formation was more pronounced in cortex of animals in contrast to the hippocampus. In the mice of increased age the Aß42 plaque distribution changed. Plaque accumulation in the brains of the air exposed as well as DEE exposed 5xFAD mice was found to be more prominent in hippocampus than in cortex.

4.2.5. No pulmonary inflammation after diesel inhalation in lungs of mice

Many studies have provided experimental support for the associations found in epidemiological studies between PM exposure and adverse pulmonary effects. Upon deposition PM can trigger inflammatory processes potentially leading to airway obstruction, compromised gas exchange and the exacerbation of pre-existing conditions such as asthma or chronic bronchitis (Dockery et al., 1993; Nel et al., 1998; Samet et al., 2000). PM exposure is associated with lung pathology including chronic obstructive pulmonary disease (COPD), bronchitis, asthma and lung cancer.
The subchronic inhalation of diesel engine exhaust particles may, in view of the applied concentrations, lead to a slight but significant inflammation in lungs of exposed animals. These possible inflammations may cause side effects which can lead to an elevation of cytokine levels in blood. Blood circulation can possibly transport inflammatory proteins to different target organs in mice where they may lead to harmful disadvantages. To evaluate whether the DEE exposure resulted in pulmonary inflammation in the 5xFAD mice and their WT littermates, lung sections were stained via HE staining. Representative pictures are shown in Figure 20. The immunohistochemical evaluation of the lungs of the animals by HE staining indicated that there were no obvious differences in tissue structure. Lung tissues of 5xFAD mice were found to be comparable to those of their WT littermates. Also the exposure to DEE nanoparticles, for 3 as well as 13 weeks, did not cause structural changes within the pulmonary tissues. The deposition of clustered diesel particles can be clearly detected in the lungs of the DEE exposed mice after 3 and 13 weeks of exposure (black arrows). However, these depositions do not lead to obvious development of structural changes within the lung tissues of treated animals and no pathological changes were detected.

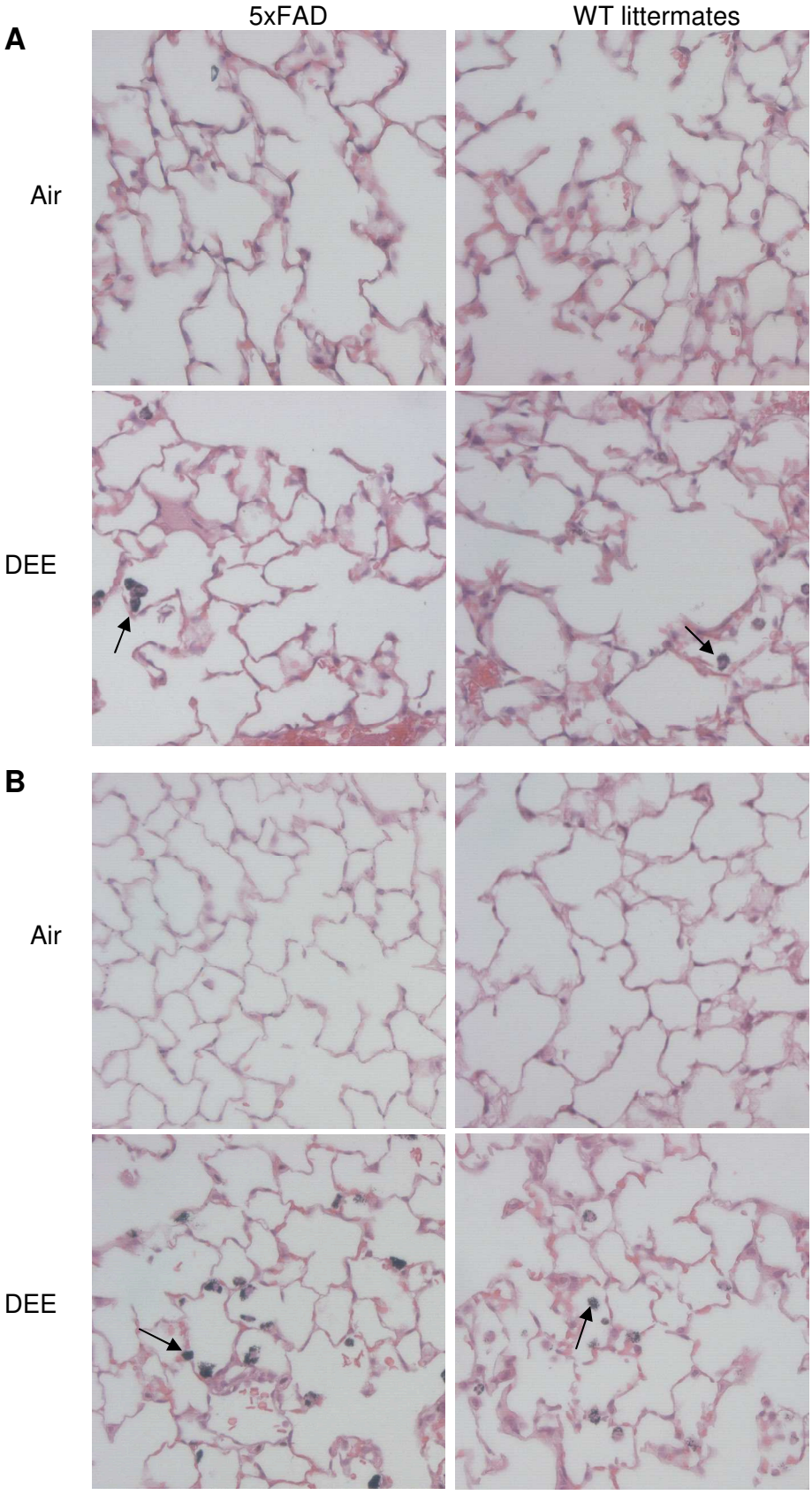

Figure 20: Haematoxylin-Eosin staining in 5xFAD and WT mice

Representative pictures of lung sections after HE staining to evaluate tissue modifications between different background and treatment of mice (400 x magnification). (**A**) After 3 weeks DEE or air exposure (**B**) After 13 weeks DEE or air exposure

5. Discussion

Since the earliest stages of humanity, people are exposed to particulate matter. It is becoming increasingly clear that inhalation exposure to PM can lead to or exacerbate various diseases, which are not limited to the lung but extend to the cardiovascular system and possibly other organs and tissues.

Regarding the investigation of the effects of inhalation exposure to PM, both long-term and acute effects need to be assessed. Periods with pronounced acute particulate air pollution due to specific climatologic circumstances are known to lead to augmented mortality rates and increased incidence of cardiopulmonary diseases. Aside from such acute events, it is important to consider that people are in fact also chronically exposed to lower concentrations of particulate air pollution in ambient air. Importantly, high acute exposures to particulate matter also contribute to chronic adverse health effects (Pope, 2000).

The central theme of the present thesis was the investigation of the potential contribution of nanoparticle-inhalation to the acceleration of Alzheimer's disease as well as the evaluation of the relevance of oxidative stress as a potential underlying mechanism in neuroinflammatory responses triggered by nanoparticles. Therefore, two experimental approaches were followed with the use of two specific mouse models. The effects of a controlled acute exposure to carbon nanoparticles (CNP) on mouse lung and brain were investigated in the $p47^{phox}$ knockout mouse model (STUDY 1). For the investigation of the effects of long-term exposure, the 5xFAD mouse Alzheimer's disease model was used in a 3 and 13 weeks exposure scenario to diesel engine exhaust particles (STUDY 2).

STUDY 1

There were two principal reasons to select the $p47^{phox}$ knockout mouse model for the first study. On the one hand, it has been revealed from a large number of studies that the toxic effects of inhaled ambient PM and nanoparticles, are driven by the generation of ROS and the associated induction of oxidative stress (reviewed in Donaldson et al., 2003; Li et al., 2003; Unfried et al., 2007). Apart from radical-generating processes due to intrinsic chemical properties of particles, ROS are also known to be generated from cellular sources upon direct interaction with particles.

Herein, the classic NADPH oxidase enzyme complex (NOX2) in professional phagocytic cells has been recognised as a major source and driver of pulmonary lung diseases like silicosis and cancer (Castranova et al., 1996; Knaapen et al., 2004). On the other hand, increased oxidative stress has been implicated in various diseases of the central nervous system including Alzheimer's disease (Quinn et al., 2006). Also, in the brain, oxidative stress and associated neurotoxic effects are considered to be mediated to significant extent by the generation of superoxide ($O_2^{\bullet -}$) from microglial NADPH oxidase activity (Block and Hong, 2005). Taken together, it is therefore highly justified to investigate the potential contribution of NAPDH oxidase to the oxidative stress responses in the brain in response to inhalation of nanoparticles.

The relevance of the phagocyte NADPH oxidase in nanoparticle-induced neurotoxicity has emerged from a number of recent *in vitro* studies. Long and co-workers investigated the potential neurotoxic effects of TiO_2 nanoparticles, a type of nanomaterial which has found its way already in many nanotechnological applications (Long et al., 2007). They exposed neurons *in vitro* to these nanoparticles, either in the presence or absence of microglia, and revealed that TiO_2 was non-toxic to isolated neurons alone. However, in the presence of microglia damage to neurons was observed, and in parallel experiments it was also demonstrated that the TiO_2 nanoparticles stimulate microglia to produce ROS. In a similar investigation, the contribution of microglia in particle-induced neurotoxicity could also be shown for diesel exhaust particles (DEP) (Block et al., 2004). Neuron-glia cultures from NADPH oxidase deficient ($phox^{-/-}$) mice showed no neurotoxic effects after treatment with DEP in contrast to the treated cultures from the wildtype mice. With the subsequent use of the phagocytosis inhibitor cytochalasin, the investigators could demonstrate that DEP selectively damage dopaminergic neurons through the phagocytic activation of microglial NADPH oxidase (Block et al., 2004).

An important drawback of the aforementioned *in vitro* studies is that unrealistically high particle concentrations were used. The kinetic studies performed by the groups of Oberdorster (Oberdorster et al., 2004; Oberdorster et al., 2002) and Kreyling (Kreyling et al., 2002) clearly demonstrate that nanoparticles can translocate into the central nervous system. However, the actual amount of particles that reached this target was found to be only a minor proportion of the exposure dose and the calculated amount that will initially deposit in the upper airways (including the nasal

passages) as well as in the distal lung (Oberdorster et al., 2004). Moreover, *in vitro* studies with brain cells also do not take into account that effects of particles may also be driven in an indirect manner i.e. inhaled nanoparticles may act “at a distance” as discussed for other secondary targets of the body like the cardiovascular system. Therefore, it is necessary to assess effects of nanoparticles beyond the respiratory tract, as well as their potentially underlying mechanisms of action, through *in vivo* studies. In the field of inhalation toxicology, for mechanistic investigations often the methods of intratracheal instillation or pharyngeal aspiration are applied (e.g. Tsurodome et al., 1999; Schins et al., 2004). Two of the main reasons for this are, that inhalation studies are much more expensive and complicated to design and perform in a controlled manner. The major disadvantage of intratracheal application studies is that the materials enter the lungs in a non-physiological manner and as a bolus unlike inhalation approaches. Moreover, this method bypasses the nasal compartment and thus excludes the potential olfactory pathway mediated effects. To investigate brain effects some investigators have therefore used intranasal application. Although this approach has proven to be useful for basic mechanistic investigations (e.g. Elder et al., 2006), it is faced with similar dosimetry and physiology limitations and therefore obscures quantitative interpretations of the biological significance of findings on nanoparticle-induced neurotoxicity. Thus, for the investigation of effects of inhaled nanoparticles on the brain, controlled inhalation studies are pivotal.

In previous investigations in our laboratory, it could already be demonstrated that a 2 h inhalation exposure to diesel engine exhaust particles causes increased mRNA expression of the oxidative stress marker gene HO-1 in rat brain (van Berlo et al., 2010a). However, the role of NADPH oxidase derived ROS in these effects remained to be investigated. Therefore, in the present study, a short term nose-only inhalation exposure study was performed in $p47^{phox-/-}$ mice, in which the phagocytic NADPH oxidase function is abrogated, versus wildtype mice. As an exposure system an electric spark discharge device (Palas Soot Generator) was used. With this method, elemental carbon nanoparticles were generated from graphite electrodes. Combined with the nasal-exposure facilities, this allowed for a highly controlled nanoparticle dose delivery to the animals. Importantly, the exposure apparatus and method of spark discharge that was used was identical to those that were used in the kinetic studies performed by Oberdorster and colleagues (Oberdorster et al.,

2004).The detailed particle characterisation in the animal exposure unit showed that the exposure conditions were rather similar in the respective studies. In the Oberdorster study the average diameter of the aerosols was 35 to 37 nm, and animals were exposed for 6 hours at a concentration of 150 to 170 µg/m^3 air. In the present study, the soot generator was set in such a manner that similar mass exposure levels were created (i.e. 154 µg/m^3), close to the maximum that could be reached in the exposure facilities without shifting the particle size distribution too strongly to the right, ensuring exposure in the nano-size range. In the present condition this resulted in slightly larger particles (i.e. average of 58.8 nm) compared to those that were generated in the Oberdorster laboratory. The major difference between both studies is that for the current mechanistic investigations a mouse model was used, whereas the kinetic study was performed in rats (Oberdorster et al., 2004). Because of the obvious differences in respiratory tract morphology and physiology between both species, no quantitative statement can be made concerning actual translocation rates of the nanoparticles into the brains of the mice. However, the principal shape and chemical composition of the nanoparticles does not differ for both studies and translocation of this type of nanoparticles, as demonstrated for the rat, can therefore also be assumed in a qualitative manner in present mouse study.

The main outcome of the short term inhalation study was that the mRNA expression levels of the selected marker genes of oxidative stress, inflammation and/or oxidative DNA damage were not altered in the brains of the mice after the carbon nanoparticle exposure. Neither in the WT mice nor in the $p47^{phox-/-}$ mice significant changes in the mRNA expression of the selected genes could be detected. In a previous study, we could observe enhanced mRNA expression of HO-1 and COX-2 in specific brain regions of the rat (wildtype) after a 2 h nose-only inhalation to diluted DEE (van Berlo et al., 2010a). There are several likely reasons for the observed differences between both studies. Probably of most importance, in the previous study the dose (i.e. 1.9 mg/m^3) as well as the type of nanoparticle exposure (i.e. diesel engine exhaust) was different. Diesel particles contain considerable amounts of metals and organics attached at their carbonaceous core. Therefore, this was not a “pure” nanoparticle exposure, unlike the discharge generated carbon nanoparticles, which are known to be virtually free of contaminants. Moreover, the exhaust of diesel engines, apart from particles, also

contains various non-particulate compounds such as carbon monoxide (CO), nitric oxides (NO, NO_2), sulphur dioxide (SO_2), hydrocarbons, formaldehyde and transition metals (Scheepers and Bos, 1992). Although the rats were exposed to highly diluted diesel exhaust, a contribution of non-particulate compounds cannot be entirely ruled out. In future, studies may be designed that compare the potential neurotoxic effects of volatile versus particle-bound components in rodent models. This can be achieved with the use of HEPA filter systems.

Another major reason for the observed contrast between present and previous study (van Berlo et al., 2010a) is that experiments were carried out in two different species. The different responses may relate to the variations in respiratory tract morphology and physiology and associated differences in nanoparticle deposition and translocation between rats and mice. Moreover, it may be that rats respond more strongly to nanoparticles after inhalation than mice, as is known for chronic high exposure studies (Bermudez et al., 2004).

Finally, the differences in post-exposure times for both studies should be considered. The mRNA expression effects in the brains of the mice were determined with an average 3 h post-exposure recovery time after the 4 hours exposure. In the rat study effects were evaluated 4 h and 18 h after a 2 hours exposure. It might be argued that the average time interval between initial exposure and the dissection (i.e. 7 hours) of the mouse brains was not long enough to cause sufficient translocation of the nanoparticles. However, in the rat study HO-1 upregulation was observed within 6 hours (van Berlo et al., 2010a).

Whereas the mRNA expression changes were absent in the mouse brains, in the lungs a significant induction of Ogg1 as well as of APE/Ref-1 could be observed. In line with present findings, Risom and colleagues found that short-term exposure to diesel exhaust particles resulted in an enhanced expression level of Ogg1 in mouse lungs (Risom et al., 2003). In a recent study in our laboratory, a 4 h exposure to carbon nanoparticles was found to cause a significant upregulation of APE/Ref-1 in rat lungs (Wessels et al., 2011). The enzymes Ogg1 and APE/Ref-1 represent two major components of the base excision repair pathway (BER) of oxidative DNA lesions (Klungland and Bjelland, 2007; Marnett, 2000; Tell et al., 2005). Alterations in the mRNA expression of these genes have also been proposed as sensitive markers for the detection of oxidative stress (Albrecht et al., 2005; Rusyn et al., 2004;

Tsurudome et al., 1999). Interestingly, the mRNA levels of both Ogg1 and APE/Ref-1 were found to be only increase in the nanoparticle exposed WT mice and not in the $p47^{phox-/-}$ mice. As such, this would indicate that CNP exposure leads to a mild induction of oxidative stress in a p47phox NADPH oxidase dependent manner in the lungs of mice. Since Ogg1 and Ape/Ref-1 mRNA expression levels were not affected in the brains of the same animals, this suggests that the applied particle dose in present study was to low to trigger oxidative stress in this distant organ. Nevertheless, the relevance of the p47phox NADPH oxidase in nanoparticle-induced oxidative stress could be shown for the first time. This also provides further support for the relevance of the present knockout mouse model in future nanoparticle toxicity studies.

A final interesting observation in the current animal study was the significantly lower constitutive protein expression of IL-1ß in the mouse brains of the $p47^{phox-/-}$ mice. IL-1ß is constitutively expressed in the normal brain (Vitkovic et al., 2000a; Vitkovic et al., 2000b) and it has been shown that IL-1ß modulates hippocampal functions including memory and learning and neural plasticity (Avital et al., 2003). In contrast, high levels of IL-1ß have been implicated in impaired memory functions (Barrientos et al., 2009) and have been associated with various different neurodegenerative disorders like Alzheimer's disease (Griffin et al., 1989). However, in the present study, CNP exposure did not change the protein level of IL-1ß. These findings are in line with the absent mRNA expression changes in the mouse brains. However, the over all absence of CNP-triggered brain effect may also be due to the applied inhalation protocol. The mice were subjected to a nose-only inhalation, which requires fixation of the animals in tubes for the whole exposure duration. Exposure to acute stress has been described to increase the expression of various pro-inflammatory cytokines in brain (Goshen and Yirmiya, 2009). It has been shown that immobilization of rats for several hours in plastic restrainers resulted in an increase of IL-1ß levels in hypothalamic of these stressed animals (Shintani et al., 1995). Such a stress effect may also have overlaid the CNP-exposure effects. This aspect was taken into consideration in the design of the second study that was performed in this thesis. Instead of nose-only inhalation, the subchronic inhalation study with the 5xFAD mice was performed in whole body exposure units.

Further studies are needed to determine the effect of inhaled CNP to the brain. Also the role of the NADPH oxidase system in this context has to be evaluated in additional studies. As mentioned before, the observations in the lung have demonstrated the principle suitability of the model. However, for future studies with NADPH oxidase deficient versus proficient mice, exposure to a potentially more toxic type of nanoparticle, e.g. DEE particles or CAPS should be considered as well as prolonged exposure and/or post-exposure times. However, the NADPH oxidase deficient mice are prone to bacterial infections and granuloma formation and hence can be maintained only under strict SPF conditions (Quinn et al., 2006). Because of these intrinsic difficulties in the handling of this mouse model, subchronic inhalation studies with real life ambient particles will be involve tremendous experimental adaptations.

STUDY 2

The focus of the second study was on the investigation of adverse effects in the brain tissues of mice after subchronic inhalation to diesel engine exhaust, representing the predominant source of environmental NP in urban environments. The used 5xFAD mouse model mimics various features of AD development and progression in humans. The study hypothesis was that a subchronic inhalation exposure to environmental NP would accelerate the age-related onset of AD-like pathology in this mouse model.

The whole body and organ weights of mice were determined at the end of the 3 week as well as the 13 week exposure. This was done to evaluate whether the genetic background and/or the DEE exposure would lead to differences in phenotypes resulting in changes in body and organ weights. All assessed organs showed more or less the same weights. Evaluation of bodyweights can give information about physical activity of animals as it is well known that genetic background and physical activity itself has influence on this parameter. The response to exercise can be measured by change in body weight. Physical activity is beneficial in reducing the weight and also associated with health problems often experienced by individuals as they age (Nehrenberg et al., 2009). Exposure to DEE particles led to a decrease in bodyweight after 3 weeks of particle exposure. However the results

from the Y-maze and X-maze tasks revealed no significant differences in exploring activity (i.e. total number of arm entries). As such, the reduction of body weight may be the result of a reduced food intake. However, since food was given ad libitum and its consumption not measured, no statement can be made on its role. A study aspect that needs to be taken into account was that the whole body exposure causes deposition of diesel particles onto food pallets in the experimental cages. Thus, the taste of food has possibly changed and perhaps resulted in a decreased food intake even if food pallets were changed 3 times a week. Adaption to the changed taste may also explain that differences in bodyweight became absent after 13 weeks of exposure. No differences between non-transgenic and transgenic mice in bodyweight were detected at this time point. Other labs observed phonotypical differences after 9 month of age (Jawhar et al., 2012).

Diesel exhaust particles (DEP) form a special group among the various types of particles that are used to address mechanisms of toxicity of PM. They contain considerable amounts of metals and organics onto their carbonaceous core, and can be generated and subsequently sampled in a highly controlled and reproducible manner for toxicology studies. Accordingly, while representing a dominant component of PM in urban environments, DEP are typically used a model particle and lots of studies have been performed investigating the contribution of diesel engine exhaust to the adverse health effects that are attributed to air pollution. Traditionally, these studies have focused on respiratory tract diseases (e.g. Mauderly, 2000; Marano et al., 2002; Rao et al., 2005), but in more recent years on extrapulmonary effects, e.g. cardiovascular diseases (Mills et al., 2007). Chronic inhalation to DEE nanoparticles is suggested to lead to behavioural deficits like memory impairments in humans. In a cohort of elderly women, exposure to traffic-related PM was associated with mild cognitive function impairments (MCI) (Ranft et al., 2009). Concentrations of black carbon in ambient air have been associated with cognitive impairment in children (Suglia et al., 2008). Moreover, children from Mexico City, notorious for its high levels of air pollution, exhibited cognitive deficits compared to children from a less polluted city (Calderon-Garciduenas et al., 2008a).

Cognitive deficits and memory impairments in mice caused by DEE particle inhalation were not detected in this thesis. Cognitive impairments might be detected with the help of additional behavioural test. The 5xFAD mouse model demonstrated

no differences in alternation rate if compared to their littermates after 3 months of age but after 6 months of age significant differences could be detected. Alternation rate represents an indicator of spatial working memory impairments. These data correspond well with the recent study of Jawhar and co-workers, who revealed that 5xFAD mice develop an age-dependent motor phenotype in addition to working memory deficits with increasing age (Jawhar et al., 2012). In the present thesis, the transgenic mice comprised also greater deficits in motor condition. The string suspension task showed highly significant differences in clean air exposed 5xFAD mice versus the clean air exposed littermates, however only at later stage, i.e. at 6 month of age. It has been shown that motor disturbances can be identified predominantly in later stages of Alzheimer's disease (Suva et al., 1999).

A key finding of this thesis was that in response to the 13 weeks DEE inhalation exposure both the transgenic and the non-transgenic mice showed differences in the sensory-motor task in comparison to the corresponding air exposed animal groups. This deficit may occur due to axonal defects. Axonopathy and deficits like swelling of accumulated motor proteins, organelles and vesicles were detected in an APP transgenic mouse model carrying the Swedish mutation (Stokin et al., 2005). Additionally, motor impairments have been observed in various studies with AD patients (O'Keeffe et al., 1996; Pettersson et al., 2005; Wirths and Bayer, 2008). Motor dysfunctions have also been reported to occur in patients with MCI (Kluger et al., 1997) which is considered to be a preliminary stage before the onset of AD (Aggarwal et al., 2006). As such, this may provide a link between the currently observed motor impairments in DEE exposed mice, and the observed associations between chronic exposure to traffic associated PM and MCR in elderly women (Ranft et al., 2009). In recent inhalation studies with DEE region-specific inflammatory and oxidative stress responses have been identified in the rat brain (Gerlofs-Nijland et al., 2010; van Berlo et al., 2010a). Inflammation is a significant cause of axonal loss in patients suffer from AD or multiple sclerosis (Bjartmar and Trapp, 2003; Stokin et al., 2005). Interestingly, prenatal exposure to diesel engine exhaust has also been reported to lead to behavioural changes in mice, indicated by decreased spontaneous locomotion activity (Suzuki et al., 2010).

To some extent, quantitative differences are seen in the behaviour tests performed in this thesis and data reported in literature with various mouse AD models. Differences between the different models likely relate to strain specific

pathology development, but can also be influenced by laboratory specific variations. Differences between present data with the 5xFAD model and currently available data in literature for untreated mice may be explained by the specific study design. An important aspect in our study to take into account is that the control animals were housed in inhalation units (i.e. clean air exposed). This altered microenvironment may very well have influenced the onset and pace of AD features. Environmental enrichment has been shown to affect cognitive deficits as well as amyloid deposition in AD mouse models (Jankowsky et al., 2005; Lazarov et al., 2005). Another major aspect is that the behaviour findings may be influenced due to the fact that the experiments took place during daytime leading to differences in explorative behaviour. Mice are night active animals, normally sleeping over the day. In the present study an inversion of sleep-pattern design was not considered; in the field of inhalation toxicology, exposures are typically performed in sleeping animals. It will be interesting to investigate the effects of inhaled nanoparticles in mice in relation to activity, also in view of the role of circadian rhythms in brain diseases (e.g(Bedrosian et al., 2011; Vloeberghs et al., 2004).

It is hypothesized that interactions between environmental factors and genetic factors are implicated in development of AD. Traditionally, AD mouse models have been mainly used to asses potential beneficial aspects of candidate-drugs or environments; exogenous factors that may accelerate AD like pathology are less often reported. Induction of acceleration of AD like pathology in the 5xFAD transgenic mouse model was recently demonstrated by Devi and colleagues (Devi et al., 2010). Exposure to 5 days restrained stress led to an acceleration of Aβ42 levels in 3 months old 5xFAD transgenic mice (Devi et al., 2010). Aβ42 levels were significantly increased in female 5xFAD mice after stress exposure, moreover Aβ plaque formation was clearly enhanced. The results from Devi et al and from this thesis show that genetic predisposition can increase with higher vulnerability against environmental stressors, manifesting in acceleration in AD like pathology. As the 5xFAD mouse model represents a highly aggressive mouse model for AD these effects cannot longer be observed when the mice grow older. Until the age of 6 months Aβ42 levels in brain homogenates of the 5xFAD mice raise in drastically dimensions (Oakley et al., 2006). The development of the 5xFAD phenotype after 6 months of age may overlay the effects evoked by the DEE inhalation exposure. Data

of Aβ42 levels in brain homogenates of clean air exposed 5xFAD mice at 3 months and 6 months proceeded similar to those that were observed by Oakley et al., underlining the fact that the mouse model developed as expected. The data obtained in the present study has also revealed that in future (nano)particle studies with the 5xFAD model, exposures should occur in animals younger than 6 months.

Exposure to particulate matter (Laden et al., 2006; Pope et al., 2002) and diesel engine exhaust particles (Mauderly, 2000) has been associated with the increased occurrence of pulmonary diseases. Physicochemical particle properties that have been shown to contribute to the toxicity of particles include particle size, solubility as well as surface-bound organic compounds (Schins, 2002) (Donaldson et al., 2003; Donaldson and Tran, 2002). In the pathologic progression of different diseases, chronic inflammation has been highly implicated (Azad et al., 2008; Knaapen et al., 2004). Exposure to diesel nanoparticles for 3 and 13 weeks at the selected dose level did not lead to detectable lung pathology indicating that strong side effects due to chronic lung inflammation can be excluded. This raises the hypothesis that inhaled nanoparticles may affect the brain in a direct manner after they are transported to the brain (Oberdorster et al., 2004). Further studies could be considered to determine the relative impact of the olfactory versus the alveolar routes in the observed effects.

The differences in the design of the CNP study and the DEE study make it not easy to compare both. In the respective inhalation studies a difference in terms of the gender of the animals existed: in the CNP study both male and female mice were used, in the DEE study only female mice were evaluated. It is well known that the use of different genders may result in different outcomes, due to different hormonal balance in male and female animals. Recent and ongoing studies in our laboratory on pulmonary toxicity of engineered nanomaterials have for instance revealed a difference in susceptibility to develop lung fibrosis (Boots et al., manuscript in preparation). In mouse models of AD, including the 5xFAD model marked contrasts in disease development have been shown, and also in humans, it is well known that woman have a higher risk of developing AD. These sex differences still remain after age adjustment (Baum, 2005). As already discussed, also the use of different types of exposures likely causes different types and extent of diseases outcomes. The

surfaces of the spark generated CNP particles are virtually free of additional toxic components. Therefore this study represents a “pure” nanoparticle inhalation study. In contrast, the 5XFAD mice and their littermates were not exposed to clean nanoparticles, but a mixture of carbonaceous particles with a significant amount of surface-bound constituents including organics, metals and gases (Scheepers and Bos, 1992). Consequently synergistic effects can be elicited (Saldiva et al., 2002). Both, the core particles and their constituents are relevant for PM-elicited effects (Knaapen et al., 2002; Li et al., 2003; Shi et al., 2003). A further aforementioned difference between both studies involved the method of inhalation. The p47$^{phox-/-}$ mice were exposed via nose-only inhalation, whereas whole-body inhalation was used in the 5xFAD animals. Therefore, to some extent the 5xFAD mice may also be exposed in a more significant way to particles via the oral route. After nose-only inhalation, only a proportion of inhaled particles will reach the gastrointestinal tract via the mucociliary clearance route (Oberdorster et al., 2004; Oberdorster et al., 2002). During whole body exposures particles may also be taken up into the gastrointestinal tract after their deposition onto food pellets. Moreover, mice also extensively clean their fur, by which they can swallow further numbers of particles that deposit on their hair.

Obviously, the use of different time points also represents a key difference. In the p47$^{phox-/-}$ study, the animals were sacrificed few hours after the exposure ended. It may be that the nanoparticles may need more time to reach certain target organs to evoke effects. On the other hand, this study allowed for the evaluation of immediate effects. In contrast, in the 5xFAD study some resolution of reversible effects could have taken place. The extensive behavioural tests were performed in during several days after the last exposure, and the animals therefore had a post-exposure time of 10 days before sacrificing. A highly interesting approach for future research would be a long term inhalation study in p47$^{phox-/-}$ versus wildtype mice, eventually on a 5xFAD background. In a recent study in Alzheimer mice, daily application of the NADPH oxidase inhibitor apocynin during four months by oral gavage resulted in a reduction of plaque size and cortical microglia numbers (Lull et al., 2011). As such, it could be evaluated whether the DEE-induced effects in the subchronic study are mediated by NADPH oxidase generated ROS. However, the p47$^{phox-/-}$ mice appeared to be extremely complicated to handle. Because of their deficiency, the mice are extremely

prone to infection and associated disease developments, and therefore long-term whole body inhalation studies will be difficult to perform.

In conclusion, since the identification of the hazard posed by environmental particulates by epidemiological research (e.g. (Pope, 2000), a large body of scientific data has been generated investigating its nature, extent and mechanisms. Epidemiological studies have provided insight into novel risks posed by ambient particles (e.g. cardiovascular effects), while animal studies and *in vitro* investigations have contributed to mechanisms and particle components involved in adverse health effects. The emerging associations between exposure to (nano)particles and the central nervous system found in epidemiological studies should also be substantiated by plausible biological mechanisms identified in experimental research. However, when addressing neurotoxic effects of particles, one should be critical of the interpretation of *in vitro* toxicity studies in relation to the dose applied. When using pulmonary epithelial cells as a model to study pulmonary toxicity, it is relatively easy to compare the applied *in vitro* concentrations with deposition estimates in relation to ambient exposure levels and particle properties including size and surface composition. However, the relevance of the translocation rates of nanoparticles along the olfactory system or the alveolar region of the lung is still poorly understood.

The results from the inhalation studies described in this thesis are in support of the ***central nervous system as a major target of environmental nanoparticles***. They also indicate that ***long term inhalation exposure may accelerate the pathogenesis of Alzheimer's disease***. Further studies are needed to determine the relevance of the applied animal models and exposures for humans in relation to realistic exposure conditions. However, it is also important to consider the potential health risks of novel NP types and routes of NP exposure as they will become increasingly relevant. In recent decades, ambient exposure levels to PM have been substantially improved in outdoor environments. However, in recent years there has also been an increasing focus on the potential toxicity of (nano)particles from indoor sources, in the knowledge that people spend most of the time in indoor environments (Afshari et al., 2005; Samet and Spengler, 2003). Moreover, engineered NP are gaining more and more interest in different industry branches like the food sector, cosmetic industry and the medical field. Evaluation of effects of engineered NP to the

CNS after chronic exposure via the oral route depicts a highly novel and innovative field of investigation in nanotoxicology research (Chaudhry et al., 2008; Nogueira et al., 2012). The experimental approaches described in this thesis represent relevant tools to address the potential impact of novel engineered nanomaterials on the central nervous system.

6. Summary

In recent years there has been increasing concern that inhalation exposure to ambient particulate matter (PM) can lead to or exacerbate various diseases, which are not limited to the lung and the cardiovascular system, but also extend to other organs and tissues. Several research groups have shown with rodent inhalation studies that PM, and more specifically the fraction of nanoparticles (NP) (<100 nm) within this mixture, may cause adverse health effects in the brain via the induction of oxidative stress and inflammation.

The central focus of in this thesis has been on the hypothesis that inhaled NP play a crucial role in inducing or worsening oxidative stress and inflammation in the CNS, and thereby may contribute to brain pathologies. Oxidative stress and mildly increased inflammation in the brain, which are commonly observed in animal experimental studies, are characteristic for normal brain aging processes. However, they are also featured in various diseases of the CNS including the common neurodegenerative disorders Alzheimer's disease (AD).

The first study addressed the effects of short term (4 h) nose-only inhalation exposure to model carbon NP generated by spark discharge in the lungs and brains of wildtype (WT) and $p47^{phox-/-}$ mice. The $p47^{phox-/-}$ model was used to investigate the role of NADPH oxidase mediated superoxide formation in NP-induced oxidative stress and inflammation. It could be shown that the lungs of $p47^{phox-/-}$ mice are less responsive to NP inhalation than lungs of WT animals. Lung tissue mRNA expression levels of the DNA base excision genes OGG1 and APE-1 were increased upon NP exposure in WT animals but not in the of $p47^{phox-/-}$ mice, reflecting enhanced NADPH oxidase dependent stress and possible DNA damage. However, for the brain, no clear NP-exposure associated contrasts in mRNA or protein marker changes could be detected. The constitutive protein expression of the pro-inflammatory cytokine interleukin-1ß was found to differ between the brain homogenates of the sham-exposed wildtype and knockout mice.

The second study of the project involves a subchronic (i.e. 3 or 13 week) whole-body inhalation study to DEE in an Alzheimer mouse model. For this investigation it was decided to work with B6SJL-Tg (APPSwFlLon, PSEN1*M146L*L286V)6799VAS

mice (also referred to as “5xFAD mice”). The female mice of this strain are characterised by the development of histological, neurological as well as functional impairments which are considered to mimic the development and progression of AD in humans. Our study hypothesis is that a subchronic inhalation exposure to combustion derived NP (DEE) leads to an acceleration of these AD-like effects in this mouse model. Therefore, groups of animals were exposed to diluted DEE or HEPA-filtered air for 3 or 13 weeks, each 5 days per week, 6 hours per day. At the end of the 3 weeks and 13 weeks exposures, animals were subjected to a series of behavioural tests, i.e. the Y-maze task and X-maze task (both to assess the spatial working memory of mice reflected by the spontaneous alternation) and the string suspension task (to evaluate motor coordination and grip strength). Following, animals were sacrificed and brains were analyzed using histopathological, biochemical and molecular-biological techniques. The hypothesis of this study was that subchronic exposure to NP leads to an acceleration of the age-related onset of AD-like pathology. The results demonstrated deficits in motor condition and/or grip strength as well as increased brain tissue homogenate protein levels of β-amyloid 42 in the NP-exposed 5xFAD mice. Acceleration of β-amyloid 42 plaque deposition in the cortex and hippocampus was also detected.

The results of the behaviour tests and histopathology findings indicate, in line with the expectations, the typically age-dependent behavioural deficits and marked plaque-formation in the 5xFAD mice.

In conclusion, the results of this thesis are in support of the central nervous system as a major target of environmental nanoparticles, and indicate that long term inhalation exposure may accelerate the pathogenesis of Alzheimer’s disease.

7. Zusammenfassung

Die Sorge, dass inhalierte Feinstäube aus der Außenluft bereits vorhandene Krankheiten verschlimmern, die nicht nur die Lunge und das kardiovaskuläre System, sondern auch darüber hinaus andere Organe und Gewebe betreffen, ist in den letzten Jahren angestiegen. Verschiedene Forschergruppen konnten mit Hilfe von Inhalationsstudien an Nagetieren zeigen, dass die Nanopartikelfraktionen (<100 nm) von inhaliertem Feinstaub zu einer Induzierung von oxidativen Stress und Entzündungsreaktionen führen und somit auch gesundheitsschädigenden Auswirkungen auf das Gehirn haben können.

Der zentrale Fokus dieser Arbeit basiert auf der Hypothese, dass inhalierte Nanopartikel eine kritische Rolle in der Induzierung von oxidativen Stress und Entzündungsreaktionen innerhalb des zentralen Nervensystem spielen und daraufhin zu verschiedenen Gehirnpathologien beisteuern. Oxidativer Stress und ein leichter Anstieg von Entzündungsreaktionen im Gehirn, welche schon häufig in Tierversuchen nachgewiesen werden konnten, sind Charakteristika der normalen Hirnalterung. Des Weiteren sind sie Merkmale für verschiedene Krankheiten des zentralen Nervensystems, wie auch zum Beispiel für die weit verbreitete neurodegenerative Erkrankung Morbus Alzheimer.

Die erste Studie untersuchte die Effekte einer 4stündigen „nose only“ Inhalation mit Funken generierten Karbon-Nanopartikeln in Lungen und Gehirnen von Wildtyp (WT) und $p47^{phox-/-}$ Mäusen. Das $p47^{phox-/-}$ Maus Model wurde eingesetzt um die Rolle der NADPH Oxidase vermittelten Superoxid-Formation, in einer durch Nanopartikel induzierten oxidativen Stress- und Endzündungsreaktion, zu ermitteln. Es konnte gezeigt werden, dass Lungen der $p47^{phox-/-}$ Maus weniger Reaktion auf die Inhalation von Nanopartikel zeigten als solche von WT Tieren. Die mRNS Expressionslevel von DNS Basenexzision Genen wie OGG1 und APE/Ref-1 waren in Lungengeweben von WT Mäusen nach Belastung mit NP erhöht, nicht jedoch in den $p47^{phox-/-}$ Mäusen. Dies deutet auf einen NADPH-Oxidase abhängigen Stress und DNS Schädigung hin. Im Gehirn hingegen konnten keine klaren Nanopartikel-assoziierten Unterschiede im mRNS- oder Markerproteinen-Level detektiert werden. Die konstitutive Proteinexpression des proinflammatorischen Zytokins Interleukin-1β in Gehirn-

Homogenaten unterschied sich in nicht-belasteten Wildtyp Tieren und p47$^{phox-/-}$ Mäusen.

Die zweite Studie umfasste eine subchronische (3 bzw. 13 Wochen Belastung) „whole-body“ Inhalation mit Dieselruß Partikeln in einem Alzheimer Mausmodell. Hierfür wurde eine B6SJL-Tg (APPSwFlLon, PSEN1*M146L*L286V)6799VAS Maus verwendet, auch bezeichnet als 5xFAD Maus. Die weiblichen Mäuse dieser Linie zeichnen sich dadurch aus, dass sie histologische, neurologische und funktionale Auffälligkeiten entwickeln, welche die Entwicklung der Alzheimerschen Erkrankung im Menschen imitieren sollen. Die Hypothese dieser Studie ist, dass eine subchronische Belastung mit Dieselruß Partikeln zu einer Beschleunigung der auftretenden Alzheimer Effekte im Maus Model führen kann. Hierfür wurden die Tiere für 3 bzw. 13 Wochen mit Dieselruß Partikeln oder gefilterter Luft, an 5 Tagen der Woche für jeweils 6 Stunden, belastet. Anschließend erfolgte die Durchführung einer Reihe von Verhaltenstests. Der „Y-maze task“ und „X-maze task“, mit welchen man das räumliche Gedächtnis so wie das Arbeitsgedächtnis testet und der „string suspenstion task“, mit welchem man motorische Fähigkeiten und Muskelkraft misst. Anschließend wurden die Tiere getötet, die Hirne wurden mittels hostopathologischen, biochemischen und molekularbiologischen Methoden analysiert.

Die zugrundeliegende Hypothese dieser Studie war, dass eine subchronische Belastung durch Nanopartikel zu einer Beschleunigung der altersabhängigen Alzheimer-Pathologie führt. Die Ergebnisse zeigten Defizite im Bereich der motorischen Fähigkeiten und der Muskelkraft auf. Des Weiteren konnte ein erhöhtes Level an β-Amyloid 42 Protein in Gehirn-Homogenaten in mit Nanopartikel belasteten 5xFAD Mäusen detektiert werden. Eine Beschleunigung der β-Amyloid 42 Plaquedeposition im Cortex und Hippokampus konnte ebenfalls beobachtet werden.

Die Ergebnisse der Verhaltenstests und der histopathologischen Untersuchung zeigen die erwarteten und typischen altersabhängigen Defizite und Plaque Formatierungen in den 5xFAD Mäusen.

Zusammenfassend kann gesagt werden, dass die Ergebnisse dieser Thesis darauf hinweisen, dass das Zentrale Nervensystem ein wichtiges Zielorgan für Umweltpartikel darstellt. Belastungen über einen länger anhaltenden Zeitpunkt könnten zu einer Beschleunigung der Alzheimerpathologie führen.

8. References

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10. Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner versichere ich, dass ich weder an der Heinrich-Heine-Universität Düsseldorf noch an einer anderen Universität versucht habe, diese Doktorarbeit einzureichen.

Ebenso habe ich bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 30. Oktober 2012

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Maja Hullmann