General introduction
Introduction

Chemotherapy is a frequently used treatment strategy for cancer, often given in combination with surgery, radiation, and/or hormonal treatment. It is used to treat cancer with agents that can be prescribed as adjuvant, neoadjuvant, curative or palliative treatment strategy. There are approximately 50 different cytostatic agents which can be classified according to their working mechanism. Generally a number of cytostatics from different classes are combined in a chemotherapy cocktail. Treatment with this chemotherapy cocktail is given in a number of repeating cycles within a certain time period. Besides affecting cancer cells, cytostatic agents also affect healthy cells in the body which leads to a number of side effects. Some of the short-term effects are diarrhea, hair loss, myelosuppression, immunosuppression, and gastro-intestinal tract toxicity. These short-term effects generally disappear over time after the treatment has ended (Schagen et al., 2002; Sieklucka-Dziuba et al., 1998; Verburg et al., 2000). However, there are also a number of long-term side effects associated with chemotherapy, of which cognitive impairment is one. Cognitive impairment can range from very subtle to more severe and the predominantly affected cognitive functions are memory, processing speed, and more complex aspects of attention (Correa and Ahles, 2008; Wefel et al., 2008). The decline in cognitive functioning is often noticed around two years after treatment although variation in this first awareness occurs (Ahles et al., 2002).

The first clinical studies exploring cognitive impairment after adjuvant chemotherapy were performed in the early 1980s and these first findings led to an increasing awareness of this long-term side effect. Nowadays much attention is being paid to the quality of life after cancer and cancer-treatment. Despite the large increase in the number of clinical studies, cognitive decline is difficult to investigate in patients, partly due to methodological issues, such as relatively small sample sizes, differences in age of the patients, nature and location of the tumor(s), and intensity of the adjuvant treatment. The main problem is that for a proper investigation of cognitive decline prospective studies are needed with cognitive evaluation starting shortly before the occurrence of cancer and cancer treatment and repeated measurements during the recovery period and long afterwards. An additional difficulty is that not every cancer survivor suffers from cognitive decline, with estimations of approximately 17 - 34 % affected, suggesting a treatment and/or individual factor involvement, which may be related to IQ or level of education. Furthermore, psychological and emotional status and various genetic factors can play a role in cognitive decline (Ahles et al., 2002, Ahles and Saykin, 2007). There is also a large variation in the neuropsychological tasks used in the different studies, and in the criteria used to determine cognitive impairment. Moreover, not every study used appropriate control groups, study designs, or statistical measures (Schagen and Vardy, 2007). This has led to an urgent need for animal studies objectively exploring cognitive impairment after peripheral cytostatic treatment and the underlying mechanism(s).

An animal approach allows a systematic study of the underlying physiological mechanisms involved in the cognitive decline. Moreover, it allows a systematic analysis of the variables involved such as type of cytostatic, age, gender, etc. Increasing our knowledge of potential mechanisms involved in cognitive impairment is essential for the improvement of chemotherapeutic strategies. However, the number of animal studies is still surprisingly scarce and the results from the various studies are inconclusive. Most studies showed in one or more tests cognitive impairment (Eijkenboom et al., 1999; Foley et al., 2008; Gandal et al., 2008; Konat et al., 2008; Li et al., 2008; MacLeod et al., 2007; Madhyastha et al., 2002; Mustafa et al., 2008; Phillips et al., 1986; Reitiz et al., 2006; Seigers et al., 2008; Seigers et al., 2009; Sieklucka-Dziuba et al., 1998; Winocur et al., 2006; Yanovski et al., 1989), but this appears to be highly dependent on treatment protocol and the learning task. One study even reports positive learning effects of cytostatic treatment (Lee et al., 2006). This introduction will give an overview of animal studies exploring the effect of cytostatics on cognition and neurobiological processes. However, since there are many cytostatics that have been used for cancer treatment, it is
impossible to describe all substances in detail. Furthermore, only a small number of the available cytostatics have been extensively studied for its effects on cognition and the brain. Therefore, we will first give a brief overview of the different cytostatics and their working mechanisms, based on the classification of DeVita and colleagues (DeVita et al., 2005) with the most frequently studied cytostatics and their effect on cognition. We will further review potential mechanisms that may underlie the cognitive impairment as seen in patients after adjuvant chemotherapy.

Cognitive and neurobiological effects of cytostatic agents in animal studies

Alkylating agents
Alkylating agents alkylate electron-rich atoms to form covalent bonds and the most important antitumor activities are reactions with DNA bases. Monofunctional alkylating agents react with only one DNA strand, whereas bifunctional agents react with an atom on each DNA strand to produce cross-links. This reaction of the alkylating agents with the DNA will prevent the cell from replicating (DeVita et al., 2005). Of the alkylating agents, the effect of cyclophosphamide on cognitive performance is most frequently described in the literature.

Cyclophosphamide-associated cognitive impairment has been explored in a number of animal studies. In these studies mice or rats were treated with cyclophosphamide (Reiriz et al., 2006), in combination with doxorubicin (Konat et al., 2008; MacLeod et al., 2007), or 5-FU (Lee et al., 2006). Cyclophosphamide did not affect anxiety behavior (Konat et al., 2008; Reiriz et al., 2006) or cued fear (MacLeod et al., 2007) in rats or mice. Treatment with cyclophosphamide did, however, impair learning behavior in rats in a passive avoidance task (Konat et al., 2008) and a contextual fear conditioning task (MacLeod et al., 2007), and in mice in memory retention in a step-down inhibitory avoidance conditioning task (Reiriz et al., 2006). Surprisingly, Lee and colleagues found that female rats showed improved cognition measured in a Morris water maze and a Stone 14-unit T-maze seven weeks after treatment with cyclophosphamide and 5-FU. This effect was gone seven months after treatment. The authors suggest that this unexpected beneficial effect of cyclophosphamide and 5-FU on learning behavior can be explained by the estrogen cycle. Cytostatic treatment causes pre-mature menopause, and whereas lowered estrogen levels have a negative effect on cognition in humans, it has a positive effect on learning behavior in rats (Lee et al., 2006).

There are a number of animal studies that explored the effect of alkylating agents, and in particular cyclophosphamide, on neurobiological processes. These processes included the induction of oxidative stress (Bhatia et al., 2006; Konat et al., 2008; Manda and Bhatia, 2003; Oboh and Ogunraku, 2009), HPA axis activity (Navarra and Preziosi, 1997), neurogenesis (Dietrich et al., 2006; Mignone and Weber, 2006), and apoptosis (Maslinska, 1986; Rzeski et al., 2004; Wick et al., 2004). A potentially confounding factor, however, is that cyclophosphamide is a prodrug and that both the activation of this drug as well as its metabolism to its potentially neurotoxic product acrolein (Lovell et al., 2001) is mediated by several cytochrome P450 enzymes that differ in expression between strain and species. There is only one animal study available that has addressed the causal relationship between a neurobiological effect, oxidative stress, and the behavioral changes (Konat et al., 2008).

Cisplatin and its analogues
Cisplatin and its analogues form a variety of monofunctional and bifunctional adducts which may lead to the formation of intrastrand or interstrand DNA cross-links. This adduct formation interrupts certain cellular processes, such as separation, replication, and transcription of the DNA strands. This leads to either DNA repair or cell death (DeVita et al., 2005). The platinum drugs have not yet been extensively studied for their effects on cognition in animal models. Some unpublished work has shown that oxaliplatin impairs novel object recognition, spatial reference memory, and contextual fear condition
Antimetabolites

Antimetabolites are structural analogs of essential metabolic substances and their presence disturbs the biosynthesis or the function of nucleic acids and impairs the formation of new DNA or RNA, which leads to an arrest in the cell cycle (DeVita et al, 2005). The most frequently studied antimetabolite in relation to cognitive behavior is methotrexate (MTX). MTX is an inhibitor of dihydrofolate reductase, an important enzyme in folate metabolism. This enzyme maintains the intracellular tetrahydro-folate pool required for the synthesis of thymidylate, purine nucleotides, and certain amino acids (DeVita et al, 2005). MTX has frequently been used in animal studies exploring behavior and cognition (Foley et al, 2008; Madhyasta et al, 2002; Mullinex et al, 1990; Phillips et al, 1986; Seigers et al, 2008, Seigers et al, 2009; Siecklucka-Dziuba et al, 1998; Stock et al, 1995; Yanovski et al, 1989) often in combination with 5-fluorouracil (5-FU) (Foley et al, 2008; Gandal et al, 2008; Winocur et al, 2006A). MTX decreases explorative behavior in rats, which was seen in a dark-bright arena (Madhyasta et al, 2002), in a novel environment (Mullinex et al, 1990), and cage exploration (Phillips et al, 1986). However, Gandal and colleagues showed that mice treated with MTX and 5-FU show more exploration behavior in a novel object recognition task (Gandal et al, 2008). Also increased anxiety behavior was seen in mice treated with MTX and 5-FU in fear conditioning (Gandal et al, 2008). MTX was also shown to induce cognitive impairment in a number of learning tasks. Rats treated with MTX showed impaired learning in a Morris water maze and novel object recognition (Seigers et al, 2008), in conditioned emotional response (Yanovski et al, 1989), and on operant learning (Foley et al, 2008). MTX also impaired the ability to consolidate a previously learned memory when given directly after Morris water maze learning or contextual fear conditioning (Seigers et al, 2009). No cognitive impairment was seen in appetitive Pavlovian tasks (Stock et al, 1995). Learning impairment in a conditioned taste aversion task lasted for at least two weeks after exposure (Yanovski et al, 1989). However, in a different study no effect was seen in this task nine weeks after treatment (Stock et al, 1995). Mice treated with MTX showed impaired learning in a passive avoidance task (Siecklucka-Dziuba et al, 1988), and after treatment with MTX and 5-FU in a Morris water maze, non-matching to sample learning, and delayed non-matching to sample learning (Winocur et al, 2006).

The neurobiological effects of MTX include reduced neurogenesis (Seigers et al, 2008; Seigers et al, 2009), and blood flow/ glucose metabolism (Mizusawa et al, 1988; Phillips et al, 1989; Seigers et al, in press B), but also neurotoxic effects/ apoptosis (Billingsley et al, 1982; Gregorios et al, 1989; Igarashi et al, 1989; Madhyastha et al, 2002; Morris et al, 1995; Phillips et al, 1989; Silverstein and Johnston, 1986), oxidative stress (Rajamani et al, 2006; Uzar et al, 2006), and white matter damage (Gilbert et al, 1989; Gregorios et al, 1989; Seigers et al, 2009).

Another frequently studied antimetabolite in relation to cognitive behavior is cytarabine. The metabolites of cytarabine are incorporated into DNA; this incorporation inhibits DNA polymerase, which in turn interferes with DNA chain elongation during both DNA replication and DNA repair (DeVita et al, 2005). Cytarabine can very easily cross the blood-brain barrier and cerebrospinal fluid levels can rise up to 50% after intravenous administration (Schiff et al, 2008). Not many studies explored the effect of cytarabine on cognition. However, it was seen that cytarabine impaired remote, but not short-term, memory in a Morris water maze (Li et al, 2008) and in a conditioned taste aversion test (Wang et al, 2003). Oxidative damage (Geller et al, 2001; Koros et al, 2007; Koros and Kitraki, 2009; Li et al, 2008), reduced neurogenesis (Kokoeva et al, 2003), and apoptosis (Ahlemeyer et al,
2003; Courtney and Coffey, 1999; Jung et al, 2004; Koros and Kitraki, 2009; Lau et al, 2009) are neurobiological effects caused by cytarabine treatment.

A third frequently studied antimetabolite in relation to cognitive behavior is 5-fluorouracil (5-FU). 5-FU enters the cell through the uracil transport mechanism and interferes with DNA biosynthesis and repair via thymidylate synthase inhibition. Another cytotoxic effect of 5-FU is the incorporation of its metabolite in nuclear and cytoplasmic RNA which interferes with RNA processing and functioning (DeVita et al, 2005). 5-FU is known to induce cognitive impairment in animals (Mustafa et al, 2008), or in combination with MTX (Foley et al, 2008; Gandal et al, 2008; Winocur et al, 2006). Rats treated with 5-FU showed impaired learning in an object location task (Mustafa et al, 2008), and in an operant learning paradigm (Foley et al, 2008). Mice treated with 5-FU and MTX showed no impairment in a novel object recognition task but were impaired in contextual fear conditioning (Gandal et al, 2008), in a Morris water maze, non-matching to sample learning, and delayed non-matching to sample learning (Winocur et al, 2006A). As mentioned earlier, Lee and colleagues showed that female rats treated with cyclophosphamide and 5-FU showed improved learning seven weeks after treatment and no difference in learning behavior seven months after treatment (Lee et al, 2006). 5-FU has been shown to impair neurogenesis (Han et al, 2008; Mustafa et al, 2008), induce apoptosis (Han et al, 2008; Yamaguchi et al, 2009), and cause white matter damage (Han et al, 2008).

**Topoisomerase interactive agents**

DNA topoisomerases change the topology of DNA by forming single- (type I topoisomerases) or double-strand (type II topoisomerases) breaks in the double helix. This relaxes the torsional stress that occurs when the DNA double helix unwinds when DNA and RNA polymerases access the DNA. Type II topoisomerases also untangle and separate the replicated DNA during cell division. When topoisomerases are absent, the torsionally strained supercoiled DNA accumulates which will interfere with vital cellular functions. Topoisomerase interactive agents cause accumulation of DNA cleavage complexes of protein-linked DNA strand breaks. These lesions in the ongoing DNA replication or RNA transcription lead to cytotoxic DNA damage, causing cell-arrest, apoptosis, or cell necrosis (DeVita et al, 2005).

Of the different topoisomerase interactive agents, doxorubicin is the most studied agent for its effect on cognition given alone (Sieklucka-Dziuba et al, 1998), or in combination with cyclophosphamide (Konat et al, 2008; Macleod et al, 2007). When doxorubicin was administered alone it did not impair learning in a passive avoidance task (Sieklucka-Dziuba et al, 1980). Together with cyclophosphamide, doxorubicin did not affect anxiety (Konat et al, 2008), but did impair context- but not cue-specific memory of fear in rats (Macleod et al, 2007). Konat and colleagues found impaired passive-avoidance learning after treatment with doxorubicin and cyclophosphamide in rats which could be reversed by treatment with the antioxidant N-acetyl cysteine (Konat et al, 2008). Doxorubicin is associated with oxidative stress (Joshi et al, 2005; Montilla et al, 1997; Öz and İlhan, 2006), and neurotoxicity/ apoptosis in brain areas without a blood-brain barrier (Bigotte and Olsson, 1984), which suggests that possible effects of compounds like doxorubicin are indirect. Although doxorubicin is extensively distributed to tissues, the brain penetration of doxorubicin is low (Bigotte and Olsson, 1984). In part this is due to the fact that doxorubicin is a good substrate for P-glycoprotein. However, in P-glycoprotein deficient mice the brain levels were only moderately (3-fold) higher than in wildtype controls and still 20 to 100-fold lower than in tissues like liver and kidney (van Asperen et al, 1999). The poor penetration of doxorubicin in brain areas with a blood-brain barrier suggests that possible negative effects of the compound on brain areas that are involved in cognitive processes like the hippocampus and cortical regions are not directly mediated by the cytostatic compound.
Antimicrotubule agents

Microtubuli are dynamic structures formed from tubulin and create the mitotic spindle which is necessary for the separation of replicated DNA. Consequently, disruption of the dynamics of microtubuli by antimicrotubule agents interferes with cell division and proliferation. Furthermore, antimicrotubule agents may disrupt many of the nonmitotic functions of microtubules, such as chemotaxis; membrane and intracellular scaffolding; transport, secretion, and/or anchorage of organelles and receptors; adhesion; locomotion; and mitogenic signaling (DeVita et al., 2005).

Paclitaxel is an antimicrotubule agent that has been associated with cognitive impairment in patients (Hurria et al., 2006; Tchen et al., 2003). However, similar to doxorubicin, paclitaxel does not cross the blood-brain barrier very well (Schiff et al., 2008). Paclitaxel is a very good substrate for P-glycoprotein as shown in studies with P-glycoprotein deficient mice where the brain exposure was more than 10-fold higher than in wildtype controls (Kemper et al., 2003). When rats were treated with paclitaxel during the training phase of a five choice serial reaction time task, no cognitive impairment was seen. The authors suggest that the treatment protocol was possibly responsible for the lack of significant differences, since a single cytostatic was given during the training phase, whereas in the clinic multiple cytostatics are given. This might be interpreted as an indication that paclitaxel is not affecting cognitive functionality. In this study, however, effects of paclitaxel on cognition were explored acutely after administration whereas effects on cognitive performance might show up for the first time after longer delay periods (Boyette-Davis et al., 2009). The HPA axis (Navarra and Preziosi, 1997) and the immune system (Cata et al., 2008) appear to be important for the neurotoxicity/apoptosis effects (Rzeski et al., 2004; Wick et al., 2004) of the antimicrotubuli agents.

Neurobiological processes involved in cognitive impairment

The blood-brain barrier

Before addressing the neurobiological processes involved in cognitive impairment, we need to consider that during our day-to-day life, the brain is in fact quite effectively protected against potentially harmful compounds by the blood-brain barrier (BBB). This BBB is formed by the capillary endothelial cells of the brain, which are closely linked by tight junctions. Moreover, brain endothelial cells lack fenestrations and have low pinocytic activity and together these characteristics build a rigid wall. On top of this physical architecture the BBB is equipped with a range of efflux transporters that restrict the BBB penetration of drugs that might otherwise be able to accumulate. The best known and most dominant drug transporter is ABCB1 (also called P-glycoprotein), but other ABC-transporters such as ABCG2 and ABCC4 are also involved. Only drugs that are sufficiently lipophilic to allow passive diffusion and/or able to (ab)use an inward directed transport system and that are also not recognized by any of the efflux transporters will penetrate the brain in appreciable amounts (De Vries et al., 2006). Nevertheless, all drugs, even those that do not fulfill these criteria may accumulate into the brain to some extent and it will depend on their potency to perturb the delicate processes involved in neuronal activity whether these, even at such low concentrations, may negatively affect cognitive functioning. For example, the levels of MTX in the brain are approximately 5-10% of the plasma levels (Schiff et al., 2008). There is, however, a large effect of the schedule used to administer the cytostatic compound. Penetration into brain tumors is significantly enhanced if MTX is administered as i.v. bolus injection as compared to i.v. infusion (Dukic et al., 1999; Dukic et al., 2000). However, there is little known about the transporters that are responsible for the low penetration of MTX in brain tissue, but it is suggested that the ATP-binding cassette (ABC) transporters Bcrp1, Mrp2, and Mrp3 play an important role in the excretion of MTX (Vlaming et al., 2009A and B).

Even though MTX does not penetrate the brain very well, the drug is associated with cognitive impairment in the clinic when given in chemotherapy cocktails (Schagen et al., 1999; Schagen et al,
Chapter 1

2002; Scherwath et al, 2006) as well as in animal studies (Foley et al, 2008; Seigers et al, 2008; Winocur et al, 2006) and is known to disrupt several neurobiological processes (Leke et al, 2006; Mizusawa et al, 1988; Phillips et al, 1989; Seigers et al, 2008; Seigers et al, 2009; Seigers et al, in press B; Sieklucka-Dziuba et al, 1998; Silverstein and Johnston, 1986). This suggests either that a low level of a cytostatic agent in the brain is sufficient to cause damage, or that the behavioral and neurobiological effects of cytostatic agents such as MTX are indirectly induced. This may involve secondary, perhaps peripherally released, mediators. It is important to realize that substances that do as well as substances that do not cross the blood-brain barrier according to the literature are capable of inducing similar neurobiological processes that may result in cognitive impairment. There are a number of complicating factors that hamper the comparison of the outcome of the studies performed. In total, the amount of published articles describing cognitive and neurobiological effects of cytostatics in animal models is relatively small. In these publications there is a large variety in the cytostatics tested. Another complicating factor is that substances are frequently applied in combinations as in chemotherapy cocktails which makes it impossible to get a clear insight in the cognitive aspects of each cytostatic compound by itself. This shows the urgent need for models in which separate cytostatic agents are tested for their effect on different neurobiological processes, which was the basis for this thesis. In the section below, the effect of cytostatic agents on the brain will be briefly discussed by first describing neurobiological processes affected by chemotherapy as described in the literature (oxidative stress and HPA axis reactivity), followed by the processes explored in this thesis (white matter damage, inflammation, blood flow, neurogenesis, and the role of cancer).

Oxidative stress
Oxidative stress is caused by the formation of reactive oxygen species (ROS) which are mainly produced by the respiratory chain of mitochondria. The formation of ROS can lead to mutations in the mitochondrial DNA; in turn leading to errors in the mitochondrial DNA coded proteins, altered electron transfer, and eventually again ROS generation, in a vicious circle (Lenaz et al, 1999). Since cytostatic agents in general disturb DNA, one can expect that mitochondrial DNA is also altered by chemotherapy treatment leading to ROS formation and oxidative stress.

The presence of oxidative stress after cytostatic treatment has been shown for a number of cytostatics, including carboplatin (Geller et al, 2001; Husain et al, 2001; Husain et al, 2003), cyclophosphamide (Oboh and Ogunraku, 2009), cytarabine (Geller et al, 2001; Koros et al, 2007; Koros and Kitraki, 2009), and doxorubicin (Joshi et al, 2005; Montilla et al, 1997; Öz and Ilhan, 2006), and MTX (Rajamani et al, 2006; Uzar et al, 2006). Besides causing oxidative stress by DNA mutation, doxorubicin can also produce hydroxyl radicals, hydrogen peroxide, and superoxide anions by the conversion of the cytostatic drug into a free radical (Öz and Ilhan, 2006). Konat and colleagues explored the causal role of oxidative stress in the development of cognitive impairment after cyclophosphamide and doxorubicin. Rats showed impaired learning behavior after the treatment with the cytostatic agents, which was absent when these substances were given in combination with the antioxidant N-acetyl cysteine (Konat et al, 2008). This suggests that oxidative stress indeed plays an important role in the development of cognitive impairment after treatment with these substances and possibly after treatment with other substances that cause oxidative stress.

HPA axis
The HPA axis appears to play an important role in the tolerability of several cytostatics since animals that received hypophysectomy or adrenalectomy were more susceptible to the lethal effects of 5-FU, busulfan, carmustine, cyclophosphamide, and vindesine, possibly due to the reduced corticosterone level. Corticosterone inhibits nuclear factor kappaB (NF-κB), a transcription factor associated with apoptosis. This inhibition can occur via binding of the corticosterone-cytoplasmic receptor complex to NF-κB, or corticosterone can up-regulate synthesis of NF-κB inhibitors (Navarra and Preziosi, 1997).
This is consistent with the observation that the toxicity of MTX in rats appears to be dependent on the corticosteroid plasma levels. MTX toxicity was decreased when supplementary corticosterone was given, whereas a low level of corticosterone resulted in increased toxicity (English et al., 1987).

**White matter**

5-FU has been shown to decrease myelin sheets and deregulate Olig2 expression, crucial for generating functional oligodendrocytes, in the corpus callosum of rats (Han et al., 2008). Furthermore, 5-FU, carmustine, cisplatin, and cytarabine all affected oligodendrocytes precursors in vivo (Dietrich et al., 2006). MTX is also associated with degeneration of white matter and white matter necrosis (Gregorios et al., 1989). In one of our studies, we measured the thickness of the lateral corpus callosum which was lastingly reduced after MTX treatment (Seigers et al., 2009). White matter and oligodendrocytes are important for neuronal impulse conduction, and damage to white matter may explain the reduced speed of information processing noticed in patients after adjuvant chemotherapy (Han et al., 2008).

**Immune system/ (neuro) inflammation**

Cytostatic agents may also indirectly affect cognition through their action on the immune system, since activation of the immune system is associated with cognitive impairment (Banks et al., 2002). Because cytostatics reduce cell proliferation in the gastrointestinal mucosa there is a decreased barrier function and an enhanced risk of developing infections caused by micro-organisms originating from the intestines. Indeed, various cytostatic agents are associated with mucositis (DeVita et al., 2005). Mucositis is associated with elevated cytokine release (De Koning et al., 2006). Peripheral cytokines can induce inflammation and cytokine release in the central nervous system in various ways: by diffusion into the brain through the circumventricular organs which lay outside the blood-brain barrier; by activation of sensory afferents of cranial neurons (vagal and glossopharyngeal nerves); or by active transport via a saturable transport system; and by secretion of immune-active substances (e.g. cytokines and prostaglandins) by cells from the blood-brain barrier (Seruga et al., 2008; Wilson et al., 2002). This cytokine release in the central nervous system caused by peripheral sickness behavior can lead to central sickness behavior which, as mentioned previously, is associated with cognitive impairment (Banks et al., 2002). Central cytokine release can activate microglia (Hanisch and Kettenmann, 2007; Seruga et al., 2008) possibly leading to neuroinflammation, which is associated with cognitive impairment as well (Wilson et al., 2002). Furthermore, neuroinflammation and microglia activation are also known to have a negative effect on neurogenesis (Das and Basu, 2008; Ekdahl et al., 2003) which is thought to play an important role in learning and memory (Gould et al., 1999). On the other hand, it is known that chemotherapeutic compounds such as MTX have a strong inhibitory effect on stem cells in bone marrow which is the reason that these substances are used as potent immunosuppressors (Pannacciulli et al., 1982).

This indirect route of cytostatic compounds via peripheral cytokines that may facilitate the process of neuroinflammation has hardly been explored. We showed that MTX activates microglia; however, this activation was not associated with neuroinflammation, since no effect was seen in the uptake of a tracer for peripheral benzodiazepine receptors or in central cytokine levels. This suggests that cognitive impairment after MTX treatment is not caused by neuroinflammation (Seigers et al., in press B).

**Blood flow**

A reduction in blood flow or damage to blood vessels can result in altered neuronal functioning and impaired cognition. It is known that chemotherapy reduces local cerebral blood flow (Mizusawa et al., 1988) and has a negative effect on cerebral glucose metabolism in patients (Silverman et al., 2006) as well as in rats (Phillips et al., 1989). This effect may be caused by the anti-vascular effect of cytostatic agents which can also induce vascular toxicity (De Vos et al., 2004). We showed that MTX indeed reduces the density of blood vessels in the hippocampal area. This reduced blood vessel density may be
related to the decreased central glucose metabolism measured with $[^{18}F]$FDG PET (Seigers et al, in press B). Vascularization and neurogenesis are closely related as shown in a paper of Palmer and colleagues. BrdU positive cell clusters are often found near small capillaries, possibly due to a need of extra energy supply. Furthermore, brain microvascular endothelium secretes brain-derived neurotrophic factor which promotes the survival and differentiation of neuronal precursors. Up to 37% of the BrdU positive cells is positive for endothelial markers, suggesting an even closer relationship between neurogenesis and angiogenesis/vascularization (Palmer et al, 2000). This suggests that the reduction in blood vessel density with the accompanying reduction in energy supply and proliferative signals may be the cause of the decreased hippocampal cell proliferation which was seen in a number of studies (Dietrich et al, 2006; Han et al, 2008; Mignone and Weber, 2006; Mustafa et al, 2008; Seigers et al, 2008; Seigers et al, 2009).

**Neurogenesis/ neurotoxicity**

Since cytostatics are aimed at the inhibition of the process of cell division, they will probably also affect cell proliferation in the brain if they are capable to pass the blood-brain barrier. It has been accepted for a long time that glial cells in the brain can divide thereby continuously producing new cells. More recently it has been recognized that stem cells can also produce new neurons which can be integrated in specific brain regions in the process of neurogenesis. The most prominent regions in which neurogenesis occur are the subventricular zone, which lines the lateral ventricles, and the subgranular zone of the hippocampal dentate gyrus (Gould et al, 2000; Kempermann et al, 2004). Cells produced in the subventricular zone migrate to the olfactory bulb where they probably are functionally involved in the perception of taste and odor (Whitman and Greer, 2009). Chemotherapy possibly affects the neurogenesis this brain area, since many chemotherapy treated patients report alterations in the perception of taste and odor after the start of adjuvant therapy (Steinbach et al, 2009).

Since the hippocampal formation is well known for its involvement in learning and memory processes, it is suggested that neurogenesis in this brain structure plays a functional role in cognitive performance. Animals with lowered neurogenesis perform worse in hippocampal-dependent tasks and exhibit a learning impairment (Gould et al, 2000), whereas an enriched environment enhances neurogenesis, and increases learning capacity (Kempermann, 2002; Madsen et al, 2003). Carmustine (Dietrich et al, 2006), cisplatin (Dietrich et al, 2006), thiopeta (Mignone and Weber, 2006), and 5-FU (Han et al, 2008; Mustafa et al, 2008) have all been shown to decrease neurogenesis and/or hippocampal cell proliferation. Furthermore, our studies have shown that MTX also decreases hippocampal cell proliferation (Seigers et al, 2008; Seigers et al, 2009). However, the functionality of neurogenesis is highly debated and the role of the newly formed neurons in cognitive behavior is far from clear. While a number of studies reported that learning increases neurogenesis (Gould et al, 1999) and that neurogenesis plays a role in learning (Bendel et al, 2005; Bruel-Jungerman et al, 2005; Van der Borght et al, 2007; Wati et al, 2006), other studies report a change in hippocampal cell proliferation with only a partial or no effect on learning and memory (Madsen et al, 2003, Raber et al, 2004; Shors et al, 2002; Snyder et al, 2005; Winocur et al, 2006B, Wojtowicz et al, 2008). Therefore, more neurobiological processes, such as altered blood flow or oxidative stress, are possibly involved in the development of cognitive impairment after chemotherapy which is supported by clinical studies showing that cognitive impairment is mostly noticed in non-hippocampal dependent tasks (Ahles and Saykin, 2007).

The decrease in neurogenesis has often been shown to correlate with an increase in cell death in the hippocampus after carmustine, cisplatin, and 5-FU (Dietrich et al, 2006; Han et al, 2008; Wick et al, 2004). Cytostatic agents can also induce apoptosis and cell death in other brain regions and cell types (Courtney and Coffey, 1999; Koros and Kitraki, 2009; Rzeski et al, 2004; Wick et al, 2004). Astrocytes are also known to be vulnerable to the negative effects of cytostatic agents. Cytostatics can activate astrocytes with phenotypical changes from epithelial-like to process-bearing cells and an
increase in astrocyte activation markers. This phenotypical change impairs the ability of astrocytes to maintain physiological levels of glutamate and to express several glutamate degrading enzymes, leading to excitotoxic glutamate levels. This excessive accumulation of extracellular glutamate is a contributing factor in acute and/or chronic neuronal damage, since the activation of astrocytes impairs their ability to protect neurons against excitotoxic injury (Ahlemeyer et al., 2003). This disruption of the glutamatergic buffering may also be caused by oxidative stress. Oxidative stress leads to decreased glutamate transporter activity, resulting in damaged astrocytes, which further disturbs glutamatergic synapses leading to possible neuronal damage (Leke et al., 2006). These negative effects of cytostatics on various brain cells may lead to histopathological changes (Igarashi et al., 1989; Phillips et al., 1989; Silverstein and Johnston, 1986) and lesions in the brain (el-Badawi et al., 1990; Maslinska, 1986; Rzeski et al., 2004).

Cancer

Almost all animal experiments described in this overview made use of healthy animals to test the effects of several cytostatics on cognition and biological processes. However, one should not forget that in the clinic, cytostatics are prescribed as an adjuvant treatment of cancer. Furthermore, cognitive impairment can also be noticed after the diagnosis of cancer and before the onset of any systemic treatment (Hermelink et al., 2007; Wefel et al., 2004 A, B). Therefore, we measured the effect of MTX on hippocampal cell proliferation in tumor-bearing animals. The presence of a tumor did appear to decrease the number of proliferating cells in the hippocampus, suggesting that this may contribute to the cognitive impairment observed in cancer patients before any treatment is initiated (Seigers et al., in press A). However, in patients, additional explanations for this early cognitive impairment can be found in diagnosis related emotional stress, or DNA damage/deficiencies in DNA repair mechanisms (Hermelink et al., 2007; Wefel et al., 2004 A, B) with the latter two being linked both to the development of cancer and neurodegenerative disorders.

Concluding remarks

Cognitive impairment is a long-term side effect of adjuvant chemotherapy which can have a large impact on the quality of life after cancer. Many clinical studies have been performed to elucidate this cognitive impairment. However, clinical studies have strong limitations with respect to exploring possible mechanisms and their causal involvement due to, for example, methodological and ethical constraints. Furthermore, not every cancer survivor suffers from cognitive impairment and the severity of the impairment also differs. This suggests individual differences in the vulnerability to develop cognitive impairment after chemotherapy. These differences may include genetic variation in genes encoding for example for the functionality of the efflux pumps in the blood-brain barrier, DNA repair mechanisms, telomere length and activity, cytokine regulation/proinflammatory cytokine levels, neural repair, and neurotransmitter activity (Ahles and Saykin, 2007).

Due to the impact of cognitive impairment on the quality of life and the individual variation in the occurrence and severity of this phenomenon, there has been an increase in the number of animal studies performed and subsequently the number of published studies during the last years. In these animal studies, several possible pathways that may contribute to the cognitive impairment observed after chemotherapy were explored. These potential pathways include inhibition of neurogenesis (Dietrich et al., 2006; Han et al., 2008; Mignone and Weber, 2006; Mustafa et al., 2008; Seigers et al., 2008; Seigers et al., 2009), oxidative damage (Geller et al., 2001; Husain et al., 2001; Husain et al., 2003; Joshi et al., 2005; Konat et al., 2008; Koros et al., 2007; Koros and Kitraki, 2009; Montilla et al., 1997; Oboh and Ogurnuku, 2009; Öz and İlhan, 2006; Rajamani et al., 2006; Uzar et al., 2006), decreased HPA axis activity (English et al., 1987; Navarra and Preziosi, 1997), reduced brain vascularization/blood flow
(Mizusawa et al, 1988; Phillips et al, 1989; Seigers et al, in press B), and white matter damage (Dietrich et al, 2006; Gregorios et al, 1989; Han et al, 2008; Seigers et al, 2009). While the studies are based on a variety of cytostatic agents, this overview indicates that each of these pathways may contribute to the behavioral consequences of chemotherapy. Furthermore, it is hard to conclude which pathways are primarily affected by the effect of the cytostatic agent in the brain and which are secondarily affected via changes in e.g. vascularization or peripheral factors signaling the brain. There is a clear lack of systematic studies exploring effects of single cytostatic compounds on a range of neurobiological mechanisms.

A major problem when comparing the animal studies performed is the difference in the models that are used. These differences included species (rat or mouse), gender (male, female, or both), age of the animals (young, old, or intermediate), cytostatic used (different cytostatics, one, or multiple), treatment strategy (dosage, single, or multiple treatment), route of administration (intraperitoneal, intravenous, intrathecal, or intraventricular), time between treatment and testing (before, during, or after), and the different tasks used. All these differences created the need for a more concise animal model, to facilitate comparisons between the studies, which was the basis for this thesis. In this thesis several potential neurobiological processes that may play a role in the development of cognitive impairment after adjuvant chemotherapy were explored. One animal model was used in which male rats were treated with a single, high dose of MTX. MTX was chosen since this cytostatic agent is associated with cognitive impairment in both clinical and animal studies. The animals received a high dosage of MTX, since in the clinic a low MTX dosage, 25 mg/ week as prescribed to patients with chronic inflammatory diseases such as rheumatoid arthritis, is not associated with cognitive impairment (Lems and Jansen, personal communication). This may be related with previous reports that show that high plasma concentrations of MTX as observed after i.v. bolus injections result in a 3-5 fold increase in MTX concentration in brain tumors as compared to i.v. infusions (Dukic et al, 1999; Dukic et al, 2000).

The neurobiological processes studied in this thesis are hippocampal cell proliferation (chapter 2 and 3), white matter damage (chapter 3), blood flow and glucose metabolism (chapter 5), and inflammation (chapter 5), since these processes can all affect cognition, as previously described. Furthermore, we explored the role of cancer on hippocampal cell proliferation (chapter 6), since cancer in itself can also influence cognitive function. We also explored if MTX in our animal model induced cognitive impairment, by studying cognition shortly (chapter 2) and long-term (chapter 4) after MTX treatment and on the consolidation of a previous learned memory (chapter 3).

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