Methotrexate decreases hippocampal cell proliferation and induces memory deficits in rats

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Abstract

Methotrexate (MTX) is a cytostatic agent used in adjuvant chemotherapy for treatment of breast cancer and is associated with cognitive impairment in a subgroup of patients. The aim of this paper is to test whether MTX can rapidly affect various brain structures resulting in decreased hippocampal cell proliferation and white matter damage. We also studied whether cell death occurs in the hippocampus following MTX. All these processes may contribute to the memory deficits reported in patients.

The first study explored the effect of an intravenously injected high-dose MTX (250 mg/kg) on hippocampal cell proliferation, white matter, and cell death. Proliferation was not significantly decreased one day after administration of MTX, although a high individual variation was seen. However, seven days after MTX treatment hippocampal cell proliferation was significantly lower compared to control animals. White matter density was decreased in the lateral corpus callosum of animals treated with MTX, one day, one week, and three weeks after treatment. MTX did not induce hippocampal cell death at any of the time intervals after treatment.

The second study examined the effect of MTX on memory by subjecting animals to a learning task directly followed with MTX treatment. In both learning tasks, memory was impaired in treated animals. In the Morris water maze, animals treated with MTX spent significantly less time in the correct quadrant compared to control animals during the probe trial which was performed one week after training and treatment. In contextual fear conditioning, animals treated with MTX showed significantly less freezing behavior compared to control animals, four weeks after training and treatment.

These studies suggest that the negative effect of MTX on hippocampal cell proliferation and white matter density may be part of the mechanisms underlying the cognitive impairment observed as side effect after cytotoxic treatment in humans.

Introduction

Adjuvant chemotherapy is frequently applied in the treatment of cancer. The survival rate of patients treated with cytostatics is high; however, the treatment is associated with both short- and long-term side effects. One of the potential long-term effects is cognitive impairment, mostly noticed as a decrease in attention/ concentration, speed of information processing, and memory (Kreukels et al, 2005).

There is an increasing number of human studies exploring the effect of chemotherapy on cognitive impairment (Brezden, et al, 2000; Kreukels et al, 2005; Schagen et al, 2002; Shilling and Jenkins, 2005), while animal studies providing insight in the mechanism behind this phenomenon are scarce. In the few studies performed, methotrexate (MTX) is a frequently used cytostatic. For example, Winocur and colleagues found impaired learning in BALB/C mice treated with several intraperitoneal injections of MTX and 5-fluorouracil in a Morris water maze, in a non-matching-to-sample test (NMTS), and in a delayed-NMTS of non-spatial memory (Winocur et al, 2006). Madhyasta and colleagues found learning impairment in Wistar rats treated with multiple intracerebroventricular MTX injections in a two-compartment conditioned avoidance task (shuttle box test) and altered locomotor and exploratory behavior in a dark-bright arena test (Madhyastha et al, 2002). Sieklucka-Dziuba and colleagues studied the effect of a single intraperitoneal injection of MTX on learning behavior in male and female Albino Swiss mice. Animals treated with MTX showed impaired learning behavior in a passive avoidance task compared to control animals (Sieklucka-Dziuba et al, 1998).

Our recent study suggests that the effect of MTX on cognition might be mediated by its effects on hippocampal cell proliferation. Male Wistar rats were intravenously injected with a high-dose of MTX (250 mg/kg) which resulted in a significant decline in performance in a Morris water maze and in a novel object recognition test, three and four weeks after treatment respectively. We also showed a long-
lasting dose-dependent decrease in hippocampal cell proliferation three weeks after treatment with MTX (Seigers et al., 2008). Since hippocampal cell proliferation is thought to play an important role in learning and memory (Kempermann, 2002; Madsen et al., 2003), the negative long-term effect found in our study may explain the cognitive impairment seen in people treated with adjuvant chemotherapy.

A substantial number of clinical studies observed cognitive impairment several years after completion of chemotherapy treatment (Brezden, et al., 2000; Kreukels et al., 2005; Schagen et al., 2002; Shilling and Jenkins, 2005), but there are also several studies that show the occurrence of cognitive impairment directly after treatment (Shilling and Jenkins, 2005; Tchen et al., 2006; Wefel et al., 2004; Wienke and Dienst, 1995) indicating that cytostatics may rapidly affect brain structures involved in cognitive performance. Therefore, in this study, we explored the hypothesis that the inhibitory effect of MTX on hippocampal cell proliferation will show up rapidly after treatment. Since several cytostatics are associated with decreased presence of myelin in white matter and increased cell death (Dietrich et al., 2006; Han et al., 2008), we also studied the effect of MTX on white matter and cell death. Damage to white matter was explored by measuring the thickness of the corpus callosum, the fiber structure connecting left and right hemispheres. Cell death was studied in the hippocampus using a silver nitrate staining visualizing degenerating neurons (Tashlykov et al., 2007).

Since damage to the hippocampal area is known to cause retrograde amnesia (Maren et al., 1997), we expect that the MTX induced lowering of hippocampal cell proliferation on the short-term will affect a previous learned memory. To test this, animals were given two learning tasks (Morris water maze and contextual fear conditioning), after which they were treated with MTX. This study will not only give us insight in the mechanism behind cognitive impairment, it will also give insight in which aspects of learning and memory are affected by MTX.

**Methods**

Adult (3 months of age) male Wistar rats (Harlan, Zeist, the Netherlands, average body weight at the start of the experiment 326 gram ± 3.5 SEM) were housed in groups of 3 or 4 animals in clear Plexiglas cages (58 x 38 x 20 cm) on a layer of wood shavings with a fixed 12:12 h light:dark cycle (with lights on at 08.00 a.m.) and food and water *ad libitum*. All animals in one cage received the same treatment; meaning that MTX treated animals and control animals were housed in separate groups. Experiments started 2 weeks after arrival of the animals according to the protocol described below. All experiments were approved by the Animal Experimentation Committee of the University of Groningen.

Rats were injected with saline or a high-dose of MTX (250 mg/kg, 100 mg/ml solution, Pharmachemie BV, Haarlem, the Netherlands) in the tail vein under a short-lasting (< 3 minutes) mild \( \text{O}_2 \)-isoflurane anesthesia. After the intravenous MTX injections animals received repeated intra-peritoneal injections of the tetrahydrofolate calcium leucovorin (10 mg/ml solution, Pharmachemie BV, Haarlem, the Netherlands), which was administered in a protocol similar to the application in patients. Eighteen hours after the injection of MTX, leucovorin was administered in a concentration that was 8% of the injected MTX dose; after 26, 42, and 50 hours, the administered concentration was reduced to 4%. Leucovorin is clinically used as a so-called rescue therapy in combination with the cytotoxic agent. Tetrahydrofolate (THFA) is a co-factor in DNA synthesis; MTX is an inhibitor of the enzyme THFA reductase and depletes the pool of tetrahydrofolates. Leucovorin is a tetrahydrofolate that does not require activation by THFA reductase (Genestier et al., 2000; Huennekens, 1994). Pilot studies revealed that leucovorin itself does not have an effect on neurogenesis and that high-dose MTX without leucovorin is lethal, due to severe diarrhea and weight loss.

**Immunohistochemistry**
Short-term effect of high-dose MTX on hippocampal cell proliferation

Male Wistar rats were injected with either saline or MTX (250 mg/kg) as previously described. The animals were sacrificed 1 or 7 days after the injection through transcardial perfusion with saline followed by 4% paraformaldehyde. Brains were removed and placed in 30% sucrose solution at 4°C. Microtome sections of the hippocampus (40 µm) were stored in 0.01 M PBS including 0.1% azide until immunohistochemical staining.

From the serial sections, every twelfth section from each animal was selected and immunohistochemically stained for Ki-67 using a slightly adapted standard protocol (Kee et al., 2002). In brief, free-floating sections were pre-treated with 0.4% H₂O₂ for 30 minutes, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Francisco, California). Subsequently, sections were incubated with mouse-anti-Ki-67 (1:200, MONX10283, Monosan, Uden, the Netherlands) for 48 hours at 4°C. After a second blocking step, sections were incubated with a biotinylated secondary antibody (1:400, goat-anti-mouse, Jackson, Wet Grove, PA, USA) for 2 hours at room temperature. This was followed by incubation in an avidin biotinylated peroxidase complex (1:400, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Labeled cells were visualized with 0.15 mg/ml diaminobenzidine (DAB) and 0.003% H₂O₂ solution.

After mounting of the sections onto glass slides for microscopic analysis, sections were counterstained with a Mayer-haematox solution for 30 seconds. Counting of Ki-67 positive cells in both hemispheres of the dentate gyrus was performed under a light microscope with a magnification of 400x. Counting was performed in the subgranular layer of the dentate gyrus, the border of the area that was quantified was defined as the subgranular layer having a thickness of two cell diameters. All cells were counted in the subgranular layer of the dentate gyrus from top to bottom of the 40 µm thick sections, and counts in both blades were summed. Because every twelfth section of the brain was stained, the number of positive cells was multiplied by 12 for the estimated total amount of Ki-67 positive cells in the hippocampus.

Effects of high-dose MTX on white matter

Brain sections were also analyzed with the Quantimet 550 IW image analysis system (Leica, Cambridge, UK) for changes in white matter. White matter damage was not only studied 1 day and 7 days after MTX treatment but also 3 weeks after MTX administration. For this last group sections were used from a previous experiment (Seigers et al., 2008). From each animal, thickness of the corpus callosum was measured in the middle and 3.5 mm bilaterally of a section at Bregma -3.30 mm.

Effects of high-dose MTX on cell death

Every twelfth section from serial sections of animals sacrificed 1 day, 1 week, or 3 weeks after treatment with saline or MTX was selected and immunohistochemically stained with silver nitrate using a slightly adapted standard protocol (Ter Horst et al., 1995). In brief, free-floating slices were rinsed in 4.5% sodium hydroxide/ 0.6% ammonium nitrate solution. After rinsing, the slices were impregnated for 10 minutes with a mixture of 7% sodium hydroxide, 8% ammonium nitrate, and 50% silver nitrate. After rinsing the slices in a mixture of 0.5% sodium carbonate and 0.012% ammonium nitrate, development took place with 0.006% ammonium nitrate mixed with developer mix, after which the slices were fixated with 37.5% sodium thiosulfate silver fixation solution. The sections were rinsed with distilled water and mounted onto glass slides. Analysis of cell death was performed under a light microscope by scoring the number of cells that had absorbed the silver nitrate, which is considered to be a measure for cell death (Tashlykov et al., 2007; Ter Horst et al., 1995).
Chapter 3

Behavioral tests

*Morris water maze*

The Morris water maze (MWM) was performed in a circular black pool (Ø 140 cm) with a black platform. The pool was filled with water, with a temperature of 26 ± 1 °C, in such a way that the platform was approximately 1 cm below the water surface. The pool was surrounded with external constant cues, and the observer was always positioned at the same location. The task consisted of 4 training days with 4 trials per day, with an intertrial time of 1 hour. One trial lasted for 3 minutes or until the rat found the platform and sat on it for 10 seconds. If a rat did not find the platform within 3 minutes it was guided by hand. Animals were divided into 2 groups based on learning behavior on the last day of the MWM and were injected with either saline or MTX (250 mg/kg) within 1 hour after the last trial as previously described.

Body weight of all animals was measured on a daily basis. Body weight gain will be expressed as the percentage of the body weight on the day of the injection. One week after the injection, the animals were placed in the pool without the platform for 1 minutes (probe trial).

Behavior of the animal was tracked by using Ethovision 3.0 and analyzed for escape latency in the learning phase, being the time from the beginning of the trial until the rat sat on the platform. During the probe trial the time the animal spent in the right quadrant, average swim speed and total distance moved was analyzed.

*Contextual fear conditioning*

The contextual fear conditioning task was performed with a different group of animals than the Morris water maze. The test was performed in a passive avoidance box composed of black Plexiglas (42 x 42 x 42 cm) with a grid floor to establish a dark safe area, with an open top for observation. One of the walls contained a sliding door. On the other side of this door, a small Plexiglas platform (30 x 10 cm) was attached; this platform was lit to form a light, unsafe area. The construction was placed in such a way that the platform was located 70 cm above the floor of the experimental room, while the closed black area was situated on a table. The animals were given 1 habituation session per day for 2 days, to explore the light and dark area freely. Each session lasted 3 minutes and started on the lit platform. On the third day the door was closed after the animal entered the dark compartment and a foot shock was given of 0.8 mA for 3 seconds. After the shock the animals remained in the dark area for 30 seconds before they were placed back into their home cage. The animals received either saline or MTX (250 mg/kg), as previously described, within 1 hour after the foot shock. One month after the foot shock and treatment the animals were placed in the dark compartment for 3 minutes, with the door closed. The session was recorded and analyzed for immobility behavior.

*Statistics*

Body weight was analyzed using repeated measure ANOVA. Light-microscopic counts of Ki-67 positive cells, the density of white matter, and the behavioral tasks were analyzed using one-way ANOVA with treatment as between-subject variable. LSD or Tukey post hoc tests were performed when the ANOVA test was significant. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.
Results

Immunohistochemistry

Short-term effect of high-dose MTX on hippocampal cell proliferation
The total number of Ki-67 positive cells was 5283.0 ± 293.6 SEM for control animals sacrificed 1 day after injection; 4132.0 ± 1093.8 SEM for animals treated with MTX sacrificed 1 day after injection; 5176.8 ± 713.0 SEM for control animals sacrificed 7 days after injection; 2167.5 ± 520.3 SEM for animals treated with MTX sacrificed 7 days after injection (figure 1). The total number of Ki-67 positive cells significantly differed between the groups, $F_{3, 22} = 3.952, P < 0.05$ for one-way ANOVA. Post hoc test revealed that the total number of Ki-67 positive cells of animals sacrificed 7 days after treatment with MTX was significantly lower than both control groups ($P = 0.01$).

![Figure 1. Total number of Ki-67 positive cells in the hippocampus of control rats, perfused 1 day after treatment (open bar, n = 4); animals treated with MTX, perfused 1 day after treatment (light grey bar, n = 6); control animals, perfused 7 days after treatment (dark grey bar, n = 5); and animals treated with MTX, perfused 7 days after treatment (black bar, n = 8). Data are represented as mean with standard error of the mean. Scatter plot represents the individual data per group. The total number of Ki-67 positive cells significantly differs between the groups, $F_{3, 22} = 3.952, P < 0.05$ (one-way ANOVA). The total number of Ki-67 positive cells 7 days after treatment with MTX was significantly lower than both control groups ($P = 0.01$).](image)

Effect of high-dose MTX on white matter
The density of the lateral corpus callosum was 180.28 µm ± 6.6 SEM for control animals; 151.37 µm ± 6.1 SEM for animals treated with MTX sacrificed 1 day after injection; 148.77 µm ± 6.9 SEM for animals treated with MTX sacrificed 7 days after injection; and 163.53 µm ± 5.2 SEM for animals treated with MTX sacrificed 3 weeks after injection. The density of the lateral corpus callosum, was significantly different between the groups, $F_{3, 31} = 5.547, P < 0.005$ (figure 2). Tests for contrasts revealed that the lateral corpus callosum was significantly less dense in all MTX treated animals compared to control animals ($P < 0.001$ for all groups). No effect was seen in the density of the corpus callosum in the center of the brain (data not shown).
Figure 2. Density of the lateral corpus callosum, measured above the middle of the left and right hippocampus of control animals (open bar, n = 12); animals treated with MTX, sacrificed 1 day after treatment (light grey bar, n = 7); 1 week after treatment (dark grey bar, n = 7); and 3 weeks after treatment (black bar, n = 8). Data are represented as mean with standard error of the mean. The density of the lateral corpus callosum significantly differed between the different groups, $F_{3, 31} = 5.547, P < 0.005$. Tests for contrasts revealed that the lateral corpus callosum had significantly less density in all MTX treated animals compared to control animals ($P < 0.001$ for all groups).

Effect of high-dose MTX on cell death
Every twelfth section from animals sacrificed 1 day, 1 week or 3 weeks after treatment with either saline or MTX was selected and stained with silver nitrate to visualize cell death. The number of cells that had absorbed the silver nitrate was very low in all groups and no differences were seen in silver nitrate absorption between the different groups (data not shown).

Figure 3. Body weight gain during and after the Morris water maze for control animals (open circle, n = 8), and animals treated with MTX (black circle, n = 8). The white bar on the x-axis represents the 4 days of the learning phase of the MWM, which lasted from day -3 till 0. The animals were treated with either saline or MTX after the last trial on day 0, which is shown with an arrow. The grey bar represents the probe trial which was given on day 7. Body weight before the injection (day 0) is expressed as 100%, bars represent standard error of the mean. Repeated measures ANOVA for day 0 till day 8 revealed a significant difference between the control animals and the animals treated with MTX ($F_{1, 13} = 8.290, P < 0.001$).
Behavioral tests

**Morris water maze**

The Morris water maze was performed on day -3 to 0, with day 0 being the day of the injection. Body weight gain of the animals was measured on a daily basis (figure 3). Body weight gain significantly decreased in rats treated with MTX compared to control animals, when analyzed from day 0 till day 8 ($F_{1, 14} = 8.290, P < 0.001$). However, at the day of the probe trial (day 7), the decrease in body weight gain was not significant anymore indicating recovery.

Figure 4A shows the daily average escape latency during the training period of the MWM before treatment. The animals were divided in 2 groups, based on learning behavior on day 4 of the MWM in such a way that there was no difference in learning capacity between the groups to prevent learning bias. Escape latency of both groups significantly improved during the learning phase, with $F_{1, 3} = 12.596, P < 0.01$ and $F_{1, 3} = 15.039, P < 0.01$ for control animals and animals treated with MTX respectively. The difference on day 1 did not influence learning behavior of the animals and is caused by random swimming behavior during the first trials.

The animals received a probe trial of 60 seconds 1 week after the last trial and treatment. There were no significant differences between the control animals and the animals treated with MTX for average swim speed or total distance traveled (data not shown). However, animals treated with MTX spent significantly less time in the quadrant where the platform used to be compared to control animals (33.9% ± 2.7 SEM for control animals and 25.0% ± 3.2 SEM for animals treated with MTX), $F_{1, 14} = 5.466, P < 0.05$ (figure 4B).

![Figure 4](image-url)
Contextual fear conditioning

Contextual fear was tested 1 month after the food shock and treatment by placing the animals in the dark compartment where they had received the foot shock. Animals treated with MTX showed significantly less freezing behavior compared to control animals (59.9% ± 8.8 SEM for control animals and 20.4% ± 5.7 SEM for animals treated with MTX), $F_{1, 15} = 16.290, P = 0.001$ (figure 5).

Discussion

This study investigated the effects of methotrexate on hippocampal cell proliferation, white matter, and cell death on several time points after MTX administration. Memory performance was tested in two different behavioral tasks. The results show that hippocampal cell proliferation is significantly reduced seven days after treatment with MTX compared to control animals. No significant group effect was seen in animals sacrificed one day after treatment. However, the large standard error of the data obtained at one day after treatment suggests a large individual differentiation. Indeed, some animals treated with MTX had an equal number of Ki-67 positive cells compared to control animals (n = 2), whereas others showed a major decrease in the number of Ki-67 positive cells (n = 4). This finding suggests that there is an individual difference in the speed of response to MTX, resulting in a general decrease in hippocampal cell proliferation one week after treatment. Furthermore, treatment with MTX decreased the thickness of the lateral corpus callosum shortly after treatment (one day) as well as on the long-term (three weeks after treatment). No cell death was noticed after treatment with MTX at any time point studied (one day, one week, or three weeks after treatment).

A short-term effect of a chemotherapeutic drug on hippocampal cell proliferation was also found in a study of Mignone and Weber in C57BL/6J mice. The animals received three daily injections of either thioTEPA or 5-fluouracil (5-FU), and the last injection was directly followed by an injection of BrdU. Two hours after the BrdU injection, the mice were sacrificed and the effect of the cytostatic agents on cell proliferation was studied. ThioTEPA significantly decreased hippocampal cell proliferation in a dose-dependent manner, whereas 5-FU did not induce an effect, possibly due to insufficient penetration in the brain (Mignone and Weber, 2006).

The effect of cytostatic drugs on hippocampal cell proliferation, white matter density, and cell death was also studied by Noble and colleagues (Dietrich et al., 2006; Han et al., 2008). CBA mice received three injections for three consecutive days of either BCNU or cisplatin. The animals were
sacrificed one day, days days, or 42 days after the last injection. A different group of animals received three injections of 5-FU every other day, and were sacrificed one, seven, fourteen, 56 days or six months after the last injection. BCNU significantly decreased cell division up to 42 days after treatment. Cisplatin showed similar effects, but the number of dividing cells returned to normal levels in the subventricular zone and the dentate gyrus six weeks after treatment. 5-FU also decreases cell division for up to six months in the subventricular zone, for up to 56 days in the corpus callosum, and only on the long-term (starting at day fourteen) in the dentate gyrus. Cell death was measured with a TUNEL staining and all cytostatic drugs induced cell death up to ten days for BCNU and cisplatin and fourteen days for 5-FU. A decrease in myelin sheaths and a deregulation of Olig2 expression, crucial for generating functional oligodendrocytes, was found in the corpus callosum of animals treated with 5-FU which suggest that 5-FU causes damage to white matter (Dietrich et al., 2006; Han et al., 2008).

Several papers of Palmer and colleagues also show radiation negatively affects neurogenesis and induces cognitive impairment. Radiation increased the presence of reactive oxygen species (ROS) and induced increased apoptosis in primary neural precursor cells of the rat (Limoli et al., 2004). Radiation has also been shown to decrease the number of proliferation cells in the hippocampus of C57/BL/J6 mice (Mizumatsu et al., 2003) and to inhibit neural precursor cell proliferation (Monje et al., 2002). Furthermore, radiation alters the cell fate profile, resulting in a decrease of neuron production, and an increase in oligodendrocyte production and in proliferation of activated microglia (Monje et al., 2002).

We also showed in this paper with the results from the behavioral tests that MTX affects memory when given directly after the learning task, shown in the Morris water maze and contextual fear conditioning. The impaired learning causes by MTX could be caused by consolidation deficits or through retrograde amnesia. The memory process consists of four different stages, being encoding, storage, consolidation, and retrieval (Akbari et al., 2006; Riedel et al., 1999; Robertson, 2002). Short-term consolidation can be affected by treating animals directly after training with, for example, convulsive shocks, brain stimulation or drugs. The memory trace is not stored into long-term memory when the consolidation process is disrupted (Riedel et al., 1999). Since we treated animals with MTX directly after the training period it is likely that we disrupted the consolidation process. This is supported by a number of articles (Akbari et al., 2006; Kinney et al., 2003; Riedel et al., 1999). Riedel and colleagues performed a study to see how the consolidation process of a memory trace could be impaired. Male Lister hooded rats were subjected to a Morris water maze and glutamatergic synaptic transmission was blocked at different time points. When neural activity was blocked chronically for seven days, with the start of the treatment either directly after training or five days later, consolidation of the memory trace was blocked. This suggests that consolidation of a memory trace takes a certain amount of time and that neural activity is necessary for all stages of the memory process (Riedel et al., 1999).

Similar effects were shown in a study by Kinney and colleagues. In this paper, male Sprague-Dawley rats, implanted with a cannula in the left lateral ventricle, were subjected to a Morris water maze with administration of galanin at different time points. Galanin is a neuropeptide that inhibits the release of several neurotransmitters, for example glutamate, norepinephrine, serotonin, and acetylcholine. When the substance was given 30 min or 3 hours after the training, it disrupted the consolidation of the memory trace (Kinney et al., 2003).

Retrograde amnesia is the loss of memories acquired before the onset of amnesia and is frequently associated with the consolidation process, since it often affects memories close to the time of the amnesia inducing incident, with sparing of remote memories (Meeter and Murre, 2004; Wiig et al., 1996). Winocur and colleagues examined the role of the hippocampus on retrograde amnesia. Male Long-Evans rats were given food preference training, which was followed immediately, two, five or ten days later with a lesion in the dorsal hippocampus. The time between the lesion and the food preference training influenced memory. Animals that received a lesion immediately after the test did
not show food preference, whereas animals that received the lesion ten days later showed equal preference as sham operated animals (Winocur et al., 1990).

Anagnostaras and colleagues explored the effect of electrolytic lesions of the dorsal hippocampus on remote and recent memories. Female Long-Evans rats received two contextual fear conditioning tasks in two different contexts, 50 days apart. The animals received a lesion the day after the second contextual fear conditioning task and were retested ten days after the surgery. Recent, but not remote, memory was impaired in animals with a lesion compared to control animals, meaning that the animals showed no freezing behavior in the recent context and freezing behavior in the remote context (Anagnostaras et al., 1999).

In our opinion, animals treated with MTX in our study suffered from retrograde amnesia due to decreased hippocampal cell proliferation caused by MTX, since hippocampal cell proliferation is thought to be involved in learning and memory (Gould et al., 1999; Kempermann, 2002; Kempermann et al., 2004). Evidence for this hypothesis is provided by many papers describing memory impairment after irradiation which strongly inhibits hippocampal cell proliferation (Madsen et al., 2003; Raber et al., 2004; Rola et al., 2004).

Combining our data and the literature, we can conclude that disruption of the hippocampal function, either chemically or surgically, leads to retrograde amnesia and impairs the ability to consolidate a memory. Since cognitive impairment is a side-effect in a sub-group of patients after adjuvant chemotherapy (Brezden, et al., 2000; Kreukels et al., 2005; Schagen et al., 2002; Shilling and Jenkins, 2005), we hypothesize that cytostatics, such as MTX, can also disrupt the hippocampal function. Whether this cognitive impairment is solely caused by the negative effect of MTX on hippocampal cell proliferation or if other mechanisms also play a role needs further exploration.

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