KU LEUVEN

FACULTEIT PSYCHOLOGIE EN

PEDAGOGISCHE WETENSCHAPPEN

Preclinical Mouse Study on Maternal Separation Impacting Childhood Chemotherapy-induced Cognitive Impairment

Masterproef aangeboden tot het verkrijgen

van de graad van Master of Science in de

Psychologie: Klinische en

Gezondheidspsychologie door

Sien Verelst

promotor: Prof. Rudi D'Hooge

m.m.v: Livine Craeghs

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Summary

Modern science has already made great progress when it comes to treatment of cancer. Children with cancer now have an 81% chance of 5-year survival. However, survival does not come without cost. The long-term consequences of methotrexate, a key aspect of cancer treatment and one of the most used active chemotherapeutic agents, are not to be underestimated. Neurocognitive difficulties and decline are unfortunately common among childhood cancer survivors, and they can experience complications well into later life. Additionally, during treatment, the children are separated from their parents for significant periods of time, which can disrupt the attachment process. To prevent such adverse outcomes, we must first gain insight to which cognitive and social domains are affected and how.

This study aimed to address the influence of methotrexate on a developing brain, whilst exploring the role of attachment as well, using a juvenile mouse model. We hypothesized that methotrexate would cause long-term neurocognitive and social difficulties, and that maternal separation during the nurture period would make the pups more prone to the effects of chemotherapy.

The pups were subjected to either maternal separation or handling from postnatal day 10 to postnatal day 18. Next, they received three injections, on postnatal days 21, 28 and 35, with either methotrexate or vehicle. After the maternal separation, as well as after the injections, the pups were subjected to the maternal preference test to assess the bond between pup and dam directly after the experimental manipulations. In the next stage, long-term effects were determined using a behavioral test battery, containing the open field, elevated

plus maze, flipped social exploration, sociability/preference for social novelty test, automated tube test, forced swim test and the Morris water maze.

Our results showed that, although there was no immediate effect in maternal bonding found during the pup phase, mice who were subjected to maternal separation and received methotrexate appeared to have behavioral complications. They exhibited significantly slower movements and displayed possible impairment in memory. Stranger mice seemed to be less interested in interacting with them in comparison to the other experimental groups. However, anxiety-related and depression-like behavior seemed unaffected.

Due to unforeseen small sample sizes, the results in this thesis should be considered preliminary results. They are not conclusive, but they do give a direction. Hopefully they inspire other researchers to unravel the precise mechanisms of neurocognitive decline, together with the link between chemotherapy and attachment, and significantly improve the quality of life for childhood cancer survivors.

Acknowledgements

First, I sincerely would like to thank some people without whom the realization of this thesis would not have been possible. To begin with, I would like to thank Prof. Dr. Rudi D'Hooge, the promotor of my thesis. During the selection procedure, I gave preference to many of his research proposals. For my thesis, I thought it was important to have the opportunity to carry out every part of the research process myself. Due to the COVID-19 pandemic, I learned that many thesis proposals involving human subjects could only partially proceed as planned. This appeared not to be the case for animal research, and because I valued the learning process, I decided to give it a try. Although I never had a particular interest in animal research before, I noticed I developed a fondness for these kinds of studies. I've always liked animals and as it turns out, I do really enjoy animal research. I believe I treat all animals with utmost respect. On one hand because I believe it is normal to do so, on the other hand because it benefits the results. Furthermore, I have experience with cancer in my own personal environment, so the purpose of the study appealed to me as well. I am grateful for the opportunity to be a part of such a meaningful research project.

I would like to extend a big thank you to Livine Craeghs as well, my daily supervisor. Despite having a lot of work herself, she was always available for questions and advice. For several months, we worked closely together to gather the experimental data. She showed me the ins and outs of an experimental research lab and she patiently and accurately explained the protocols and behavioral tests to me. Also, the administration of compounds via intraperitoneal injection was something I was allowed to do myself, and I took great care in this. I truly appreciate the responsibility I received during this study. Aside from that, I also

enjoyed getting to know her as a person. In addition to the order of the day, there was always room for humor or a personal conversation about weekend plans or hobbies. She introduced me to the *Potterless* podcast, for which I am grateful. It proved extremely helpful to stay alert during the Morris Water Maze test! Since I am considering continuing in research in the future, she is willing to answer all my questions and to make some recommendations if necessary. Livine, thank you so much for all your efforts to help me, guide me, educate me... I know you had a lot on your plate yourself, a PhD is extremely challenging. Yet, aside from that, you made a real effort to read and improve my thesis and to transform me into a (hopefully) competent researcher. I cannot thank you enough and appreciate everything you have done for me. It was a real pleasure working with you and getting to know you!

Additionally, I sincerely would like to thank my social network as well. For starters, my family. They provided me with unconditional support throughout my whole academic journey. My parents often told me they did not understand what I was doing exactly, but their support meant a lot to me, nevertheless. It has been a challenging year for me. I have a disability student status at KU Leuven, and the combination of an internship and a thesis has been very demanding for my physical health. I am known for not giving up easily and I can work very hard for things that are close to my heart. Obtaining this degree is one of them. Although I am very independent and try to limit asking for help, my parents helped and supported me where they could and gave me the space and mental support I needed. I am also thankful for my siblings, Joren and Floor, who provided the necessary empowerment and distractions, and even read some parts of my thesis. I could not have asked for better 'cheerleaders'. My whole family showed me how proud they are of me and my efforts, and I

am beyond grateful for them. Finally, I am active in the local youth movement, and I teach gymnastics, so I would like to thank everyone involved for their patience with me and my academic obligations this year. I required a lot of space to focus on my internship, thesis and my health and I am grateful to have been able to do that without quitting all my hobbies. My roommates, who ran errands and cooked and cared for me during the busiest times, meant a lot for me as well. I truly appreciate all the support I got, sometimes without even asking for it. Thank you!

Approach and Input

At the beginning of the master's program, Prof. D'Hooge introduced himself, his PhD students, and the lab. During the research, I aimed to work as independently as possible. The lab setting was new to me, however I received great guidance at every step from Livine. Before my own experiments started, I analyzed mice videos for another one of Livine's studies. It seemed interesting and a good way to familiarize myself with the project. During the second semester last year, I spent most of my time in the lab. Livine explained every manipulation and experiment in detail. After going through the protocols, I was allowed to quickly perform these tasks independently. The maternal separation, monitoring the weights, the injections, the cleaning of the cages and taking care of the animals, the behavioral tests… Occasionally we worked during weekends as well. Livine was always present in the building and available for questions. Sometimes, I asked my questions to Dr. Zsuzsanna Callaerts-Vegh, who was often present in the lab. Other researchers and students gladly explained their own projects to me as well. All the contacts were very pleasant. For the statistical analysis, Livine thoroughly explained to me how to use the Graphpad Prism software and which variables to analyze. Dr. Callaerts-Vegh also gave a useful presentation about this for the lab's students. Next, I performed all analyses, made the accompanying figures, and reported the results. During the last semester this year, Livine and I had a weekly meeting. The content of the thesis was written by me and provided with feedback and advice multiple times by Livine. Prof. Rudi D'Hooge also read the thesis. The online 'thesis café' meetings, hosted by

Leercentrum Agora, were helpful to me as well. I had a great deal of responsibility regarding this project, I learned a lot, and could count on help when necessary.

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List of Abbreviations

ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
CNS	Central nervous system
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
E	Empty
FPGS	Folylpolyglutamate synthase
FR	Folate receptors
MRD	Measurable residual disease
MS	Maternal separation
MTX	Methotrexate
MTX_{Glu}	Polyglutamated methotrexate
MWM	Morris water maze
P	Postnatal day
PBS	Phosphate buffered saline
PCFT	Proton-coupled folate transporter
RFC	Reduced folate carrier
RNA	Ribonucleic acid
SD	Standard deviation
SPSN	Sociability/preference for social novelty test
STD	Standard
Str1	Stranger mouse 1

Str2	Stranger mouse 2
THF	Tetrahydrofolate

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Introduction

Childhood Cancer

Cancer has been one of the most common diseases for centuries. Essentially, cancer is the result of changes in the genome, i.e., an error in the management of cells, causing a process of progressive, uncontrolled proliferation of cells, also called neoplasms or tumors. In western countries, it is the second most important cause of death. Data suggests that the chances of developing some type of cancer increase exponentially with age, even more so in men (Dupont, 2014). However, these numbers apply to the adult population. Childhood cancers are quite rare and are very unlikely to occur before the age of 20 (Steliarova-Foucher, 2017). Yet, it is paramount to have a keen insight in the prevalence, development, course, treatment, and consequences of cancer during childhood, mainly due to the significantly higher number of post cancer life-years for children in comparison to adults. Additionally, their major life milestones (education, career, whether to have a family of their own or not, etc.) have yet to take place (Askins & Moore, 2008).

According to literature, the global prevalence of childhood cancer is 140.6 children per million a year, aged 0-14 years. Slightly more boys than girls are diagnosed, with an incidence sex ratio of 1.17 (Steliarova-Foucher, 2017). These numbers can vary depending on age, type of cancer and country. Childhood cancer is a high-impact disease, which is why societies with high socio-economic development are very dedicated to contributing to the development of diagnostic procedures, therapeutic strategies, and relevant health policies

(Kaatsch, 2006). An analysis of the database of the Automated Childhood Cancer Information System (ACCIS) reveals a steady 1.1% increase in childhood cancer incidence in Europe per year in the 1978-1997 period (https://accis.iarc.fr; Kaatsch, 2006).

Due to substantial increase in cancer research and development of effective treatments, the chances of surviving childhood cancer nowadays are high, especially in comparison to adults. Gatta et al. (2014) conducted a study about childhood cancer survival using the EUROCARE-5 database, which is a project involving an alliance of public health organizations across Europe concerning population-based cancer survival. Their studies suggested that from 1995-2002 all-cancer survival for 5 years was 81% in children and 56% in adults. This advancement was primarily due to improvements in treatment regimens (Askins & Moore, 2008). However, Gatta et al. (2014) observed large gaps between countries.

Acute Lymphocytic Leukemia

Leukemia is a cancer of the early blood-forming cells and refers to an excess of white blood cells in the body (Blackburn et al., 2019). Leukemia can be divided into four subcategories: acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia and chronic lymphocytic leukemia. Every type can be even more differentiated based on specific morphological features, cytogenetic abnormalities, immunophenotype and other clinical features (Blackburn et al., 2019). Leukemia is one of the most common and, fortunately, the most curable of all childhood cancers (Foster et al., 2018; Gatta et al., 2014). According to Steliarova-Foucher (2017), 36.1% of all cancers in children from age 0 to 4, are cases of leukemia. Over the last three decades, the prevalence of leukemia in Europe showed a significant increase of around 0.8% (Kaatsch, 2006).

ALL, the focus of this thesis, accounts for 60% to 74% of all leukemias diagnosed in children between 0-19 years of age (Blackburn et al., 2019). ALL originates due to a clonal proliferation of lymphoid precursors, or lymphoblasts, in the bone marrow, and it ultimately leads to impairments of normal blood cell production and causes organ infiltration by leukemic cells which is greatly life-threatening (van der Plas et al., 2015). The clinical representation of ALL, according to Blackburn et al. (2019), is usually the result of the immature leukemic cells invading the bone marrow where healthy cells (red blood cells, white blood cells and platelets) are supposed to be produced. This causes symptoms like anemia, leukopenia, and thrombocytopenia, meaning low cell counts of red blood cells, mature white blood cells, and platelets, respectively. Symptoms of anemia can include dizziness, pallor, fatigue, shortness of breath, and chest pain. Symptoms of leukopenia can include fever, frequent infections, and impaired wound healing. Symptoms of thrombocytopenia can include bleeding that is difficult to stop, easy bruising, nose bleeds, petechiae (tiny bleeding spots under the skin), and longer menstruation in females.

The mechanisms and risk factors to what causes ALL, remain generally unknown. However, significant differences exist according to ethnic group (O'Brien et al., 2018). Recent demographic research revealed that leukemia is significantly less prevalent in African regions. Even in the USA, African American children are less commonly diagnosed with leukemia in comparison to other ethnic groups (Steliarova-Foucher, 2017; O'Brien et al., 2018). Different studies state that it is thus believed that Hispanic and Caucasian children seem to carry a certain risk factor since they are more commonly diagnosed (Blackburn, 2019). An increased risk for leukemia seems to be associated with various genetic factors, for example Down's syndrome or polymorphic variants in several genes (O'Brien et al., 2018;

Blackburn et al., 2019). Furthermore, it occurs more frequently in boys than in girls, with 55% of cases being boys (O'Brien et al., 2018).

Due to medical progress in the past decades, the overall prognosis for ALL is quite promising with an average 5-year survival rate of 89% for patients between 0 and 19 years of age (Blackburn et al., 2019). However, there seems to be an extensive gap compared to the prognosis for adults. For patients who are 20 years of age and older, the survival rate comes down to 35%.

ALL Treatment

The treatment of choice for ALL depends on several factors. For example, the subtype and classification of the cancer, the current overall health of the patient, risk stratification, common side effects and the goal of the patient. Multi-agent systemic chemotherapy is commonly used and consists of drugs that can cross the blood-brain barrier (Blackburn et al., 2019). A key component in the treatment of leukemia is methotrexate (MTX) (Li et al., 2010). MTX and cytarabine (a chemotherapeutic drug used to slow or stop cancer cell growth) treatment is found to significantly increase survival in children with ALL (Shuper et al., 2000). Other drugs may also be involved in various combinations, for example steroids, anthracyclines (an antibiotic that damages DNA in cancer cells), vincristine (an alkaloid that inhibits cancer growth) and cyclophosphamide (a chemotherapeutic drug that suppresses the immune system).

The most recent treatment model for ALL is described by Heyman et al. (2020). The treatment contains the following phases: (1) *induction*, which involves the administration of dexamethasone (as a steroid), vincristine and pegylated asparaginase (a chemotherapeutic drug that stops the growth of cancer cells), (2) *induction consolidation*, which exists of two

doses of cyclophosphamide and three intrathecal doses, which can be either single (only MTX) or triple (consisting of MTX, cytarabine and prednisolone), however the measurable residual disease (MRD) is assessed via bone marrow and will be taken into account before administration, (3) *CNS consolidation*, in which the patient is subjected to another bone-marrow examination to assess MRD, and the ones in remission after induction continue with standard CNS-consolidation of which the therapy is composed of low-dose 6-mercaptopurine (an antimetabolite promoting apoptosis in cancer cells) and two doses of high-dose MTX together with triple intrathecal therapy, (4) *delayed intensification*, either consisting of two doses of triple intrathecal therapy, or of extended procedures including four repeated 3-week cycles, and finally (5) *maintenance*, for which the standard protocol is to be administered until 2 years after complete remission is achieved. There are, however, always certain groups and aspects that require specific attention. Every program will be monitored closely and adjusted to the patient, among other things via the assessment of MRD. Generally, chemotherapeutic cocktails are administered during the different phases a repeated number of times spread over days or weeks. This approach of administration increases the efficacy of the effective agents by enabling multiple pathways of cancer cell division to be attacked (Seigers et al., 2011).

Former improvements to the treatment regimen reduced but did not eliminate long-term adverse effects: (1) elimination of cranial irradiation, replaced by intrathecal MTX (2) the restriction of doxorubicin as an anti-leukemic agent in standard-risk patients, and (3) addition of asparaginase to standard regimens to improve disease-free survival (Ness et al., 2015). Over the past two decades, molecular agents and antibody-based immunotherapies were developed as well, creating the potential to treat specific targets. Targeted therapies specifically for leukemia include enzyme inhibitors, cluster of designation antibodies and immunotherapy (Eryilmaz et al., 2017). Treatment regimens used on young children have

been translated to use for patients from 15 to 39 years of age as well, given the significantly higher remission rates in children (Blackburn et al., 2019).

Methotrexate

One of the most active agents in the treatment of ALL is MTX (von Stackelberg et al., 2008). Despite being effective, literature established that MTX seems to have a whole range of neurobiological consequences as well, such as reduced neurogenesis, reduced blood flow and glucose metabolism, increased neurotoxic effects and apoptosis, oxidative stress, and white matter damage (Seigers et al., 2011). High doses of MTX are administered intrathecally, intravenously and orally (Shuper et al., 2000). It is a folate-antimetabolite that prevents the formation of new DNA or RNA in the cell, causing an interruption in the cell cycle (Seigers et al., 2011). MTX is a specific inhibitor of dihydrofolate reductase (DHFR), which is an important enzyme in the one-carbon pathway (Kiani et al., 2018; Schornagel & McVie, 1983). DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate (THF). THF is an important coenzyme in several transmethylation reactions which are essential in the repair, replication, and synthesis of DNA strands (Kozmiński et al., 2020).

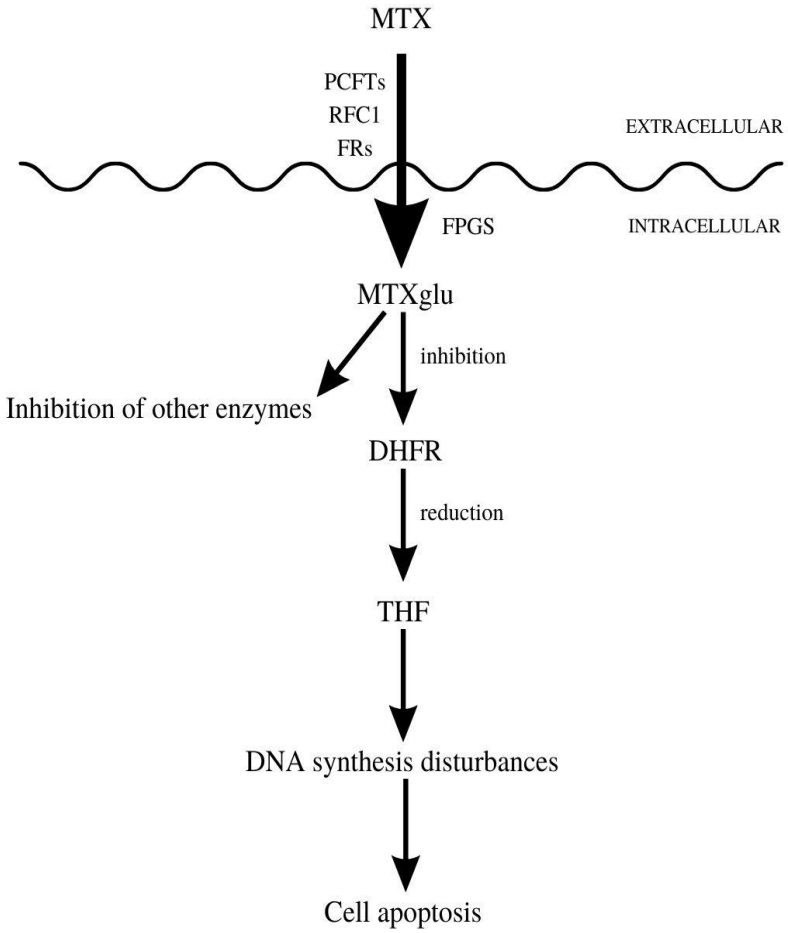
The following description of the working mechanisms of MTX is based on the work of Kozmiński et al. (2020), see Figure 1. MTX uses either proton-coupled folate transporters (PCFTs), reduced folate carrier 1 (RFC1) or folate receptors (FRs) to enter the cell. Intracellularly, folylpolyglutamate synthase (FPGS) metabolizes MTX to polyglutamate derivatives (MTX_{Glu}). Polyglutamated MTX is a superior antifolate agent in comparison to MTX since it can strongly inhibit DHFR. MTX_{Glu} also inhibits a multitude of other enzymes. Therefore, it interferes with the precursors a cell needs for the synthesis of DNA and RNA essential for cell proliferation. In the S-phase of the cell cycle, these disturbances in DNA

synthesis lead to cell apoptosis. Accordingly, the influence of MTX is most visible in rapidly dividing cells and consequently, highly proliferating cancer cells are the most susceptible to its effects.

Unfortunately, because MTX intervenes in active cell division and shows low selectivity for cancer cells, it frequently affects healthy tissues with rapidly dividing cells as well (Kiani et al., 2018). Previous research shows that higher doses of MTX have higher antileukemic effects, however, the usable dose is strictly limited since it is associated with significant toxicity as well. MTX can cause numerous side effects like nausea, vomiting, hair loss, fatigue and anemia (Bakitas, 2007). Previous research shows that the cytotoxic effects of MTX can be counteracted by activated folic acid (leucovorin). This makes it possible to partially increase the limit of the administered dose (von Stackelberg, 2008).

Figure 1

Working Mechanisms of Methotrexate



Note. Extracellularly, methotrexate (MTX) is brought into the cell using proton-coupled folate transporters (PCFTs), a reduced folate carrier 1 (RFC1) or via folate receptors (FRs). Intracellularly, MTX is metabolized to a polyglutamate derivative (MTX_{Glu}) by folylpolyglutamate synthase (FPGS). MTX_{Glu} is a strong inhibitor for dihydrofolate reductase (DHFR), which causes in its turn a tetrahydrofolate (THF) reduction. These changes create disturbances in DNA and RNA synthesis which are essential for cell proliferation. During the S-phase of the cell cycle, this will lead to cell apoptosis. Based on Kozmiński et al. (2020).

Long-Term Treatment Effects for Childhood Cancer

Although the amount of childhood cancer survivors over the past five decades is increasing, cure does not come without cost. The question arises if these adverse long-term effects are to be attributed to the cancer itself or the treatment, though many studies are convinced that the toxicity of the treatments is, in fact, the real malefactor (Wefel et al., 2012). Many cancer survivors will face somatic, psychological, social, and cognitive complications later in life due to treatment exposure to highly toxic compounds (de Fine Licht et al., 2017). Therefore, research made a focus shift from investigating death and grief to investigating the adjustment of the survivors and their families during and after fighting cancer (Van Schoors, 2019). The treatment-caused outcomes are believed to have a negative effect on the patients' mental health, school performance, job success and their quality of life (van der Plas et al., 2015). Luckily, only a minority is severely affected by these effects, the affected proportion estimated between 17% and 33% (Seigers et al., 2011). Regardless of these findings, it remains meaningful to dedicate research to this topic. Given their young age, the long-term effects of chemotherapy may have a great impact on the patients' lives and on society (Robison et al., 2005).

Neurocognitive Consequences

One of the most researched consequences of cancer treatment are the neurocognitive effects. Chemotherapy is often associated with long lasting cognitive decline, varying from subtle to severe, also known as 'chemo brain' (Seigers et al., 2011; Kannarkat et al., 2007). Neurocognitive difficulties can be found well into later life (Askins & Moore, 2008). The graduation rate of leukemia survivors is significantly lower (86%) in comparison to a sibling cohort (93%) (Robison et al., 2005). These numbers illustrate that most patients will in fact

graduate high school, but they do encourage a close monitoring of survivors to recognize early signs of learning disabilities (Robison et al., 2005). Previous studies have repeatedly shown that childhood cancer survivors show an overall reduced education attainment level, diminished cognitive functioning encompassing memory, quantitative skills, and abstract reasoning. These neurocognitive effects are mostly seen in children with acute leukemia or CNS tumors who underwent cranial radiation therapy or intrathecal therapy. Other neurocognitive skills children treated with MTX often struggle with are visual perception, nonverbal learning mechanisms dependent on attention, concept formation, short-term memory, fine motor functioning, executive functions, processing speed (Cole & Kamen, 2006; Montour-Proulx et al., 2005; van der Plas et al., 2015; Moleski, 2000; Clanton et al., 2011). Up to 23% of survivors report the use of special education services (Robison et al., 2005).

An important question to ask is through which pathways MTX achieves its neurotoxicity. In the past, the presence of seizures was considered the most prominent and observable indication of neurotoxicity. However, the research conducted by Montour-Proulx et al. (2005) revealed that a significant number of their participants experienced neurocognitive decline and white matter changes, whilst only a small number also experienced seizure. MTX is now known to cause a disease called leukoencephalopathy, which indicates the atrophy of white matter, also known as demyelination. The most notable anomalies can be found in the subcortical white matter of the cerebral hemispheres and cerebellum (Gilbert et al., 1989; Ness et al., 2015). According to Cole & Kamen (2006), white matter is notably sensitive to the impact of MTX and other chemotherapeutic agents. Research also states that an increased level of myelin basic protein in the cerebrospinal fluid, tau and choline/water ratio is related to demyelination, even though it has not yet been

causally linked to neurocognitive decline (Vezmar et al., 2003). The brain abnormalities are presumably linked to the pharmacodynamics of MTX (Krull et al., 2003). However, the exact pathophysiology of neurotoxicity attributed to MTX remains thus far unclear (Brugnoletti et al., 2008).

Risk factors for delayed neurocognitive decline are not yet absolute. There are no validated predictors that give us complete certainty about who will develop neurocognitive complications and who will not (Cole & Kamen, 2006). Nonetheless, research exists with the aim of discovering in what cases it is particularly advised to be cautious. A possible risk factor for adverse neurocognitive outcomes is sex. Young girls who receive chemotherapy and cranial radiation therapy may develop a significantly lower IQ score. Other risk factors for adverse neurocognitive outcomes are demographic and medical variables. Demographic factors include young age, which is a well-known risk factor due to the ongoing development of the nervous system at that age. Medical factors mainly refer to pre-existing complications, for example Down's syndrome, hydrocephalus, or also postoperative events like ataxia (Askins & Moore, 2008).

Finally, it is important not to lose sight of the indications that might suggest a patient is struggling with neurocognitive complications. This can be a challenge, as research points out that children often present as socially and verbally proficient during interaction (Montour-Proulx et al., 2005). Clinicians need to be aware of the risks and important to be observant during clinical visits not to miss the behavioral symptoms of neurotoxicity. Our research might be a valuable aid in this regard.

Somatic Consequences

Recent studies have shown that childhood cancer survivors are more likely to be hospitalized for other somatic diseases, unrelated to the cancer itself, later in life (de Fine Licht et al., 2017). The risk seems to gradually decrease with increasing age but remains significant in comparison to healthy controls. The diseases that cause the hospitalizations are mainly epilepsy, diseases of the nerves and peripheral ganglia, pneumonia, and pituitary hypofunction (de Fine Licht et al., 2017). Besides risks for unrelated diseases, cancer survivors showed an increased risk in hospitalization for a new cancer as well.

Psychological and Social Consequences

The late effects of chemotherapy do not exclusively situate themselves in the body. Previous research shows that the consequences can also be found in social situations and in terms of mental wellbeing. For example, childhood cancer survivors seem to be significantly more at risk for experiencing difficulties obtaining employment and insurance, and they experience an overall reduced quality of life (Robison et al., 2005). Further, research suggests that a significant number of childhood cancer survivors are more prone to developing depression, living in fear, anxiety and PTSD (Kaye et al., 2017; Iwai et al., 2017; Amatoury et al., 2018; Rourke et al., 2007). It is not yet clear which precise factors mediate the relationship between childhood cancer and the development of psychopathology. The impact of childhood cancer and its therapy on the family social environment will be discussed in the next section.

Family Consequences and Parental Bonding in Childhood Cancer

Attachment to a parent or caregiver is an important and necessary mechanism that allows a child to develop and grow up safely and successfully. The quality and timing of attachment in children could determine the quality of their later development (Malekpour,

2007). Attachment has been an object of research since the 1940s, formulated and researched by John Bowlby and Mary Ainsworth (Bretherton, 1992). After tremendous research, four major attachment styles have been described: autonomous-secure attachment, ambivalent attachment, avoidant attachment, and disorganized attachment, with the latter three being insecure attachment styles (Granqvist et al., 2017; Bretherton, 1992). Secure attachment applies to children who possess a basic confidence in their caregiver, knowing that the caregiver will behave in a responsive and comforting manner when required. They experience the caregiver as a secure base from which they can inquisitively discover and explore (Granqvist et al., 2017; Naveed et al., 2016). Avoidant attachment is an insecure form where children avoid showing their distress to the caregiver because they have learned in the past that displaying such feelings might result in rejection. Ambivalent or resistant attachment is an insecure form as well, where children exhibit signs of distress even before separation and seem to experience frustration with the caregiver's return. It is described as a distrust of the caregiver's availability even when he/she is present (Duschinsky, 2017). Disorganized attachment is a fourth style described by Main and Solomon. It is characterized by children exhibiting contradictory behavior, undirected/misdirected and interrupted movements and expressions, anomalous postures, freezing, signs of apprehension regarding the caregiver and indices of disorganization and disorientation (Granqvist et al., 2017).

Attachment is a domain of interest in this thesis because early life experiences are presumed to be associated with later adaptation and mental health. It determines the way in which children respond to stressful events and the extent to which they can draw on parental support (Fearon et al., 2010). As described in Oral et al. (2016), adverse childhood experiences potentially have harmful effects on long-term health. They defined life-threatening childhood illness and household dysfunction, like parental absence, as such

experiences. Those events can potentially increase the susceptibility to stress in adulthood (Peña et al., 2019). Given the circumstances, it may not be unlikely for a young cancer patient to develop a form of insecure attachment. The presence of such attachment styles is unfortunately not reassuring, since those children tend to have trouble with regulating emotions and interacting adequately with peers, which could all contribute to anxiety. They are also believed to have a higher risk for depression, suicide, substance abuse and smoking. Children with secure attachment styles tend to have fewer extreme reactions to stress (Naveed et al., 2016).

This way, parenting and attachment style can be a defining factor in the impact of childhood cancer treatment and its long-term consequences (Orbuch et al., 2005; Moules et al., 2016). A cancer diagnosis in children causes periods of separation (Arruda-Colli et al., 2018), posing a challenge to the attachment process. The outcomes of childhood cancer on the bond between a child and its parents is very unpredictable. Some studies report more familial closeness, others observe more strained bonds between parents and children (Orbuch et al., 2005). Orbuch et al. (2005) found that survivors who experienced a more positive relationship with their mothers, report generally higher levels of psychological well-being. Their conclusions state that the perception of the parental relationship by patients are linked to their current well-being as adolescents or young adults.

Childhood cancer has an enormous impact on the families involved and is considered one of the most stressful events you can encounter as a family (Kaatsch, 2006; Orbuch et al., 2005). To date, there are however few studies focusing on family adjustment when facing pediatric cancer. First, the existing body of literature does suggest that most families function within normal range (Van Schoors, 2019). This is hopeful news, yet it does not mean that families do not face a great deal of challenges or carry heavy burdens. Studies thus conclude

that it is not uncommon for parents to experience post-traumatic stress symptoms, emotional distress and anxiety, whereas siblings tend to worry, feel lonely, sad and experience post-traumatic stress symptoms as well (Arruda-Colli et al., 2018; Van Schoors, 2019). The burden on parents of children suffering from cancer is extremely hard. Being the mother of a child with cancer is a risk factor for family conflict. Mothers seem to experience more disruption of their social relations in comparison to fathers. A meaningful protective factor is psychological flexibility in parents, or to which extent they can accept their negative thoughts and emotions. Finally, research does show us that the impact on family decreases with time. Meaning that the more time passes since the diagnosis, the better the family can adjust (Van Schoors, 2019).

Rodent Research

The great impact of cancer and its treatment on a young human life along with all their damaging consequences has been discussed extensively in the previous parts. However, what are the exact mechanisms behind these consequences? If we are aiming to prevent the adverse effects of cancer treatments in the future, it is vital that the underlying mechanisms are well understood. It is, nonetheless, not that simple nor ethical to conduct this kind of research on human participants to gain answers. Methodological matters pose problems as well, such as the unavoidable small sample sizes in humans, differences in age, cancer, and treatment protocol (Seigers et al., 2011). Therefore, animal models are profoundly valuable in cancer research. They provide unique opportunities to identify biological changes in the nervous system associated with the administration of chemotherapeutic agents. The aim of these studies is to help design clinically relevant therapeutic interventions directed to the possible brain changes using the different mechanisms involved (Wefel et al., 2012).

Various animal studies over the past few decades have provided important information on cancer and the consequences of its treatment. Unfortunately, the results are often not aligned. While quite some studies found significant results of chemotherapy affecting cognition, there exist studies as well that failed to find an effect (Seigers et al., 2011). Different studies are also likely to use different ways of administration in animals. Some of the options are intraperitoneal injections, tail vein injections, administration in the cerebrospinal fluid, etcetera. These differences in experimental design possibly account for ambiguous results (Seigers et al., 2011).

There is, however, much less literature to be found regarding chemotherapeutic effects in pups or adolescent mice, as most animal models focus on adult rodents. The obtained insights with adult mice are extremely valuable but including pup models can provide a better approach for a childhood cancer model.

Rodent Research on Methotrexate

The neurotoxicity of MTX has been widely researched, both in vitro, in vivo and through observation of cancer patients. A great deal of animal studies has already focused on unraveling the mechanisms and consequences of MTX. However, the exact pathogenesis of leukoencephalopathy remains unknown (Gilbert et al., 1989) and given that it is a key aspect of standard chemotherapy, all additional knowledge is valuable. First, most rodent studies seem to genuinely find some effects in animals when MTX is administered. Frequently, pathological changes are found in the hippocampus and corpus callosum, which are associated with cognitive deficits (Seigers et al., 2009; Gibson et al., 2019). Also, MTX disrupts oligodendrocyte lineage dynamics and myelin in mice (Gibson et al., 2019). Myelination is the generation of oligodendrocytes and the formation of the insulating myelin sheath by

oligodendrocytes. This process spreads over more than three decades of life, which can illustrate why the consequences of chemotherapy continue well into later life. On the behavioral level, consequences are predominantly found in the domain of learning and memory (Li et al., 2010), though research including other domains, like social behavior, is rather lacking. The extent to which these effects manifest, however, varies. It is well established from a variety of studies that rats display a range of different cognitive impairments after being treated with MTX, in for instance spatial Morris Water Maze (MWM) learning, the Novel Object Recognition task (NOR), object placement recognition, conditioned emotional response and operant response learning (Seigers et al., 2011).

Gibson et al. (2019) conducted an animal study aiming to identify underlying mechanisms of chemotherapy-related cognitive impairment, using MTX. They intraperitoneally injected juvenile mice with 100 mg/kg MTX once for three successive weeks, starting on P21. Their research concludes that MTX induces chronic inflammatory microglial activation, which in turn causes astrocyte reactivity. These events dysregulate or deplete oligodendrocyte precursor cells, which can provide an explanation for white matter dysfunction and its subsequent neurocognitive deficits (Liddelow et al., 2017). Even though healthy oligodendrocyte precursor cells are known to repopulate, after exposure to MTX they fail to replenish and anomalies are still measurable in adulthood (Gibson et al., 2019). Further, they observed behavioral changes in their animals as well. The MTX exposed group exhibited motor deficits, anxiety and difficulties with attention and short-term memory.

Additionally, observations from several studies, reviewed by Seigers et al. (2011), suggest that the administration of MTX has led to a decrease in explorative behavior in rats in different contexts and it has shown to impair learning and memory abilities (Madhyastha et al., 2002). Observed mice, treated with MTX and 5-FU (another chemotherapy drug),

showing more exploration behavior. Studies have also established that mice treated with MTX and 5-FU displayed more anxiety-like behavior in fear conditioning. Further, research shows us that MTX also causes an impairment in the ability to consolidate a previous learned memory when given directly after contextual fear conditioning or MWM training. Next, mice treated with MTX displayed an impaired learning in a passive avoidance task. There are also studies that show cognitive impairment in rodents, for example, deteriorated performance in a conditioned taste aversion task two weeks after MTX treatment, but for which the effects vanish nine weeks after treatment (Seigers et al, 2011). Results like these suggest that we are required to stay attentive and keep in mind that not all impairments are permanent.

Mullenix et al. (1994) conducted a study using rodent pups. They administered MTX to six-week-old rats and then monitored their behavioral changes. They found that combined-agent therapies (for example, MTX with prednisolone) influence behavior more strongly in comparison to single-agent therapies. Another notable conclusion from this research was that females seemed to display more behavioral effects with lower doses of MTX than males. Another study found that, in neonatal rats, MTX potentially alters the development of synapses in the hippocampus (Igarashi et al., 1989). Bisen-Hersch et al. (2013) describe memory acquisition and retention impairments in mouse pups who received MTX and cytarabine on P14, P15 and P16. Li et al. (2010) compared acute and chronic chemotherapeutic administration, using MTX, in rats. They found that both groups experienced spatial memory deficits in adulthood.

Rodent Research on Maternal Separation

Mental health later in life can suffer greatly due to a disruption of the relationship between a child and caregiver early in life (Levine, 2005). Maternal separation is a model that

acts as an animal equivalent for early life stress and is often used to mimic human abuse and neglect (Nishi, 2020). However, it can also be used in the clinical context of cancer research where children are forced to spend a significant time apart from their parents.

In their research, Peña et al. (2019) aimed to identify manipulations that increase susceptibility for stress in adulthood. Early life stress and adverse life events are known to increase sensitivity for successive stress during adulthood and can thus pose a risk for depression and other mood disorders in humans. The question arises what exact manipulations and life events underlie this sensitivity. Peña et al. (2019) subjected their pups to maternal separation with limited bedding between P10-P20, which effectively caused a susceptibility to later-life stress, in contrast to maternal separation between P2-P12, suggesting a stress-insensitive period during the first 10 postnatal days (Rincón-Cortéz & Sullivan, 2014).

Research has shown that maternal separation has the possibility to modulate the hypothalamic-pituitary-adrenal (HPA) axis (Nishi, 2020). Being separated from a parent for a prolonged time can be defined as a negative event and can be a risk factor for mood disorders (Oral et al., 2016; Peña et al., 2019). They can also lead to chronic or sustained activation of the response system. Stress releases corticosterone, the rodent equivalent for the human stress hormone cortisol, which is adaptive in the short-term. However, excess corticosterone in the circulation leads to a chronic activation of the HPA axis (Dube et al., 2001), ultimately causing a lack of corticosterone (Shonkoff et al., 2012). This corticosterone deficiency eventually leads to an overactive, unnecessary inflammatory response (Dube et al., 2001).

Many studies conclude that separation of the pups from their dam increases the likelihood of developing depressive-like behaviors and anxiety-related behaviors later in life, given the fact that the maternal separation causes despair and protest in the pups. However, there is evidence as well that maternal separation early in life leads to impairments in fear

retention in adult rodents. Also, aggressive behaviors could be a consequence of maternal separation. A juvenile rat study found more vigorous fur pulling and less supine postures toward a play partner in the maternal separation group. The aggressive elements of their behavior seemed to continue well into adulthood. Also, early maternal separation can possibly cause reduced reward-seeking behavior in female mice (Nishi, 2020).

Hypothesis and Objectives

For this thesis, the aim is to explore the relationship between maternal separation and MTX, and their neurocognitive consequences. Based on the research as described above, our hypothesis includes the expectation of adverse neurocognitive effects after the administration of MTX. Additionally, we expect that maternal separation, or the quality of attachments between parents and their children, may play a crucial role in the impact of childhood cancer treatment. According to the existing body of literature, animals seem to be more prone to stress and less resilient after maternal separation, which raises the question whether they will be notably less capable of dealing with impactful events such as chemotherapy. We hypothesize that maternal separation makes the pups more susceptible to the adverse effects of MTX.

Materials and Methods

Animals

Twelve-week-old male and female C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France) and group-housed under standard laboratory conditions (07h00-19h00 light/dark cycle, ±24°C and ±40% humidity) with food and water accessible *ad libitum*. Males received standard chow, whilst females were given specialized breeding food (Ssniff,

Germany). Because of high pup mortality in litters from primiparous C57BL/6 dams (Brown et al., 1999), only females who already nurtured one successful nest were used. To obtain their second litter, females were coupled for five days (1 male, 2 females), after which they were again group-housed per gender. When showing pregnancy, the females got single-housed 17 days after coupling and kept undisturbed until P10. Eight litters, that varied from four to nine pups, were used in this experiment. At P10, all pups were tattooed on the paws (identifier) and the nests were divided into two conditions: maternal separation (MS) and control (STD). During the following nine days (P10-P18), the MS pups were separated from the dam for 3-4 hours/day, as described by Peña et al. (2019). The start time varied each day to create unpredictability. To control for any effects of handling, STD pups were all picked up individually around the same times as the maternal separation condition. At P21, the litters were weaned by removing the mother. All animal experiments were performed in accordance with the European Communities Council Directive (2010/63/EU) and approved by the KU Leuven animal ethics committee (P086/2020).

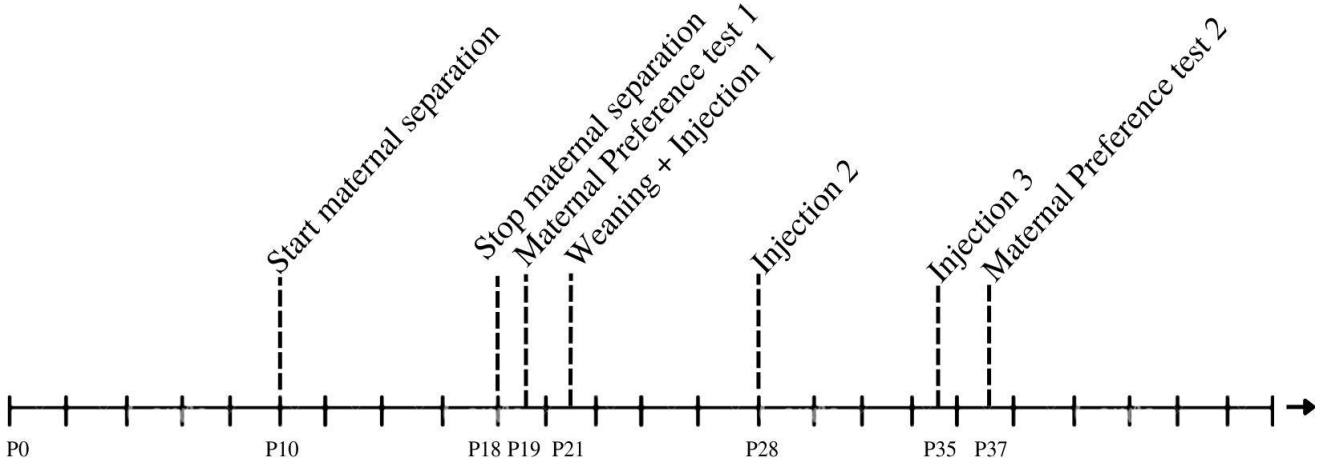
Methotrexate Injections

Prior to the injections, there were no significant differences in weight and pups were assigned randomly to treatment conditions. Half of each litter received a methotrexate (MTX, 100mg/kg) treatment, the other half a phosphate buffered saline (PBS) treatment. Each mouse received a total of three intraperitoneal injections, one each on P21, P28 and P35. When the weight of the pup was between 7g and 8g, they received a standard lower dose of 50 µl. When the weight was less than 7g, the injection was postponed a day. Their health was monitored closely and when significant weight loss occurred, pups were given peanut butter or booster gel to strengthen. The injections were given quickly but carefully, to provide minimal

discomfort to the animals. After the injections, animals were group-housed by sex, condition, and treatment. In total, we had 12 pups in the PBS+STD group, 13 in the MTX+STD group, 15 in the PBS+MS group and 16 in the MTX+MS group. Unfortunately, 13 MTX pups died due to the exposure to MTX or other complications. This left us with 12 animals in the PBS+STD group, 6 in the MTX+STD group, 15 in the PBS+MS group and 11 in the MTX+MS group. These animals completed the whole experiment. For a timeline of the pup events, see Figure 2.

Figure 2

Timeline with Events during the Pup Phase



Note. The postnatal days of the mouse pups are represented on the bottom line using 'P'. On P10 the maternal separation (MS) started where the pups of half the litters were daily removed from the mother for 3-4 hours. The pups of the other litters in the control condition (STD) were handled an equal number of times. On P18 the last MS took place. Intraperitoneal injections with methotrexate (MTX) or phosphate buffered saline (PBS) were administered on

P21, P28 and P35. On P21 the litters were also weaned. The maternal preference tests took place two days before and two days after the injections, on P19 and P37.

Behavioral assessment

When assessing the adverse effects of chemotherapy in the clinical population, there is the advantage of verbal communication. It is, most of the time, possible to let patients report in what domains they experience deterioration. Lots of neuropsychological testing involves a verbal component as well. This aspect does not exist in animal models. In animal research, we must rely on the observable. We assume that the adverse effects of chemotherapy inside the animal will become visible through behavior. This can be a demanding task. Luckily, there has been a considerable amount of research done to develop experimental paradigms which allow us to measure certain components of rodent behavior to give us an indication of their competences and well-being.

Pup Tests

Star Maze Preference Test. On P19 and P37 the Star Maze Preference Test was performed, an in-house protocol based on the Sociability/Preference for Social Novelty (SPSN) (Nadler et al., 2004). It was used to assess maternal bonding right after both our experimental manipulations, being MS and chemotherapy. The Star Maze was made of plexiglass and consisted of six arms, each 20 cm long, in the form of a star-shaped maze. The walls were transparent, and each arm had sniffing holes at the end. Four arms (numbers 1, 2, 4 and 5) had a clear plastic cage placed outside at the end to accommodate an adult mouse. The two other arms (numbers 3 and 6) were empty. The set-up was placed in a closet which could be closed so the animals could not see the researcher. The closet was lit with LED lights. The goal was to let the pup explore the maze and track where they chose to spend their time: in the

arm with their own mother, with another mother, with an unknown male, with an unknown virgin female or in the two empty arms. As mentioned before, each pup went through the test two times. The first time, at P19, the litters were not weaned yet, so the pups were still in the cage with the dams. The second time, at P37, the litters were weaned so the dams were put together in two cages, separated by condition. Before the test, the animals needed to habituate 30 minutes in the experiment room. Separate cages with bedding were used for transport.

The pup movements were tracked using the ANY-Maze computer program (Stoelting, 2019). First, the pup was gently placed in the middle of the maze. After 3 minutes, the researcher opened the closet doors, placed a clear plastic cylinder over the pup in the middle of the maze, to prevent it from wandering around, and placed the adults in their respective cages at the end of the arms. The location of the dam and the other mice were randomized to control for any preference or aversion for a specific arm. After that, the plastic cylinder was lifted, the doors closed, and the 10-minute test phase started. After the trial, the mice were placed back in their home cages and the set-up was cleaned with 70% ethanol to counter transfer of scent. The same adult mouse was used for a maximum of four trials in a row, to prevent excessive stress. This means that a maximum of four pups from the same litter could be tested in a row, after that the dam needed to have 30 minutes of rest. The parameters of interest were (1) mean speed, (2) center (2a) entries, (2b) time, (3) arm 1 (3a) entries, (3b) head time, (4) arm 2 (4a) entries, (4b) head time, (5) arm 4 (5a) entries, (5b) head time, (6) arm 5 (6a) entries, (6b) head time, (7) arms 3 and 6 (7a) entries, (7b) head time.

Adult Tests

When the animals reached adult age (10 weeks ±1 week), they went through an extensive behavioral test battery. These tests were planned in a specific order, starting with

the least stressful test, and ending with tests that are considered more stressful or could in certain ways influence the normal behavior of the animals. Handling occurred according to standardized protocols to prevent experimenter variability.

Open Field Test. The Open Field test is one of the most frequently used tests for behavioral observation and more specifically for evaluating anxiety-like behavior in rodents (Leroy et al., 2009; Nishi, 2020). It can be used to evaluate cognitive changes (Bazyar et al., 2017). The set-up for this test consisted of a plexiglass arena (50x50 cm) with transparent walls to evaluate locomotor activity, exploratory, and anxiety-like behavior. It was placed in a closet and illuminated with LED lights beneath the arena. The animals were placed inside a closed cupboard for 30 minutes for dark adaptation prior to the experiment. A transport cage with bedding was used.

Each mouse was transferred to the arena and placed in the top left corner. The researcher then closed the closet doors and started the trial. The software started automatically with a 1-minute acclimation period and then immediately started recording the 10-minute trial. Afterwards, the mouse was put back into the home cage and the arena was cleaned with ethanol for the next trial. The exploration pattern of the animals was assessed using virtual zone visits monitored by the ANY-maze software (Stoelting, 2019). The measured parameters were (1) mean speed, (2) total distance traveled, (3) center (3a) entries, (3b) time, (3c) distance, (3d) latency to first entry, (4) periphery (4a) entries, (4b) time, (4c) distance, (5) corners (5a) entries, (5b) time and (5c) distance. Total distance traveled and the number of corner crossings was used as an indication of explorative behavior. Exploration of the center area was used as an indication for more curious and less anxious behavior, whilst exploration of the sides was an indication for more anxious behavior.

Elevated Plus Maze. Further, the Elevated Plus Maze is one of the most popular tests to evaluate anxiety-like behavior in rodents as well (Nishi, 2020). It is based on mice's natural aversion for open and elevated areas, as well as on their tendency to spontaneously explore novel environments (Komada & Miyakawa, 2008). The number of open arm entries and time spent in the open arms are indications of open space-induced anxiety in mice (Komada & Miyakawa, 2008). The arena consisted of a plus-shaped maze with four arms, each 5 cm wide and 30 cm long. Two of the arms were enclosed by side walls (no roof) and two were open. There was some bedding provided in the whole maze. A lamp illuminated the set-up, and a camera was placed above to record the movements of the animals. Prior to the experiment, the animals habituated in the experiment room for 30 minutes in their home cages with filter tops. The software used for tracking the animals was EthoVision (Noldus, the Netherlands).

For the trial, the mice were placed in the middle of the maze. The software started recording immediately for a 10-minute trial. Afterwards, the mouse was put back into the home cage. Cleaning wasn't necessary since there was bedding on the ground. For this reason, males and females were tested on separate days and the bedding was replaced inbetween. The parameters measured with EthoVision were (1) total distance traveled, (2) velocity, (3) center point (3a) frequency of entries, (3b) cumulative duration, (4) open arm 1 and 2 (4a) frequency of entries, (4b) cumulative duration, (4c) latency to first entry, (5) entry zone 1 and 2 at the front of the closed arms (5a) frequency of entries, (5b) cumulative duration, (5c) latency to first entry, (6) zone 11 (the closed arms) (6a) frequency of entries, (6b) cumulative duration and (6c) latency to first entry.

Flipped Social Exploration. The set-up for this test consisted of a plexiglass arena (50x50 cm) with transparent walls and a small round barred cage in the middle. The arena was placed inside a closet. We used ANY-Maze (Stoelting,2019) to record the movements of the

animals. For this test, we also used stranger mice that were unfamiliar to the test mice. Our test mice and stranger mice habituated for 30 minutes to the experiment room in their home cages with filter tops prior to the test. Usually, the Social Exploration test places the test mouse in the plexiglass arena with a stranger mouse in the center in a small cage. This original set-up is used to examine to what extent the test mouse is interested in new mice and how much social contact they seek. In this case, we conducted the Flipped Social Exploration (Odent et al., 2021), in which we put the test mouse in the little center cage which was covered with a transparent disk to prevent escaping, while remaining visible for the camera. We examined to what extent a stranger mouse was interested in our test mouse. It is assumed that the test mouse influences the social behavior and approach from the exploring stranger mouse (Odent et al., 2021). The collected data therefore refers to the actions of the stranger mice. We wanted to make sure that any differences in data were thus due to the mouse in the center cage. A stranger mouse of the same sex was placed in the bottom left corner of the arena. As soon as the researcher left, the software started recording the 10-minute trial automatically. ANY-Maze recorded the movements of the stranger mice. Afterwards, the animals were put back into their home cages and the set-up was cleaned with ethanol for the next trial. The measured parameters for the stranger mice were (1) distance, (2) mean speed, (3) periphery (3a) entries, (3b) time, (3c) distance, (4) approach (4a) head entries, (4b) head time, (4c) head distance traveled, (5) center sniff (5a) head entries, (5b) head time, (5c) head entry latency, (6) corners (6a) entries and (6b) time.

Sociability/Preference for Social Novelty Test. The Sociability/Preference for Social Novelty (SPSN) test is a paradigm to assess the social preferences of mice, described by Nadler et al. (2004). It is specifically designed to analyze a mouse's preference for a stranger mouse versus an empty cage (sociability), as well as its preference for a newly introduced

stranger mouse versus the already familiar stranger mouse (social novelty). It allows us to determine how sociable the mice are and to what extent they prefer to spend time with a novel stranger mouse, for which social memory is essential.

The set-up for this test consisted of a narrow and rectangular plexiglass box with high walls and three compartments. The compartments were separated with transparent plexiglass walls with sniffing holes. The test mouse could freely explore the middle compartment (36.5 x 10 cm), while the other smaller compartments (10 x 10 cm) detained the stranger mice. At least two different cages with stranger mice were used, so that the two mice in opposite compartments did not smell the same. The set-up was placed in a closet with LED-lights underneath and a camera above. We used ANY-Maze software (Stoelting, 2019) to record the movements of our test mice. The test mice and stranger mice habituated for 30 minutes to the experimenter room in their home cages with filter tops prior to the test.

First, our test animal was gently placed in the middle compartment of the box. The closet doors were closed, and the researcher started a 5-minute acclimation trial on ANY-Maze. Next, the first stranger mouse was introduced. Whether the first stranger mouse appeared in the left or right compartment was alternated between trials. After the introduction of the first mouse, a 10-minute social preference trial started. At the completion of this trial period, a second stranger mouse from another cage was introduced in the remaining compartment. This started a 10-minute social novelty trial. Afterwards, all mice were put back in their home cages, the set-up was taken out of the closet to be cleaned with ethanol and everything was put back for the next trial. The measured parameters were (1) total distance traveled, (2) mean speed, (3) time spent with stranger 1, (4) time spent on the empty side/with stranger 2, (5) stranger 1 periphery (5a) head entries, (5b) head time, (5c) latency to first head

entry, (6) empty/stranger 2 (6a) head entries, (6b) head time and (6c) latency to first head entry.

Automated Tube Test. The Automated Tube Test, sometimes called the Tube Dominance Test or the Social Dominance Tube Test, is a behavioral test for mice to measure social rank (Xie et al., 2022). It measures territorial dominance without the presence of actual aggression (Pallé et al., 2019). The aim of the test is for one mouse to push the other one out of the tube and thus acquire dominance (Xie et al., 2022). The Automated Tube test consisted of a training phase and a tournament phase and took 8 days to complete. The set-up consisted of a narrow transparent tube (diameter 2.5 cm) made of plexiglass, with a small box at each end. The boxes had a small transparent sliding door, which blocked the entry to the tube. In the middle of the tube, a non-transparent black sliding door blocked the way. An automated version of the tube was used (Benedictus B.V., Rotterdam, The Netherlands), which ensured higher accuracy and standardization. The goal behavior for the mice was to walk from one box to the box at the other end through the narrow tube. The size of the tube ensured easy passage for one adult mouse whilst preventing turning around or passing another mouse. Each box was connected to a compressed air tank to administer air puffs to, if necessary, motivate the mice to enter the tube. The set-up was connected with our computer, which made sure the time measurements were very accurate. Via the computer, the doors and air puffs were manageable as well. Before the trials, the animals habituated 30 minutes to the experimenter room in their home cages with filter tops. Males and females were trained separately to avoid the transfer of scent.

First, the five-day training phase started. To begin a training trial, the mouse was carefully placed in the left box with the lid closed. Then the trial started, and the transparent door slid open to give the animal access to the tube. The mouse eventually entered the tube

and when they reached the middle, the non-transparent black door slid open as well. When it entered the end box, the transparent door from this box closed so the mouse could not re-enter the tube and the animal was returned to its home cage. The set-up was cleaned with ethanol for the next trial. When all animals completed their first trial, the test was repeated in the other direction. On the first day of training, there was no strict time limit, the mice were allowed to explore and get used to the set-up. However, if the trial took more than 3 minutes, the mice were gently encouraged to enter the tube or the end box. The following days, the training intensified. Every mouse completed four trials instead of two with a time limit of 30 seconds, alternating between left and right to start. The order of testing was reversed on a regular basis to avoid associations. To encourage a fluent and quick trial, air puffs were introduced. When the mouse stayed longer than 5 seconds in the start box, they received an air puff which stopped once they entered the tube. After five days of training, there was a resting phase of two days.

Next, the tournament phase began, which took 3 days. Every tournament day started with two training trials, starting left and right, exactly like the training phase. Afterwards, every mouse was matched against all the other mice from the same gender, except cage mates. Every mouse would participate an average of six matches a day and it was made sure that one mouse did not start in the same box every time. Before the trial, both competing mice were put in their respective start boxes. The trial started when the transparent doors slid open. Both mice needed to enter the narrow tube and reach the middle before the non-transparent black door slid open as well. Up until that point, the mice could not see each other. The mouse that was able to cross the tube to the other side was named the winner, the one that moved backwards was named the loser. When both mice were in one box, the transparent door closed again. Afterwards, they were placed back in their home cages and the set-up was cleaned with

ethanol for the next trial. The most important parameter for this test was the number of wins a mouse achieved.

Forced Swim Test. The Forced Swim Test is a behavior test that was originally designed for rats but later modified for mice (Can et al., 2012). This paradigm is used for experimental manipulations that are aimed at rendering or preventing depressive-like states (Can et al., 2012). Rodents are placed in a transparent water tank and their escape related mobility behavior is measured. The aim is to have the animal lose hope that they will escape the stressful situation, which is called 'behavioral despair' (Can et al., 2012). Immobility time is seen as an indication of a depressive-like state.

The set-up for this test consisted of a transparent tank filled with 26°C±1°C water. Rodents are natural swimmers and can stay above the water without swimming in an immobile state. By measuring how often and how long they display this action, we can assess their escape related mobility behavior. The movements of the animals were tracked, and the trials were recorded using ANY-maze (Stoelting, 2019) with one camera on the side and one above the water tank. Despite the two cameras, the quick movements of the mice were not always as clearly visible on image as we would prefer. For this reason, the amount of floating was manually registered while looking directly at the mouse in the water tank and not at the computer screen. Prior to the experiment, the animals habituated for 30 minutes to the experiment room in their home cages with filter tops. Next, the animals were picked up by the tail and gently lowered in the water tank without immersing their head, because mice cannot hold their breath. The trial took 6 minutes per mouse. Afterwards, they were carefully taken out of the water and put back in their home cage with extra paper and bedding to dry properly. In between trials, the temperature of the water in the tank was measured to make sure it didn't become too cold. During the testing day, the water was changed at least twice, so that every

mouse could do the trial in reasonably clean water. The parameter of interest during this test was primarily the time the mice spent in an immobile state.

Morris Water Maze. The Morris water maze (MWM) is one of the most researched and frequently used experiments in behavioral neuroscience to investigate spatial learning and memory in laboratory animals (D'Hooge & De Deyn, 2001). Over the years, the testing paradigm earned a core position in contemporary neuroscience (D'Hooge & De Deyn, 2001). The set-up consists of a circular pool and the goal for the animal is to efficiently learn to find a hidden escape platform in the water.

The Morris Water Maze was the longest test in our test battery and took multiple weeks to complete. The set-up consisted of a round pool (approximately 1.5m in diameter) filled with opacified water. The water should always be around 26°C. There was an escape platform present just below the surface (around 5mm) of the water, so that the animals could not see it. The goal of this experiment was for the mice to learn the position of the escape platform based on environmental cues. Around the pool, four starting points were marked with numbers. The software used to track the movements of the mice was Ethovision (Noldus, the Netherlands).

Since this was a learning experiment, the environment was of utmost importance. The experiment room, the procedure and the behavior of the researcher was always the same, so that mice could learn from spatial and behavioral cues. Prior to the experiment, the mice habituated for 30 minutes in the experimenter room. Filter tops were not used since a heat lamp was present to help the animals dry and avoid hypothermia. A swatter was used to comfortably transport the animals and to easily take them out of the pool.

Spatial learning was tested in the acquisition phase for 10 days. Every mouse swam four times a day, once from every starting point (daily randomized). To start the trial, the

mouse was taken out of its cage, put on the swatter while holding on to the tail, and carefully placed in the pool without immersing the head at the designated starting point. Then, the researcher always quietly took the same route back to a chair next to the computer while the mouse searched for the platform for a maximum of 100 seconds. When the animal found the platform or the time was up, the researcher got up and walked to the take-out position, right next to the escape platform. The swatter was gently used to guide the mouse to the platform when needed. The mouse was transported back to their home cage under a heat lamp and a new mouse was taken for the next trial. In between every trial, animals spent approximately 30 minutes in their home cage. The measured parameters were (1) total distance traveled, (2) velocity, (3) latency to find target, (4) in center zone (4a) frequency, (4b) cumulative duration, (4c) latency to first entry, (5) in periphery zone, (5a) frequency, (5b) cumulative duration, (5c) latency to first entry, and (6) time spent floating.

On day 6 and day 11, a probe trial was done to assess spatial memory. In these trials, the escape platform was removed from the pool, the mice were put in the water directly across from where the platform had been, and their exploration was tracked for 100 seconds. During these probe trials, the measured parameters were (1) velocity, (2) target, (the location where the platform used to be), (2a) frequency, how many times they swam over the target location, (2b) latency, how long it took to swim through the location of the platform, (3) the cumulative duration in the target quadrant, (4) the cumulative duration in adjacent quadrant number 1, (5) the cumulative duration in adjacent quadrant number 2, and (6) the cumulative duration in the opposite quadrant.

Statistical Analysis

The statistical analyses were executed using GraphPad Prism version 9.3.1. The significance of differences between the means of two groups were tested with an unpaired t-

test. Differences between more groups were tested with a one-way and two-way (repeated) measures analysis of variance (ANOVA). Figures were also created using GraphPad Prism.

The significance level (alpha) was set to 0.05, meaning that every test resulting in a p-value below 0.05 was considered significant. This experiment was a randomized block design since we randomly assigned treatments, MTX or PBS, within each nest of animals. Since every animal was assigned to only one treatment and one condition sphericity was assumed, meaning that we presume that the standard deviations in every group are equal.

Results

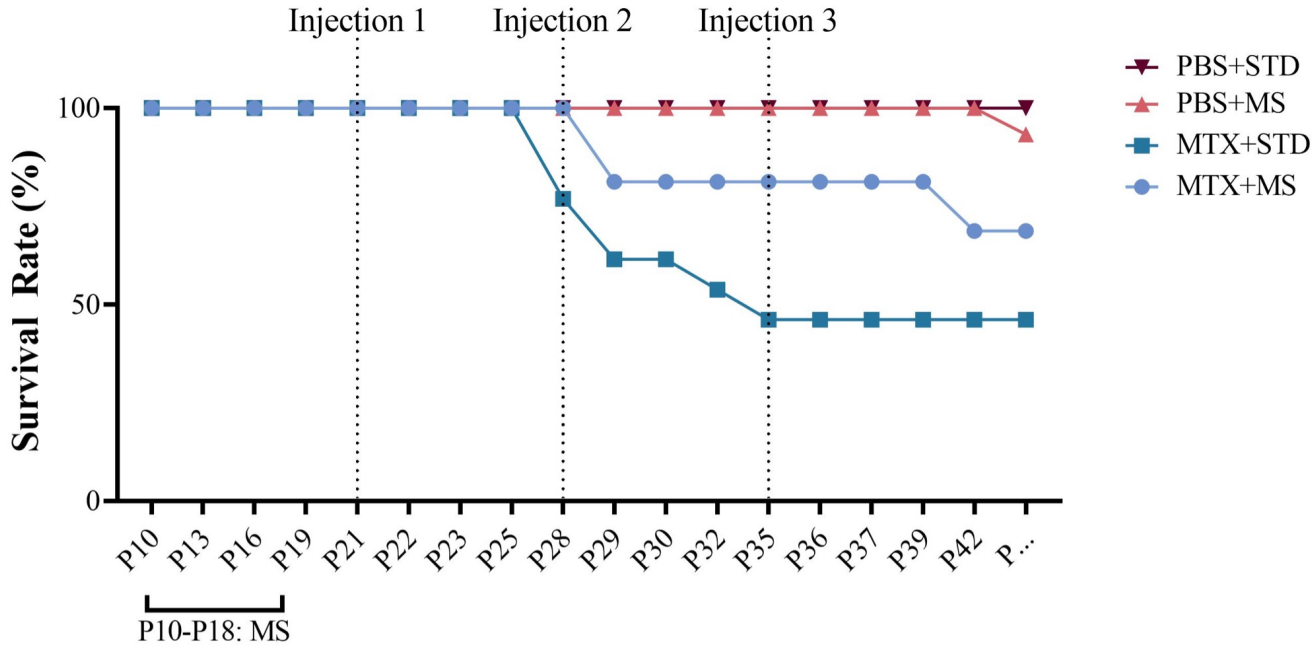
Pup Data

Survival Rate

During this experiment, there has been some animal flow. All the mouse pups were born in February. We ended up having four different groups based on treatment and condition: methotrexate + maternal separation (MTX+MS), methotrexate + standard rearing (MTX+STD), phosphate buffered saline + maternal separation (PBS+MS) and phosphate buffered saline + standard rearing (PBS+ TD). We started with 58 animals and assigned an equal number of animals to each group. Fifty-six animals completed the first Star Maze Preference Test on P19. After some losses, most likely due MTX toxicity and one PBS mortality who died due of unknown causes, our MTX+MS group ended up with 11 animals, MTX+STD ended up with 6 animals, PBS+MS with 14 animals and PBS+STD with 12 animals who completed the whole experiment (total n = 43, see Figure 3). Among them, there were 22 females and 21 males.

Figure 3

Survival Rate



Note. This figure represents the survival rate of our test animals over the course of their first weeks. Weight was closely monitored up until P42. For PBS+STD there was a survival rate of 100%, for PBS+MS it was 93.33%, for MTX+STD 46.15% and for MTX+MS 68.75%. Most deaths took place in the days after an MTX injection (P21, P28 & P35). The precise reasons why MTX seemed to be more toxic in some animals than in others, remains unknown. 'P' stands for postnatal day.

Pup Weight

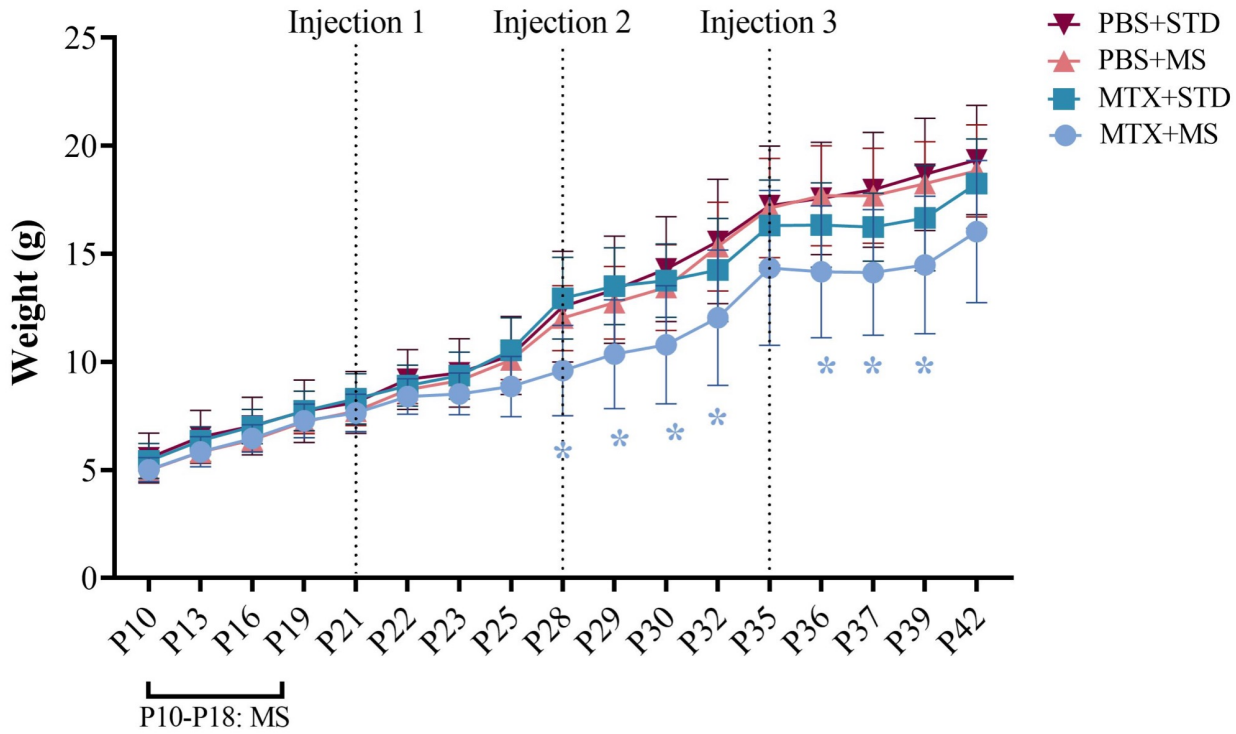
During the initial phase of the experiment, the weight of the pups was carefully monitored. There were 17 measuring moments which took place between P10 and P42, see Figure 4. A two-way ANOVA analysis revealed a main effect of time, $F(1.189,70.83) = 630.8$, $p < .0001$, meaning that the pups overall gained weight as time progressed, as

expected. There was also a main effect of group, $F(3,39) = 3.771$, $p = .0181$, meaning that the weight of the pups was significantly influenced by the experimental group they were in. The difference became significant from P28 onward. Overall, the MTX groups showed a lower weight progression, but post-hoc testing only revealed a significant effect in the MTX+MS group compared to the other groups. An interaction effect between day and group was found as well, $F(48,623) = 5.459$, $p < .0001$, meaning that on top of time, group had an additional effect on weight.

For the all-female sample, a main effect of time was found as well $F(2.539,45.54) = 598.4$, $p < .0001$. There was also an interaction effect, $F(48,287) = 2$, $p = .0003$. Even though there was no main effect for group for the female mice, group did have an additional effect on weight on top of time. For the all-male sample, a main effect of day was found $F(1.778, 30.22) = 313.6$, $p < .0001$. A main effect of group was found as well, $F(3,17) = 4.815$, $p = .0132$. There was also an interaction effect, $F(48,272) = 5.493$, $p < .0001$. Finally, a significant effect of subject was found, $F(17,272) = 37$, $p < .0001$.

Figure 4

Pup Weight Compared between Treatments



Note. This figure represents the weight of the pups compared between treatments, measured 17 times between postnatal day 10 (P10) and postnatal day 42 (P42). From P10 to P18, half of the mice were subjected to maternal separation (MS). The experimental group received injections with methotrexate (MTX), the control group received injections with phosphate buffered saline (PBS). They all received three injections: on P21, P28 and P35. Starting from P28, some significant differences in weight were found, all situated in comparisons between MTX+MS and PBS+STD. The weight of MTX+MS was significantly lower on P28, $q(20.75) = 4.314$, $p = .0289$, on P29, $q(20.76) = 4.051$, $p = .0428$, on P30, $q(20.1) = 4.574$, $p = .0199$, on P32, $q(20,36) = 3.971$, $p = .0487$, on P36, $q(19.76) = 4.046$, $p = .0443$, on P37, $q(20.35) = 4.649$, $p = .0175$, and on P39, $q(19.39) = 4.861$, $p = .0132$. Data is presented as mean with SD. (* = $p < .05$)

Star Maze Preference Test

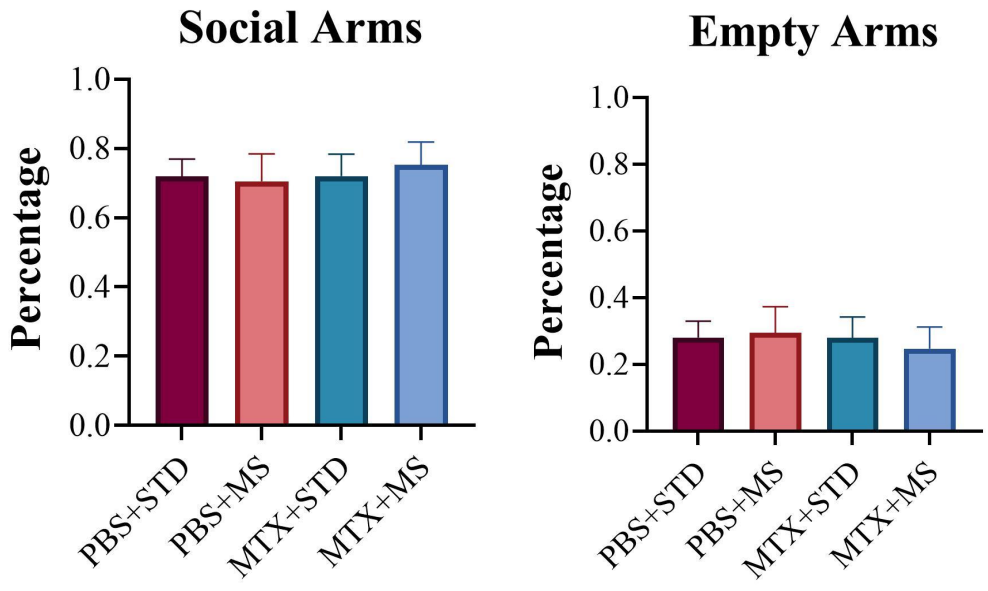
On P19 and P37 the pups completed the star maze preference test. On P19 the maternal separation period was finished but pups had not received any injections yet. On P37, they had received all three. For the analysis of the Star Maze Preference Test on P19, Student's T-tests were used to disclose any significant differences in behavior between the MS group and the STD group. None of the variables of interest showed a significant difference. All analyses were computed for the all-female and all-male sample as well, again without significant results. The pups all seemed to behave similarly on P19 and spent about the same time in each of the arms.

For the analysis of the Star Maze Preference Test on P37, one-way ANOVA analyses were used to disclose any significant differences in behavior between the MTX+MS, MTX+STD, PBS+MS and PBS+STD groups. For 'total distance traveled' there was a significant difference between the MTX+MS group and the PBS+MS group, $F(3,42) = 3.219$, $p = .0322$, meaning that the MTX+MS group covered significantly less distance in comparison to the PBS+MS group. This effect was not found in the all-female or all-male sample. A similar significant effect was found for 'average speed', $F(3,42) = 3.450$, $p = .0249$, meaning that the MTX+MS group was significantly less fast in comparison to the PBS+MS group. Again, this effect was not found in the all-female or all-male sample. Furthermore, the percentage of time spent in the social arms and the empty arms was calculated. There seemed to be no significant difference between the groups in how much social contact or how much solitude they sought, see Figure 5. There was no difference as well for the time spent in each specific arm and for the number of sniffs at the mother, the novel mother of the female. However, there was a significant effect for the time spent sniffing

the strange adult male, $F(3,42) = 3.331$, $p = .0284$. This effect was found as well in the all-female sample, $F(3,18) = 3.334$, $p = .0428$, but not in the all-male sample.

Figure 5

Percentage of Time Spent in the Social and Empty Arms during the Maternal Preference Test



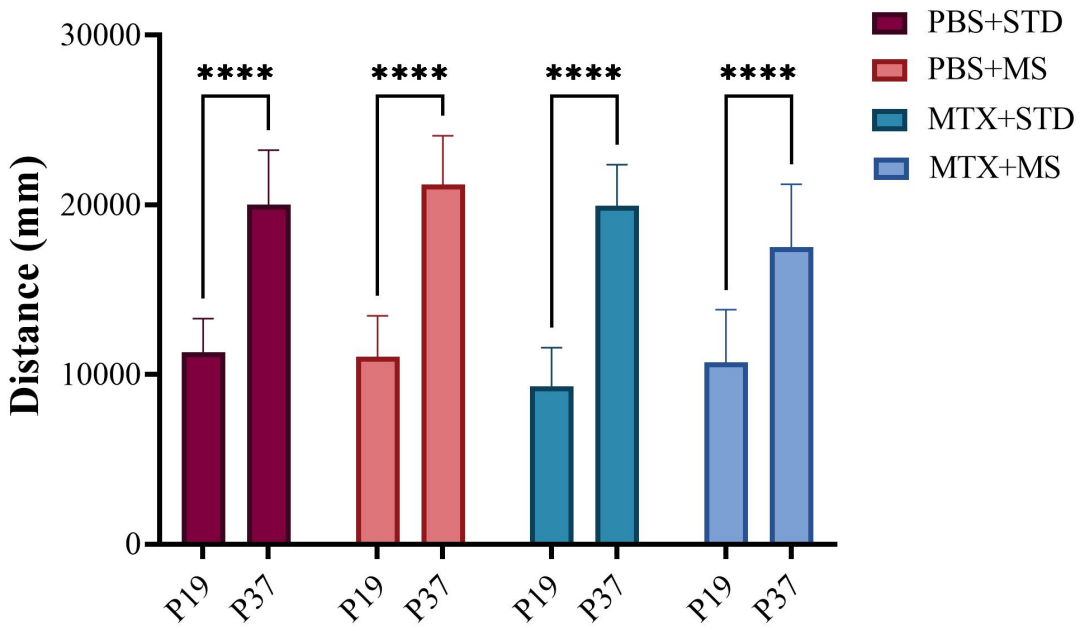
Note. This figure represents the time (in %) the pups spent in the social arms (arms 1, 2, 4 and 5) and in the empty arms (arms 3 and 6) during the Star Maze Preference Test.
Data is presented as mean with SD.

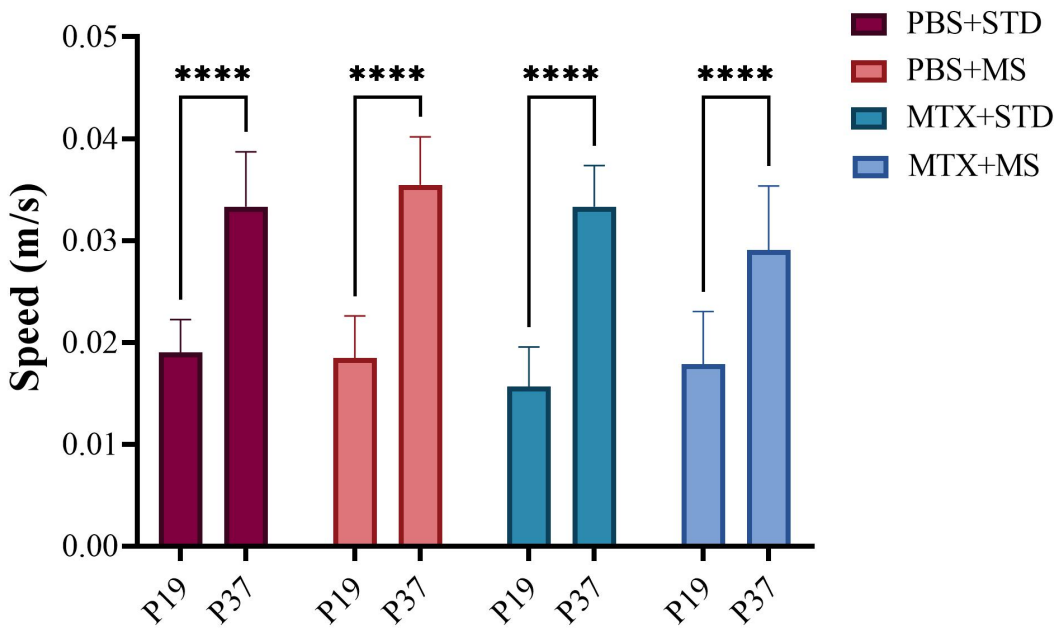
For the comparison of both tests, on P19 and P37, a repeated measures two-way ANOVA analysis was used. The data set of P19 contained more animals than the one on P37 due to some deaths. Those values were excluded for comparison to do a repeated measures ANOVA. This analysis was used to determine any differences between the groups and to check for main effects of treatment and condition. First, as expected, there were significant effects of time found, see Figure 6. For 'total distance traveled' there was an effect of time,

$F(1,42) = 260.7$, $p < .0001$. There was no significant effect of group or interaction effect. For 'speed', there was also an effect of time, $F(1,42) = 249.7$, $p < .0001$. No significant effect of group or interaction was found. The same effects were found in the all-female and the all-male sample.

Figure 6

Mean Distance and Speed of the Pups during the Maternal Preference Test





Note. These graphs display the mean distance traveled and mean speed of the pups in the Star Maze Preference Test. In every group, there is a significant effect of time, meaning that every group increased in traveled distance and in mean speed. This could indicate adequate development. There was no additional effect of group found, meaning that distance and speed between groups was similar. Data is presented as mean with SD. (**** = $p < .0001$)

For the number of times the pup went sniffing its own mother, there was a significant effect of time found in the PBS+STD group, $F(1,42) = 11.91$, $p = .0013$. All pups went to sniff their mother less often on P37 in comparison to P19, but only for the PBS+STD group, this difference was significant. There was no additional effect of group or an interaction. The same effect was found in the all-female sample, but not in the all-male sample. For the number of times where the pups went to sniff a newly introduced mother, there was a similar effect, $F(1,42) = 14.90$, $p = .0004$. This effect was only significant in PBS+STD. There was no additional effect of group or an interaction. This effect was neither found in the all-female nor the all-male sample. For the number of times where the pups went to sniff a newly

introduced female, there was a significant effect of time as well, $F(1,42) = 14.99$, $p = .0004$. This effect was significant in the MTX+MS group and the PBS+MS group. There was no additional effect of group or an interaction. In the all-female sample, no similar effects were found. In the all-male sample, only a similar effect was found in the PBS+MS group.

Adult Data

Open Field Test

The Open Field test measures anxiety-related and exploratory behavior. For the analysis, one-way ANOVA analyses were used to compare the differences between the groups in exploratory behavior and activity, as well as anxiety-related behavior. Time spent in the center, the periphery and the corners is measured. The more time they spent in the corners, the more this could indicate anxious traits. Animals who spent quite some time in the center and periphery were considered more active. The statistical analysis revealed no significant results. All four groups of mice seemed to behave similarly. There seemed to be no significant effects of treatment or condition. Analyses performed by sex revealed no significant differences either.

Elevated Plus Maze

The Elevated Plus Maze was used to measure anxiety-related behavior. For the analysis, one-way ANOVA analyses were used to compare the differences between the groups in anxiety-related behavior. Mice who are more curious are expected to spend more time in the open arms in comparison to anxious mice, who are expected to hide more in the closed arms. Frequency entries of the open and closed arms were measured, cumulative time spent in each of those arms was measured as well as latency to the first entry of each arm. The

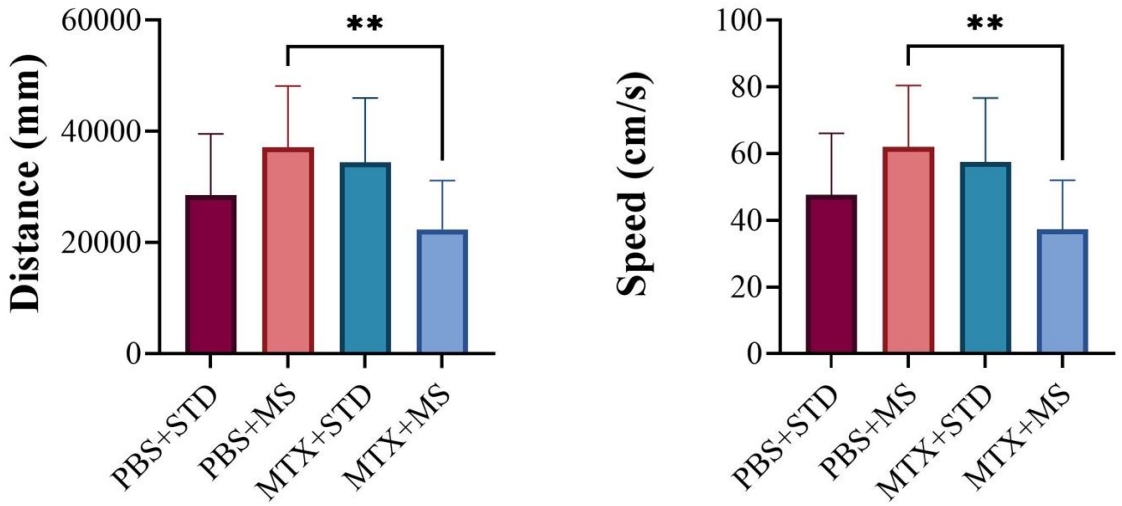
statistical analysis revealed no significant results. All four groups of mice seemed to behave similarly. There seemed to be no significant effect of treatment or condition. Analyses performed by sex revealed no significant differences either.

Flipped Social Exploration

The Flipped Social Exploration was used to assess social behavior and the interest novel mice display in the test mice. First, a significant effect of total distance traveled was found when comparing the averages in each group using one-way ANOVA analyses, $F(3,40) = 4.385$, $p = .009$, see Figure 7. Post-hoc testing revealed that the stranger mice covered significantly less distance in the MTX+MS group in comparison to the PBS+MS group. This significant difference was not found in the all-female sample, but it was found in the all-male sample, $F(3,18) = 6.953$, $p = .0026$. A significant effect of speed was found as well, $F(3,40) = 4.431$, $p = .0088$, meaning that the stranger mice had a significantly lower average speed in the MTX+MS group in comparison with the PBS+MS group. This effect was not found in the all-female sample, but again in the all-male sample, $F(3,18) = 6.948$, $p = .0026$.

Figure 7

Mean Distance and Mean Speed in Flipped Social Exploration



Note. Mean distance (mm) and mean speed (cm/s) as measured during the flipped social exploration. For both variables, there was a significant difference found between the PBS+MS group and the MTX+MS group, the PBS+MS group being significantly faster.
Data is presented as mean with SD. (** = p < .01)

For the variable 'approach: head entries', meaning how many times the stranger mouse approached the test mouse, there was no significant difference between the groups. For the all-male sample, there was a significant effect, $F(3,18) = 4.608$, $p = .0146$, meaning that for the male mice in the MTX+MS group, the stranger mice approached them significantly less in comparison to the PBS+MS and the PBS+STD groups. For the all-female sample, this effect was not found.

For 'periphery: entries' a significant effect was found as well, $F(3,40) = 4.484$, $p = .0083$, meaning that for the MTX+MS group, the stranger mice entered the periphery around

their cage a significantly lower number of times in comparison to the PBS+MS group. For the all-male sample, this significant effect was found as well, $F(3,18) = 7.972$, $p = .0014$. For the all-female sample, this effect was not found.

Finally, for the variable 'corner: entries', a significant effect was found, $F(3,40) = 4.257$, $p = .0106$, meaning that for the MTX+MS group, the stranger mice entered the corners of the arena significantly less in comparison to the PBS+MS group. This effect was also found in the all-male sample, $F(3,18) = 7.251$, $p = .0022$, but not in the all-female sample.

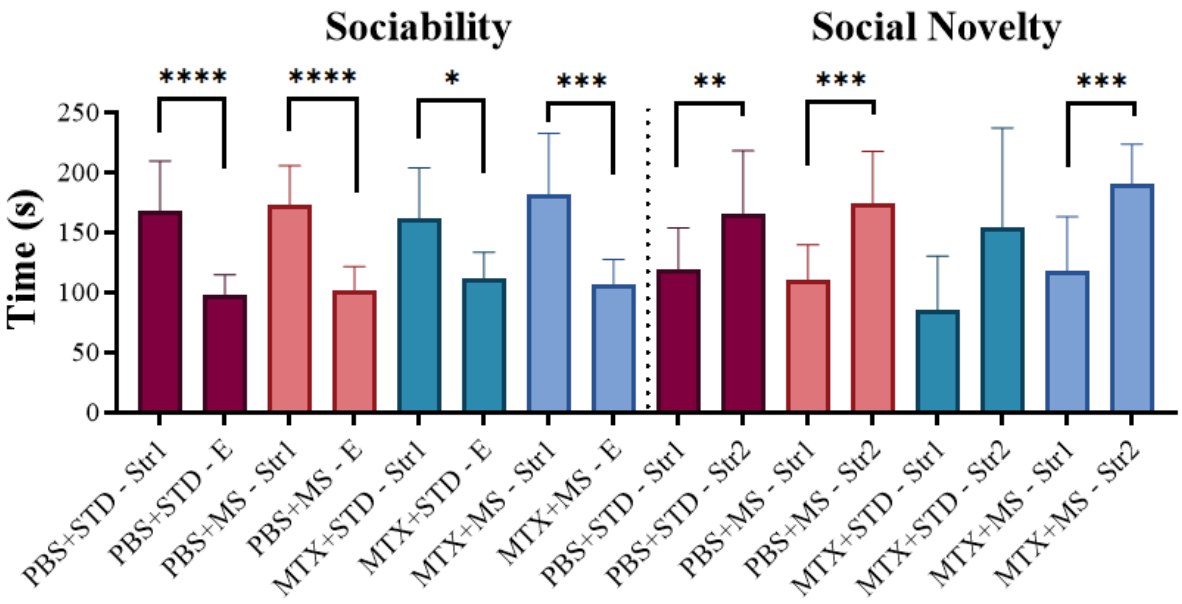
Sociability/Preference for Social Novelty Test

The SPSN test measures the degree of social preference and social memory. The social preference stage measures to which extent the test mouse prefers the social company of a stranger mouse (stranger mouse 1/Str1) over being alone. The social novelty stage measures to which extent the test mouse prefers reaching out to a new stranger mouse (stranger mouse 2/Str2) instead of already Str1, for which (social) memory is essential. For both stages, social preference and social novelty, one-way ANOVA analyses were used to reveal differences between the four groups on any of the exploratory variables. No significant results were found for total distance traveled or average speed, all mice behaved similarly in terms of sociability and social novelty. Analyses performed by sex revealed no significant differences either.

Differences in 'head time', measured in seconds, spent at each outer side of the set-up were analyzed using paired t-tests, see Figure 8. All animals preferred to spend time with Str1 instead of alone. When a novel stranger was introduced, all animals preferred to spend more time with Str2, except the MTX+STD group.

Figure 8

Head Time Spent on Each Side during the Sociability Phase and the Social Novelty Phase



Note. This figure represents the head time the animals spent on each outer side of the SPSN arena, both in the sociability or social preference condition and in the social novelty condition. 'Sociability' represents to which extent the test mice preferred to spend time with a stranger mouse (Str1) versus time at the empty side of the arena (E). Across all conditions, a significant difference was found; all mice preferred to spend more time with Str1. 'Social novelty' refers to the extent to which the test mice preferred to spend time with a newly introduced stranger mouse (Str2) versus time with an already familiar stranger mouse (Str1). Here, all mice preferred to spend more time with Str2, except MTX+STD. Data is presented as mean with SD. (* = $p < .05$, ** = $p < .01$, *** = $p < .001$, **** = $p < .0001$)

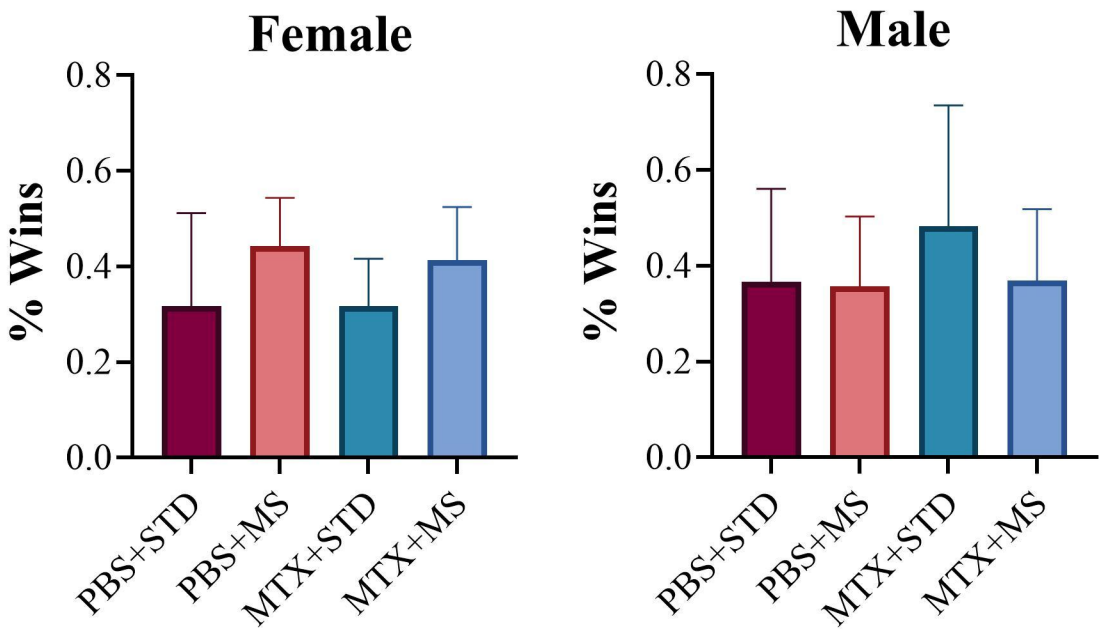
Automated Tube Test

The Automated Tube Test was used to assess dominance in mice. Every mouse competed in a tournament against all other animals from different groups from the same sex. A one-way ANOVA analysis was used to test if there was a difference between the

percentage of wins in each group. The more wins, the more the mouse would be considered dominant. The statistical analyses revealed no significant differences between the groups, see Figure 9. Analyses performed by sex revealed no significant differences either.

Figure 9

Percentage of Wins in the Automated Tube Test



Note. This figure represents the percentage of wins the mice gained during the tournaments of the Automated Tube Test. The tournaments were divided into two groups according to sex, where every mouse did a match against every other mouse from a different group. The females are represented on the left, the males on the right. One-way ANOVA tests revealed no significant differences between the groups. Data is presented as mean with SD.

Forced Swim Test

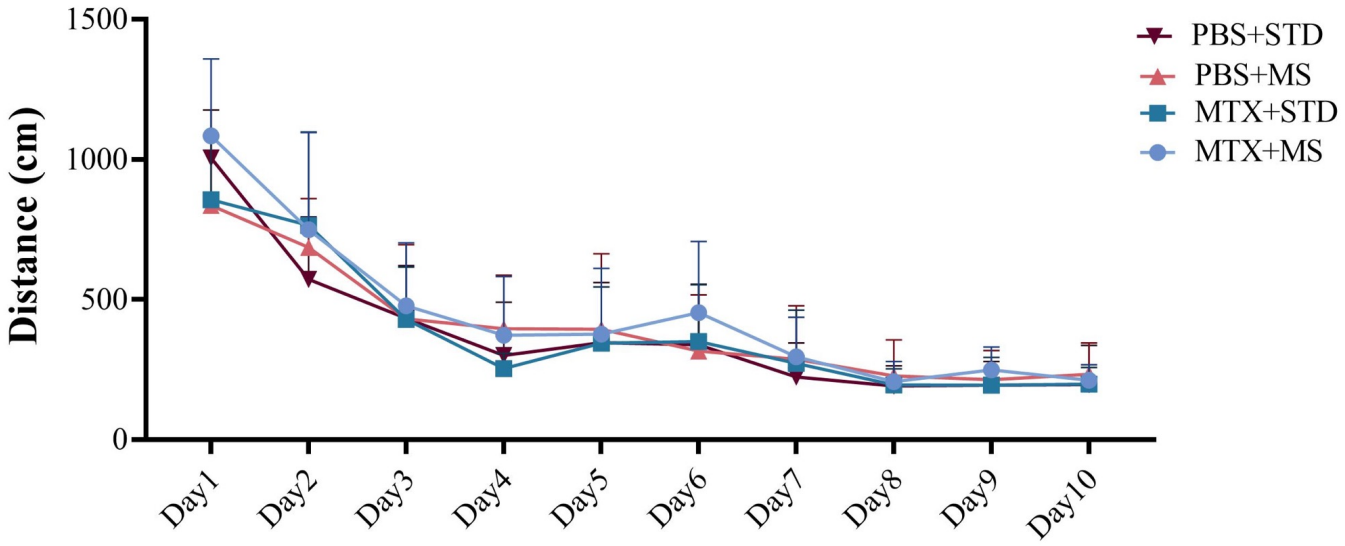
The Forced Swim Test was used to assess depression-like behavior in mice. For the analysis, one-way ANOVA analyses were used to compare the differences between the groups in floating behavior or immobility. Floating can indicate a depressive-like mood. Healthy mice often display escape-related behavior. The statistical analysis revealed no significant results. All four groups of mice seemed to behave similarly. There seemed to be no significant effect of treatment or condition. Analyses performed by sex revealed no significant differences either.

Morris Water Maze

The Morris Water Maze was used to assess spatial learning and memory. For the analysis, two-way ANOVA analyses were used. For the variable 'distance', there was a main effect of time, $F(9,351) = 65.16$, $p < .0001$, meaning that the mice covered significantly less distance as time went on. This indicates that the expected learning process took place, and the mice found the escape platform more rapidly over time, see Figure 10. However, there was no main effect of group, $F(3,39) = 1.694$, $p = 0.1841$, meaning that the difference in condition and treatment had no significant effect on traveled distance. There was no interaction effect found. For the all-female sample, a main effect of time was found as well, $F(5.062,91.11) = 26$, $p < .0001$. Also, a main effect of group was found, $F(3,18) = 3.596$, $p = .034$. This difference was found on day 4 between MTX+STD and PBS+MS, $q(7.946) = 6.712$, $p = .0064$. For the all-male sample, there was a main effect of day, $F(4.674,79.46) = 42$, $p < .0001$. There was also a main effect of subject, $F(17,153) = 1.912$, $p = .0206$. No interaction effects were found in the all-female nor the all-male sample.

Figure 10

Covered Distance in the Morris Water Maze



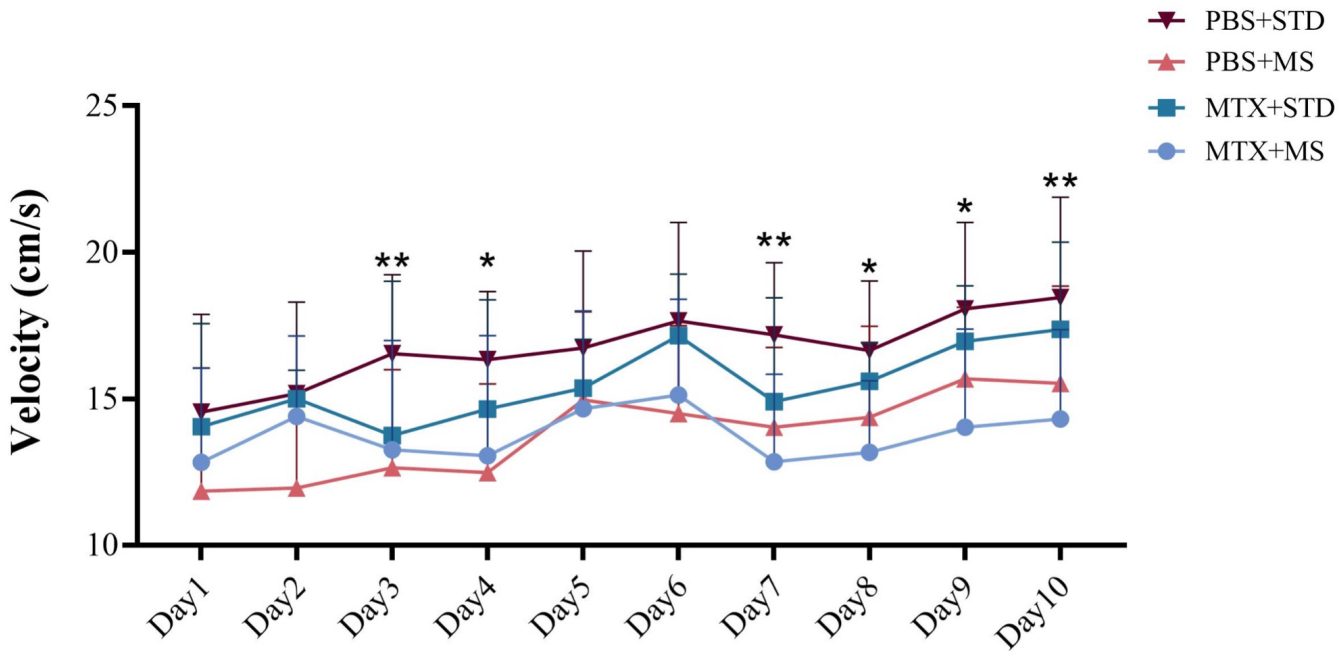
Note. Covered distance in cm during the Morris Water Maze where they were expected to find a hidden platform in opacified water for 10 days. There was a two-day break between day 5 and 6. A main effect of time was found, $F(9,351) = 65.16$, $p < .0001$. The descending trend of the graph indicates that the expected learning process took place, and less distance was needed to find the platform each day. Data is presented as mean with SD.

For the variable 'average velocity', measured in cm per second, there was a main effect of time, $F(9,351) = 9.190$, $p < .0001$, meaning over time, the mice varied in their average speed with an increasing trend, see Figure 11. There was a main effect of group, $F(3,39) = 4.544$, $p = .0080$, meaning there were significant differences in velocity depending on which group the animals were in. There was no interaction effect found. For the all-female sample there was a main effect of time, $F(2.263,40.74) = 3.841$, $p = .0252$. There was also a main effect of subject, $F(18,162) = 13.40$, $p < .0001$. An interaction effect between day and group was found as well, $F(27,162) = 1.587$, $p = .0426$, meaning that on top of the main effect

of time, the group had an additional effect. For the all-male sample, there was a main effect of time, F(2.264,38.49) = 8.205, p = .0007. There was also a main effect of subject, F(17,153) = 11.51, p < .0001.

Figure 11

Velocity of Swimming in the Morris Water Maze

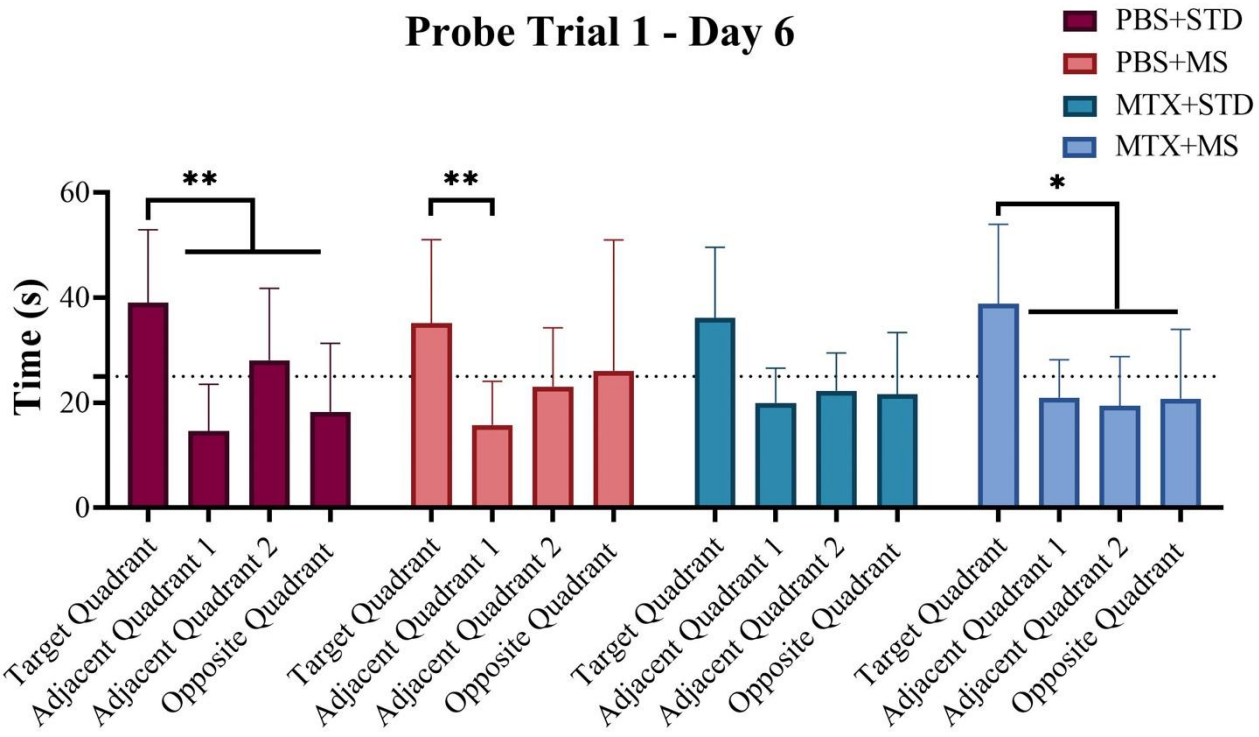


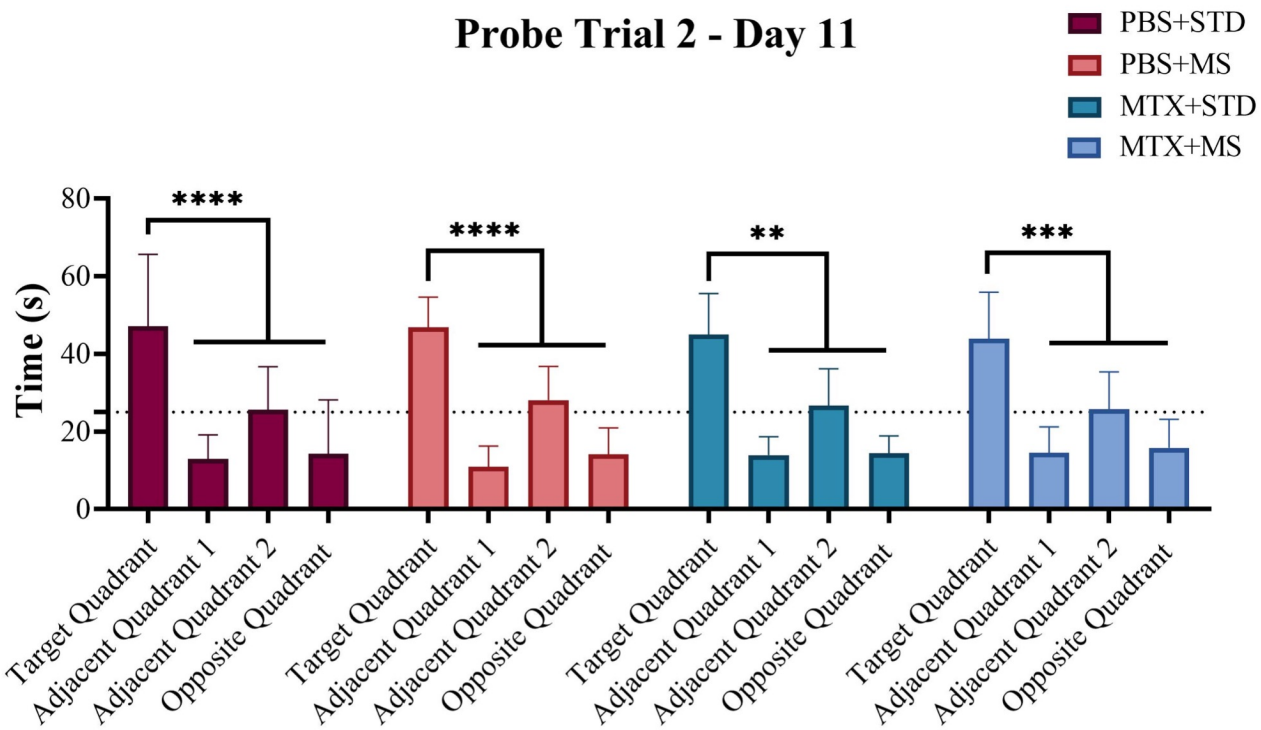
Note. This graph represents the velocity of the test mice during the Morris water maze for 10 days. Velocity is expressed in cm per second. A main effect of time was found, $F(9,351) = 9.190$, $p < .0001$, as well as a main effect of group, $F(3,39) = 4.544$, $p = .0080$. On days 3 and 4, PBS+STD was faster than PBS+MS. On days 7, 8, 9 and 10 PBS+STD was faster than MTX+MS. Velocity was slightly ascending across days and also for some days depended on which group the animals were in. Data is presented as mean with SD. (* = p < .05, ** = p < .01)

On day 6 and day 11, probe trials took place to evaluate the learning process and memory, see Figure 12. On day 6, significant differences were found in the amount of time spent in the quadrants, $F(3,156) = 15.46$, $p < .0001$. This means that after one week of learning, most mice already seemed to spend more time in the target quadrant, indicating the learning process was in progress. On day 11, the effect became more substantial, $F(3,156) = 91.78$, $p < .0001$. The same effects were found in the all-female sample on day 6, $F(3,72) = 6.530$, $p = .0006$, and on day 11, $F(3,72) = 34.56$, $p < .0001$, as well in the all-male sample on day 6, $F(3,68) = 9.065$, $p < .0001$, and on day 11, $F(3,68) = 67.94$, $p < .0001$.

Figure 12

Results of the Probe Trials





Note. This graph represents the time spent in each quadrant of the Morris Water Maze during the probe trials on day 6 and day 11. On day 6 a main effect of quadrant was found, $F(3,156) = 15.46$, $p < .0001$. On day 11, the same effect was found, $F(3,156) = 91.78$, $p < .0001$. Data is presented as mean with SD. (* = p < .05, ** = p < .01, *** = p < .001, **** = p < .0001)

Discussion

The aim of this thesis was to gain insights in the neurocognitive consequences of MTX administered in mouse pups, and whether antecedent maternal separation made the pups more prone to the adverse effects of the chemotherapy. Our experimental design was based on the research of Gibson et al. (2019) and Peña et al. (2019). To carry out this experimental manipulation, half of the pups were subjected to maternal separation for 3 to 4 hours a day, 9 days in a row. The other half was merely subjected to handling. Subsequently, all pups

received three intraperitoneal injections, half of which received 100 mg/kg MTX, half PBS (vehicle). During the pup phase, the animals were tested twice with a Star Maze Maternal Preference test to assess the maternal bond. During the adult phase, a behavioral test battery was used to establish deviant behavioral patterns. This battery included the Open Field test, the Elevated Plus Maze, Flipped Social Exploration, SPSN test, the Automated Tube test, the Forced Swim test and the MWM. With this battery, we aimed to examine exploratory behavior, anxiety-related behavior, social behavior and social memory, depression-like behavior, learning capacities and memory.

First, the weight of the animals was monitored closely. Even though most animals gained weight according to age, there was a significant difference found between the groups. This means that the weight of the pups was significantly influenced by the experimental group they were in. It is however difficult to establish whether the difference is attributable to the maternal separation or the administration of the MTX. Bridges et al. (2017) concluded in his research that MTX can cause substantial weight loss in mice. However, they conducted their study on obese, adult mice. Knowledge appears to be limited concerning the influence of MTX on the weight of mice during the growth phase. Studies focusing on the link between maternal separation and weight change exist as well, like Ryu et al. (2009). They concluded that maternal separation alone did not result in any weight change. However, in combination with another stressful event, in their case social isolation, it did cause significant weight gain. However, other literature suggested that maternal separation could eventually cause weight loss as well, if the maternal separation took place during the lactation period, like in our study. This effect has only been observed in female rats (Paternain et al., 2016). What we can say for certain, is that, based on our data, after a while both MTX groups had overall lower weights in

comparison to the PBS groups. This effect was found for the males, however not for the females.

The only behavioral test used during the pup phase was the Star Maze Maternal Preference test. There were, however, no differences found to what extent the pups showed a preference to spend time with their own dam. Based on this test, there does not seem to be an immediate effect of the experimental manipulations, being either maternal separation, MTX, or both, on maternal bonding. The Star Maze Maternal Preference test is an in-house protocol and the study conducted in this thesis could possibly have served as validation for this study. However, since the control group did not express a particular preference for spending time with the own dam either, the protocol may need some adjustments.

For exploratory and anxiety-related behavior, no remarkable features were found between the different groups in the Open Field test and Elevated Plus Maze. Both the maternal separation mice and the MTX mice did not exhibit dissimilar exploratory or anxiety-related behavior patterns. Research suggests that maternal separation in pups causes an increased amount of time spent in the closed arms of the elevated plus maze (Nishi, 2020). Additionally, rodents with high anxiety generally display the tendency to avoid the center of the open field (Crawley et al., 1997). However, we were not able to replicate these results in our data.

In terms of depressive-like behavior, all mice seemed to exhibit quite similar behavior. No remarkable conclusions could be drawn based on the Forced Swim test. The amount of time spent floating, indicating a state of hopelessness, appeared similar for all four groups. For spatial learning and memory, we failed to replicate the results stated in the literature. The female MTX+STD mice in the MWM covered substantially less distance on day 4 in comparison to the PBS+MS group. Since it appeared to be a one-time effect, it is possible that

this result was simply due to daily fluctuations in performance. However, the velocity during the MWM did differ significantly between the groups. The PBS+MS group displayed an impairment in velocity in comparison to the PBS+STD group. Additionally, the MTX+MS group displayed a substantial deterioration in velocity as well, again in comparison to the PBS+STD group. These data seem to support our claim that the maternal separation may have had an important disadvantageous effect that becomes visible in the velocity of their movements. Further research is needed to draw unambiguous conclusions. This effect was not found for the separate sexes. After chemotherapeutic treatment, research shows us that animals can potentially experience spatial memory deficits during the MWM paradigm (Seigers et al., 2011). Data shows that lesions in the hippocampus, striatum, basal forebrain, motor cortex, cerebellum, cerebral cortex, visual cortex, and cingulate cortex lead to an impaired performance (Shah et al., 2019; D'Hooge & De Deyn, 2001). This suggests that testing the spatial memory in animals could be representative for cognitive deterioration in the clinical population (Seigers et al., 2011). The paradigm is as a matter of fact frequently used in rodent models for neurocognitive disorders and its possible treatment (D'Hooge & De Deyn, 2001). There could be histological changes caused by the experimental manipulation. However, tissues were harvested post testing and they are situated outside the scope of this thesis. There could have been structural or functional anomalies in the brain, which were potentially not reflected in the measured behavior of our test battery.

In terms of social behavior, we found that, using the Flipped Social Exploration test, stranger mice seemed to be less interested in making contact with the mice from the MTX+MS condition in comparison to the interest they displayed towards mice from the PBS+MS condition. The stranger mice were also less fast in their movements for the males in the MTX+MS group in comparison to the PBS+MS group. Furthermore, it seems that for

male mice in the MTX+MS group, the stranger mice approached them and the periphery around their cage much less in comparison to the PBS+MS and the PBS+STD groups. This indicates that stranger mice seemed to be less interested in the animals from the MTX+MS group and they did not initiate contact as frequently as they would with other groups (Odent et al., 2021). Surprisingly, even though it would be expected that if the stranger mice did not spend much time near the cage, they would have spent more time in the corners, this was not the case. Apparently, the stranger mice entered the corners significantly less frequently. Further, for the SPSN test, the male mice from the PBS+STD group covered significantly more distance in comparison to the MTX+MS group. It seems they were more active during the test. The test design seemed successful, since all mice preferred to spend more head time with a novel stranger mouse instead of alone. When a second stranger was introduced, they all paid more attention to the newcomer, except for MTX+STD. However, this group consisted only of 6 animals. Despite the possible differences in head time, the results might not be significant due to small sample size. Finally, we could not find any differences in social dominance between the groups, based on the results of the Automated Tube test. A relevant question in this case is what factors determine the social rank of mice. The causal factors are not yet fully clear,however, research suggests a significant biological basis. Pallé et al. (2019) conducted a study about the individual differences that affect the emergence of a social hierarchy in rodents, using the Automated Tube test. They found that differential gene expression in the medial prefrontal cortex plays a critical role in whether the mouse becomes a dominant or a subordinate. They also concluded that social rank in mice is quite stable over time and will not be significantly influenced by basal differences in stress, mood, or physical features. Social dominance does not seem to affect stress or anxiety-like behavior (Pallé et al., 2019).

Considerations and Limitations

For all tests listed in the previous paragraph, we can wonder whether the manipulations were not strong enough to obtain an observable effect on the domains we aimed to influence, or if the domain in question is simply not susceptible to manipulation at all. Based on the literature, it seemed justified to have expected more significant results in terms of anxiety. However, our obtained results were rather subtle.

For the lack of significant results attributable to the MTX treatment, the research of Moleski (2000) might offer a partial clarification. She reviewed research about neurocognitive consequences in children with ALL who were treated with intrathecal chemotherapy and concluded that their intellectual potential undeniably suffered consequences, however considerably less severe and more subtle in comparison to the effects of cranial radiation therapy. From this point of view, we can ask ourselves if the obtained effects by chemotherapy are simply too subtle to be picked up by our behavioral test battery. Additionally, longitudinal research conducted by Copeland et al. (1996) might offer insights as well, since they included children diagnosed with cancer and receiving either intrathecal chemotherapy or no CNS therapy. Their results appeared not very compelling, concluding that chemotherapy merely had a modest effect on neurocognitive status, whilst results that were found, were limited to perceptual motor skills. Other studies, as mentioned in Madhyastha et al. (2002), simply concluded that no impairments concerning memory were found at all.

Askins & Moore (20008) stressed the use of MTX as a risk factor, especially in combination with young age. They named MTX as a predictor for poorer neurocognitive outcomes. Notwithstanding the abundant research emphasizing the neurotoxicity of MTX, there are however some other studies contradicting this statement. For example, Staff et al.

(2017) reviewed, amongst others, the work of Brugnoletti et al. (2013) and concluded that MTX seems to be rarely associated with significant neurotoxicity, except when administered intrathecally (in the space under the arachnoid membrane of the brain or spinal cord). Since our research worked with intraperitoneal administration, based on this study we should be cautious with expecting extensive effects of the MTX, even when the MTX was administered to young mice pups. It is, however, debatable which administration method is really the most toxic. Other literature suggests that intravenous administration is the most harmful after all (Rijmenams et al., 2021).

The discrepancy in results from animal models could be attributed to the fact that rodents seem to experience more toxic effects of chemotherapeutic agents when they are administered over a shorter period in comparison to animals who received the same cumulative dose over a longer period of time (Seigers et al., 2011). This has yet to be thoroughly examined and defined. All studies use their own regimen, species, and chemotherapeutic agent, which makes it quite difficult to compare different experiments and draw unambiguous conclusions.

Furthermore, the maternal separation paradigm used in this thesis was based on the research of Peña et al. (2019) and was always carried out in groups. This means that the pups who were taken away from their dam always stayed together. Studies showed that pups who were isolated individually displayed a deterioration in barrel cortex formation (a region in the somatosensory cortex to be found in rodents, which relates to the whisker path), pups separated in groups did not (Nishi, 2020). This could illustrate that, even if the maternal separation was a source of stress as intended, the proximity of the rest of the litter could have functioned as a protective factor.

Finally, lack of results from the Automated Tube Test could mean several different things. First, it is possible that the effect of MTX and maternal separation simply was not substantial enough to play part in the determination of social rank in mice. Additionally, previous mentioned research already stated that social hierarchy seems to be highly dependent on gene expression and is considered stable over time (Pallé et al., 2019). This would mean that our manipulations, both chemotherapy and maternal separation, would not qualify to affect social rank to begin with. If gene expression is conclusive and social hierarchy is rigid, it can be expected that experimental manipulations would yield no effect.

Aside from the inconclusive nature of the different discussed studies, it is important to acknowledge another decisive factor. Our sample size was too small due to a significant number of unexpected deaths among the laboratory animals, seemingly due to the administration of MTX. In the MTX+STD group, we ended up with only 6 animals instead of our goal of minimum 12 animals per group. There could be some alternative explanations, for example a weaker generation in the used strain, however we cannot know for certain. For this reason, it is important to interpret all conclusions included in this thesis as preliminary results. It is advised to use the described results and interpretations as guidelines for future research. Therefore, to draw definite conclusions, the experiment should be replicated with a larger sample size.

Translation To Real-life

Previously, we've already established that only a certain fraction of cancer survivors, between 17% and 33%, are affected by adverse long-term effects of cancer therapy. We could then wonder if this division can be expected in animal studies as well (Seigers et al., 2011). Perhaps only part of our animal population will show impairments in cognition and

neurobiological processes. Most animal studies use species that are adequately genetically homogenous, causing minimal individual interference. However, this suggests that the opposite effect could be true as well; an animal model for treatment consequences could yield no significant results due to an exceptionally resilient rodent species. In contrast to laboratory animals, the developmental history of humans is tremendously diverse, with differences in gene expression, IQ, comorbidities, and so on. All these factors could lead to differences in cognitive stability and subsequent impairment. For the course of research, it seems appropriate to examine the consequences of chemotherapy first in a homogenous animal population, before moving onto a more heterogeneous group of animals that resembles the real-life population of patients more accurately (Seigers et al., 2011).

There are undoubtedly differences in treatment strategy between the human and animal population. Whilst human cancer patients almost always receive a chemotherapeutic cocktail multiple times a day or week with numerous active agents, animals in studies are usually only treated with one or two chemotherapeutic agents for a limited number of times. Are we therefore underestimating the harm caused by the chemotherapeutic cocktails (Mullenix et al., 1994)? Perhaps so, however, administering a similar cocktail in an animal model may not be very beneficial. It would perhaps have more clinical and ecological validity, but it would be impossible to determine which neurocognitive effect is caused by which chemotherapeutic agent (Seigers et al., 2011). Also, higher administered doses increase the risk of side effects such as sickness, which could cause adverse effects on cognition. Lastly, it is also known that receiving numerous injections is highly stressful for an animal, whereby stress alone could cause adverse cognitive effects (Alzoubi et al., 2009).

Another considerable difference between the clinical population and animal studies refers to the time for cognitive deterioration. In patients who survived cancer, the adverse

neurocognitive effects are experienced up to many years after the treatment. In contrast, many animal studies conduct cognitive tests over a short period of time, fluctuating between minutes and days or weeks, after the treatment (Wefel et al., 2008; Konat et al., 2008; Madhyastha et al., 2002). It is unclear which conclusions should be drawn regarding the existence of long-term chemotherapeutic effects on cognition in rodents. Are the chemotherapeutic effects transient in rodents, or rather permanent (Seigers et al., 2011)? There are some studies that support the hypothesis that central nervous system damage caused by chemotherapeutic agents only becomes noticeable over time (Li et al., 2008). This is an important conclusion to keep in mind during the follow-up of cancer survivors.

Another big difference between the clinical population and animal models, is that the affected human patients in fact suffer from cancer. The great majority of rodents in the animal models do not, they only receive chemotherapeutic agents. There are a few studies who did include animals with tumors in their experiments investigating chemotherapeutic effects, like Seigers et al. (2010a). They did not find any interaction effect between the MTX and the presence of a tumor. This means that the cancer did not provide an additional adverse effect on top of the chemotherapy. This result suggests that it suffices to use physically healthy, cancer-free animals in research for chemotherapeutic effects. Overall, it is generally justified to use rodent models in research for cancer in humans. MTX is known to induce lowered liver folate levels (due to folate deficiency), elevated homocysteine (an amino acid influenced by vitamin B12, B6 and folic acid) and alterations in amino acid profile in humans and research shows similar biochemical changes in rats (Li et al., 2010).

Implications

Research has established that children who encounter multiple adverse childhood experiences and struggle with emotional dysregulation, may develop post-traumatic stress disorder, dissociation and increased automatic reactivity. Therefore, these patients could have difficulties in responding to traditional treatment (Oral et al., 2016). Further, since maternal separation seems a risk factor and increases susceptibility to stress later in life with all its adverse consequences, it can be paramount to invest in optimal parent-child contact, even during treatment and hospitalization. Findings like these underline the importance of involving the child's parental network during the treatment process. Hopefully, studies like this raise awareness for the patient's well-being in all its facets. Cancer treatment should no longer solely be focused on survival.

Conclusion

This thesis set out to identify the relationship between the administration of MTX and neurocognitive consequences, while investigating a possible attenuating role of maternal separation during the nurturing phase. The research showed us that mostly mice who were subjected to maternal separation, or maternal separation and MTX were significantly slower in their movements and displayed possible impairments in memory, whilst anxiety-related and depressive-like behavior seemed unaffected. In terms of social behavior, all four different treatment groups displayed similar social behavior and seemed to have preserved their natural curiosity. Stranger mice, however, seemed less interested in interacting with the animals who were subjected to the maternal separation and MTX treatment, or who solely received MTX. The initial curiosity was still present, although after a while they seemed to display some avoidant behavior to avert social contact. In this study, the administration of MTX alone

appeared not to be enough to observe visible differences in terms of behavior. However, given the fact that stranger mice displayed more disinterest not only towards groups with maternal separation, but also the mice who solely received MTX, it suggests that the mice could be noticeably affected by their treatment in a way that can be noticed by other mice in direct contact but not by behavioral tests.

Given the unforeseen small sample sizes, these results are preliminary and simply provide guidelines for future research. MTX is widely used for cancer treatment and with increasing survival rates, it is more relevant than ever to gain additional insights about the exact mechanisms and effects. If the importance of maternal separation can be confirmed soon, this can be valuable for the administration of cancer treatments to children in order to prevent worse outcomes. More dedicated research in this field will doubtlessly expand the knowledge about the difficulties childhood cancer survivors experience and give us more tools to preserve their chances at a decent quality of life.

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