rijksuniversiteit groningen

September 2012

Rats do not show cognitive impairment after breast cancer treatment with tamoxifen and methotrexate

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Abstract

Tamoxifen together with methotrexate is widely prescribed in estrogen receptor-α positive breast cancers, but reported complaints of impaired cognition after the use of these agents cause concern. Previous animal studies indicate a negative effect of methotrexate on cognition, but also negative effects of tamoxifen are reported. Tamoxifen is a so called selective estrogen modulator and acts via the estrogen receptor, either as an antagonist or agonist. Since estrogens are found to be important in cognition, the effect of tamoxifen via the estrogen receptors might explain the reported cognitive complaints. The aim of this study was to identify the effect of tamoxifen on anxiety and cognition either with or without methotrexate.

The first study explored the effect of tamoxifen per se on anxiety and cognition in ovariectomized female wistar rats. The rats were treated with three weeks of tamoxifen (1 mg/kg) or vehicle daily. During the last week of treatment anxiety and cognitive behavior was measured in the open field; the elevated plus maze; the novel- and spatial object recognition test. Tamoxifen affected neither anxiety nor cognition in these behavior tests.

In the second study the effect of tamoxifen was combined with methotrexate on anxiety and cognition in ovariectomized female Wildtype Groningen rats. The rats received an intraperitoneal (IP) injection with methotrexate (250 mg/kg) or vehicle, followed by three weeks of tamoxifen (2 mg/kg) or vehicle on a daily base. During the last week of treatment anxiety and cognitive behavior was measured in the open field and the elevated plus maze; the novel- and spatial object recognition test. Besides, spine- and synapse density in hippocampal tissue was measured through western blot analysis with antibodies against spinophilin and synaptophysin. Tamoxifen with or without methotrexate affected neither anxiety nor cognitive behavior; nor did it demonstrate changes in spine- or synapse density in hippocampal tissue.

These studies indicate that tamoxifen with or without methotrexate does not affect anxiety or cognitive behavior in rats. Western blot analysis with antibodies against spinophilin and synaptophysin demonstrated no effect of tamoxifen with or without methotrexate on spine- or synapse density in the rat hippocampus. These findings suggest tamoxifen with or without methotrexate has no adverse or beneficial effects on cognition or anxiety.

Contents

Introduction

Breast cancer (BC) is a major problem in the Western society, according to the 'Integraal kankercentrum Nederland' BC is the most common form of cancer in the Netherlands, 1 in 8 women will develop invasive breast cancer over the course of her lifetime.

Two third of the BC cases are ERα positive breast cancers (1), estrogens are found to play an essential role in the development of this type BC. In 1896 a connection between BC growth and ovarian function was first shown by Beatson *et al.* (2) who discovered that suppression of estrogen levels through oophorectomy caused a decline of metastatic BC. As reviewed by Kelsey *et al.,* current data show that the risk of BC increases through long-term combined estrogen/progestin hormone replacement therapy and long-term use of oral contraceptives increase the risk of BC (3). These studies show that estrogens play an essential role in BC. Estrogens act via mainly two types of estrogen receptors: ERα and ERβ. The growth of primary BC tumors is stimulated by the presence of estrogen. In 70 - 80% of these tumors high levels of ERα are expressed, while only 15 - 25% of the normal mammary epithelial cells express ERα. The role of ERα in BC can also be found in the association between ERα overexpression in the benign breast and an increased risk of BC (4). Additionally, in respond to estrogen mammary epithelial cells appear not to be dividing, but stimulate the growth of neighboring ERα negative epithelial cells instead (reviewed in (5)). This suggests a defect in the ERα function as a possible cause of BC. Fortunately, treatment with the anti-estrogens has successfully been used for many years as adjuvant endocrine therapy for early BC and saved many lives. Unfortunately, both chemotherapy and adjuvant hormonal treatment have been reported to induce cognitive impairment.

Chemotherapy causes cognitive impairment

The present study continues on studies suggesting chemotherapy may induce cognitive problems in patients receiving chemotherapy. Cancer patients often experience subtle changes in cognitive function after receiving their chemotherapy. This phenomenon is called a 'chemobrain' or 'chemofog' (6), which implies symptoms like memory loss, inability to concentrate, difficulty in thinking and other subtle cognitive changes which negatively affect the quality of life and functioning in activities of daily life and work according to the patients. Cognitive problems are found more severe in patients receiving adjuvant chemotherapy than in patients receiving only locoregional therapy: for instance radiation or surgery (7).

However, these cognitive complaints are not equal for all patients; some patients do not report any cognitive complaints or only moderate complaints, while others report severe complaints as a consequence of chemotherapy. The severity of the complaints depends on the dose and the duration of the chemotherapy. BC patients receiving high-dose chemotherapy were found to have a doubled risk for cognitive complaints compared to BC patient receiving the standard dose chemotherapy (8); and a higher amount of cycles of chemotherapy was associated with lower neuropsychological performance (9). Besides these methodological factors, this inconsistency can be ascribed to patient-related factors like fatigue, menopausal status, posttraumatic symptoms, baseline intelligence, education level, depression, anxiety and pre-existing knowledge of 'chemobrain' (6). Other factors can be disease progression, infection, fever, and metabolic or endocrine abnormalities (10).

Animal studies show methotrexate to decrease hippocampal cell proliferation, this might explain the cognitive impairment reported (11, 12). Moreover, in these studies methotrexate impaired cognitive

behavior in the Morris water maze and the novel object recognition (11, 12). All these studies show the negative effect of chemotherapy on cognition, several methodological factors and patient-related factors are suggested to contribute to the severity of this impairment, but the exact mechanisms is still not completely understood.

Hormonal treatment is also reported to induce cognitive impairment

Lately, more concern towards the effect on cognition of additional hormonal therapy in breast cancer patients is appearing. Tamoxifen (ICI 46474; Nolvadex) is the oldest and most used anti-estrogen in BC treatment. It is a so called selective estrogen receptor modulator (SERM), originally developed as a postcoital contraceptive (13), but is now widely ascribed to patients with ERα positive early BC. In the gastrointestinal tract and liver tamoxifen is metabolized to 4-hydroxytamoxifen (OHT) and endoxifen (13). OHT has high affinity with the ERα and acts as an antagonist on this receptor, thereby it inhibits the growth of ERα-positive and estrogen-sensitive BC cells (14). Treatment with tamoxifen for 2-3 years, followed by 2-3 years of aromatase inhibitors is found thought to be the strategy in order to improve disease-free survival and overall survival (15).

Unfortunately tamoxifen is also associated with several side effects including effects on bone density, hot flushes (50% of the women), vaginal discharge, irregular menses in pre- or perimenopausal women, changes in lipid profile, increased risk of endometrial cancer, cardiovascular disease and venous thrombosis (16, 17). These side effects show a lot of similarities with side effect seen in menopause. This may be not surprising, since tamoxifen binds to estrogen receptors inducing either an agonistic or antagonistic effect, depending on the target tissues (16).

Additionally, growing evidence suggests that tamoxifen may also negatively affect cognition, particularly (verbal) memory performance (9, 18-22). However, most studies show only small negative effects on cognition and memory caused by tamoxifen. Nonetheless, since tamoxifen is known to act via the ERs, tamoxifen may induce cognitive impairment by blocking the neuroprotective effects of estrogens (Figure 1).

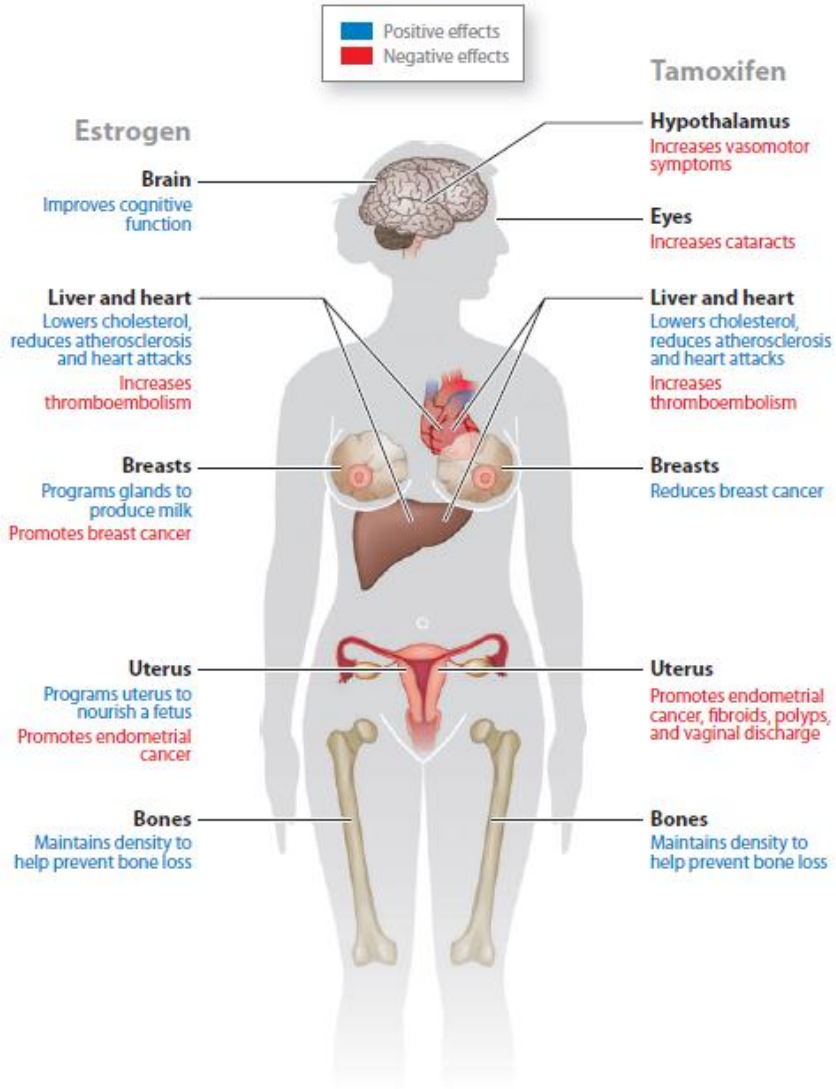


Figure 1 The positive and negative effects of estrogen and tamoxifen in various parts of the body. Tamoxifen is a so called SERM and widely used for treatment of ERα positive breast cancers. However, cognitive complains have been reported after treatment with tamoxifen. Since estrogen is known for its positive effect on cognition, the mechanism of action of tamoxifen via the estrogen receptors may explain the cognitive impairments reported **(5)**.

The neuroprotective effects of estrogen

Estrogen is known as the female sex hormone and well known for its role in reproduction. However the effects of estrogens also influence development, growth, differentiation, maturation and function in all kinds of tissues in the body, including effects on the brain (23). Besides, more and more studies show supporting results towards the neuroprotective role of estrogens. Estrogens are known to have a great contribution in anxiety and depressive-like behaviors, as well as in cognitive behaviors. Indication towards the role of estrogens in these psychological behaviors can be found from several clinical data, which point out that women are more vulnerable to develop mood disorders like anxiety disorders and major depression (reviewed in (24)). This increased vulnerability occurs postpubertally, together with the start of cyclic changes of estrogen levels (25).

The effects of estrogen on cognitive behavior differ in various cognitive tasks requiring different brain regions. In female rats estrogen improves hippocampal dependent tasks but has negative effects on striatum dependent performance tasks (reviewed in (26)). Also in prefrontal cortical dependent learning tasks, estradiol has an improving effect in female rats (27) and rhesus monkeys and women (28, 29).

Another role of estrogen in the brain is neuroprotection. The first evidence for the role of estrogen as a neuroprotective hormone was suggested in studies on sex differences in the brain. In 1991 Hall *et al.* found less severe brain damage after carotid artery occlusion in female gerbils than in same ages male gerbils (30). Later studies done in mice and rats show that female animals have a smaller infarct volume compared to the male animals after experimental stroke (31, 32). Additionally, several studies show that women are also better 'protected' against stroke than men (33). However, stroke incidence in women increases with declining estrogen levels in women. Moreover, outcomes of strokes in postmenopausal women are reported to be worse compared to age-matched men (33). These studies suggest a role in neuroprotection for estrogen, but cannot exclude other factors associated with menopause or gender to influence or cause the reported neuroprotective effects.
This suggestion is confirmed by Alkayed *et al.* (31); they showed estrogen's neuroprotective role by removing the ovaries in rats, to stop the main production of estrogen. They compared male, female and ovariectomized female rats. Female rats showed a smaller infarct size in the cerebral cortex and caudoputamen and higher blood flow during ischemia compared with male rats in both strains. Moreover, this gender effect disappeared in ovariectomized female rats. These outcomes suggest that endogenous estrogen has a neuroprotective and flow-preserving effect after stroke in rats. Furthermore, exogenous administration of estrogen should is shown to result in neuroprotection in different animals.
Exogenous administration of estrogen in ovariectomized female rats, mice and gerbils dramatically reduces infarct volume after focal or global cerebral ischemia (33). In male rats both chronic and acute administration of estrogen protect the brain after experimental stroke (34). To conclude these studies support that the differences in brain damage after ischemia between male and female can be subscribed to the neuroprotective effects of estrogen.

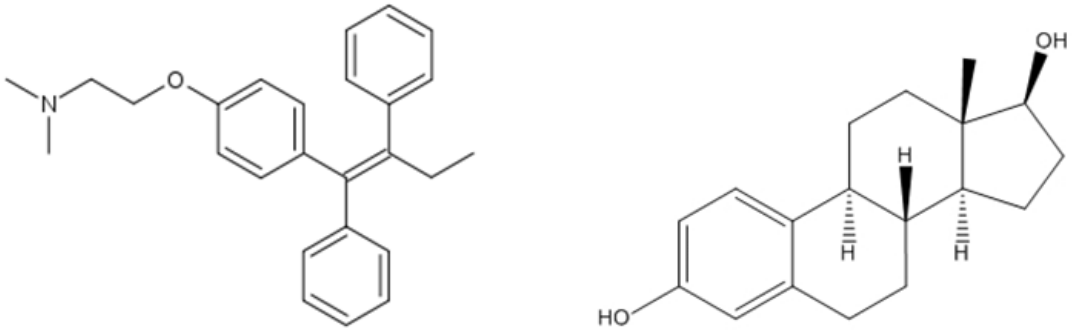
Besides protection of the brain, estrogens are known to enhance some aspects of learning and memory (35). Research into Alzheimer's disease has found estrogen to increase the level of choline acetyltransferase (CHAT), which is required for synthesis of acetylcholine, which in turn is thought to be important in memory (27, 36, 37).

On the other hand, evidence is found against the positive effects of estrogen on the brain. A double blind randomized placebo controlled trial after the effect of estrogen on dementia and mild cognitive impairment was carried out by the Women's Health Initiative Memory Study (WHIMS). A group of 4532 women aged 65 years and older were randomly divided in two groups: combined therapy (estrogen and progesterone) or placebo. Every year the women's cognition was measured through the Modified Mini Mental State Examination (3MSE). Although the trial had to end prematurely because of health risks, the already collected data showed that women that received both estrogen and progesterone had a doubled risk compared to the group receiving placebo to develop dementia. Moreover, the combined estrogen therapy could not prevent mild cognitive impairment in these women (38). A possible explanation for this somewhat unexpected result can be found in the timing of administration of estrogen. Sherwin (39) remarked that in studies where positive effects of estrogens were found, the administration of estrogen was timed at the start of menopause. Subsequently, in studies with negative effects of estrogens the administration was timed many years after menopause, which is also the case in the WHIMS. Additionally, a review by Maki also pointed out that estrogen treatment only had positive effects when administrated in younger women (<65 years) (40). In conclusion, estrogen seems to have many positive effect on cognition, however

evidence is found that these positive effect may be dependent on the timing of administration of estrogen.

Does tamoxifen mimic the neuroprotective effects of estrogen?

As described above, estrogens (Figure 2) are known for its neuroprotective effects and tamoxifen (Figure 2) is thought to mimic these neuroprotective effects. However, where estrogens have a uniformly agonistic effect on estrogen receptors (ER), SERM's including tamoxifen can have both agonistic as antagonistic effects on the ER's, depending on the type of target tissue. This is also why they are called SERM's instead of estrogen receptor modulators (ERM), what they were first called.



Tamoxifen *Estrogen*

Figure 2 Molecular structure of tamoxifen and estrogen.

The tissue selective character of tamoxifen was shown in a study in an athymic mouse model transplanted with both a breast (MCF-7) tumor on one side of mammary fat pads and an endometrial (EnCa101) tumor on the other side. Whereas tamoxifen inhibited the growth of the breast cancer, it stimulated the growth of the endometrial cancer in presence of the same metabolites (41). Whether tamoxifen acts as an agonist or antagonist depends on differential estrogen-receptor expression in a given target tissue and differential expression and binding to the estrogen receptor of co-regulator proteins (41, 42).

There is still debate about the precise mechanism of tamoxifen, according to the classical model binding of tamoxifen to the ER causes the ER to undergo a conformational change which enables it to form dimers and to interact with estrogen response elements (ERE) that are located within the regulatory regions of target genes. Via this pathway the ER regulates the gene promoters, but transcription is also influenced by the cellular and promoter environment, like the subtypes of estrogen receptor and co-regulators present (43) (Figure 3).

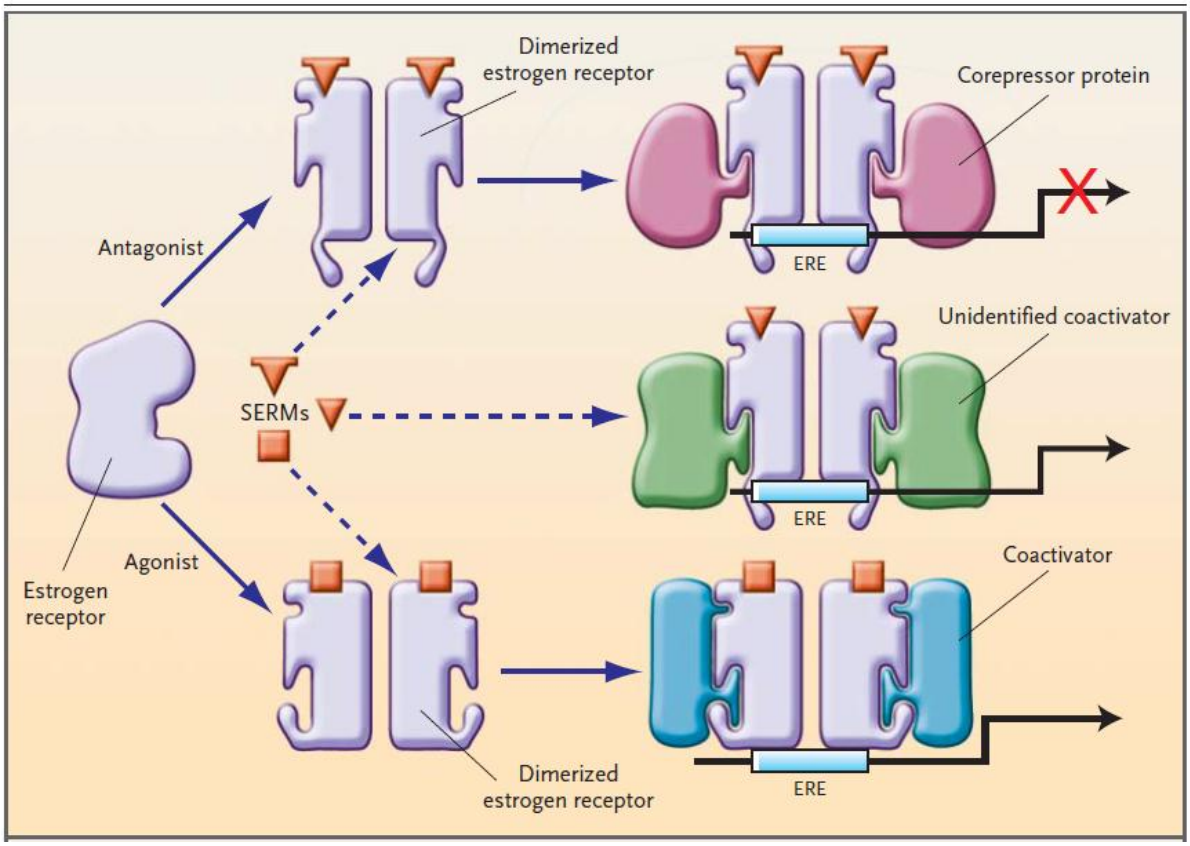


Figure 3 The mechanism of acting in SERMs binding to the ER. SERMs (orange) binding to the ER (purple) induce a conformational change of the ER that permits it to spontaneously to form a dimer with another ER. The dimer-ligand complex facilitates binding with estrogen response elements (ERE) located within target genes; and binding of various co-regulators. Dependent on the type of ligand binding this co-regulators can either act as a co-repressor (red) and increase antagonistic activity; or they can act as a co-activator (blue) and increase the agonistic activity. So the agonistic/antagonistic effect of SERMs depend on the type of SERM binding and the relative levels of expression of the co-regulators proteins present in the different target cells **(41)**.

Mainly two types of estrogen receptors are known; ERα and ERβ. Binding of tamoxifen to the ERα or ERβ induces different actions. Binding of tamoxifen to the ERα almost always results in activation, but can be inhibited through the formation of a heterodimer with the ERβ and ERα (44). An in vitro study by Hall *et al.* showed that the ability of tamoxifen to activate ERα-mediated transcription was even completely suppressed in presence of ERβ (45). So presence of ERβ may disable tamoxifen to activate ERα.

Tamoxifen bound to the ERβ acts almost always antagonistic because ERβ lacks a large part of the carboxyterminal F domain (46), which is important to induce a agonistic effect by binding with tamoxifen (47). So if the target tissue has relatively higher levels of ERα, tamoxifen would probably have a agonistic effect on this tissue. However, if the relative ERβ are higher, tamoxifen would be more likely to have an antagonistic effect. Nevertheless, the ERβ can partially replace ERα if ERα is absent (44). In that case, tamoxifen would probably also act as an agonist. Yet, SERMs can still differ in activity among tissues that synthesize the same ER subtype (reviewed in (43)), indicating that the activity of SERMs are probably not dependent on receptor selectivity only.

So different mechanisms should explain the differences in action of SERMs in different tissue and indeed the agonistic/antagonistic effect of tamoxifen and other SERMs also depends on ER-mediated gene transcription. Two distinct transactivation domains on the ER facilitate the interaction of the ER with the transcription apparatus: AF-1 and AF-2 (48). These activation domains are important for

maximal transcription, but in some contexts activity of only one of the domains can be sufficient (48). Besides, in the ERβ the AF-1 region the activity is found negligible compared to the AF-1 activity in the ERα (49), so ERα activity would dominate ERβ activity in genes that require both activation domains. Tamoxifen is found to act as an antagonist on genes that require only the AF-2 domain and as a partial agonist in genes where AF-2 is not required (50). Therefore, it is thought that in breast tissue, where tamoxifen acts as an antagonist, AF-2 activity is required. Conversely, AF-1 activity alone is thought to be sufficient on tissues where tamoxifen acts as an agonist, like in the uterus, the bone, the liver and the cardiovascular system (Figure 4) (43, 51).

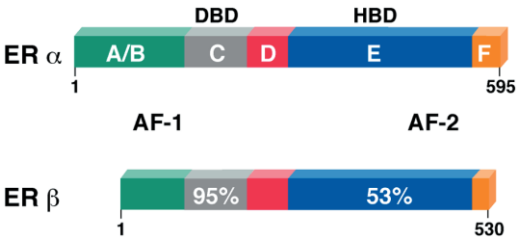


Figure 4 Structure of ERα and ERβ. Tamoxifen almost always acts as an antagonist bound to the ERβ, because ERβ lacks a large part of the F domain (orange), which is important to induce a agonistic effect by binding with tamoxifen. Furthermore the two distinct transactivation domains AF-1 and AF-2 are important for the agonistic/antagonist effect of tamoxifen. The AF-1 domain in the ERβ is negligible compared to the AF-1 domain in the ERα. Tamoxifen acts as an antagonist on genes that require only the AF-2 domain and as a partial agonist where AF-2 is not required **(50)**.

But there are also proteins found that influence the effects of SERMs, called co-regulators. More than 20 co-regulators are found to bind to the ER and modulate their function, either positive (co-activator) or negative (co-repressor) (reviewed in (44). Depending on the ligand binding, a unique ER conformation is formed, which enables variable combinations of regulator proteins to interact with the ER and modulate the function of the ER-SERM complex. Some of these co-regulators can modulate the ER by co-repressor recruitment that increases the antagonistic activity, while others favor co-activator recruitment, this increases the agonistic activity. The type of co-regulator binding depends on the type of SERM that is bound to the ER and the relative levels of different co-regulators present in the different target cells.
Steroid receptor co-activator protein 1 (SRC-1) is an example of a co-activator. Overexpression of the gene encoding for this co-activator is found to enhance the agonistic activity of tamoxifen in target cells (52). Hence, the effect of the SERM tamoxifen also seems to depend on the relative levels of co-regulator proteins in the target cell (reviewed in (42)).

On top of that, there also other pathways proposed to explain the mechanism behind SERM's: through activating protein 1 (AP-1) and by transcriptional repression through binding to the p65 subunit of the nuclear factor (NF)-κB (53), but these mechanisms will not be described in detail in this report.

Literature shows diverse effects of tamoxifen on cognition

So the effect tamoxifen has on the brain depends on whether tamoxifen antagonizes or agonizes the ERs in the brain. Many studies suggest an estrogen-like effect of tamoxifen in the brain; improvement in attention, learning, and memory has been demonstrated after hormone replacement therapy (HRT) in postmenopausal women (reviewed in (10)). However, some studies suggest an inhibiting effect on neuroprotection for HRT. Co-treatment with tamoxifen was found to remove the increase of the 5-hydroxytryptamine2A receptor (5-HT2AR) and serotonin transporter (SERT) seen after estradiol treatment (54). Also some clinical studies find that tamoxifen can induce memory problems (20) and lower processing speeds in cognitive and neuropsychological tests (55). Yet it should be noted that the latter study had a very small sample size of only 46 women and had no baseline data of cognitive function, which can have influenced the study results. Other clinical

studies, on the other hand, could not find any effects of tamoxifen on cognitive function (56, 57). To conclude, studies after the effects of tamoxifen on brain and cognition are contradicting. Hence, more research is needed in order to make a clear conclusion about the effects of tamoxifen on cognition.

Cognitive effects of tamoxifen can be suggested by means of the distribution of the two ER types found in the brain. Both ER types are found in several areas in the brain important in cognitive function, including the cerebral cortex, the prefrontal cortex, hypothalamus, amygdala, and hippocampus (reviewed in (10, 21, 58). Since tamoxifen is shown to be able to cross the blood-brain barrier (59), this supports the thought that tamoxifen may have an effect on cognition. In studies performed in rodents and monkeys, ERα is mostly found in the hypothalamus and amygdala (60) and is also shown to be expressed in limbic-related areas of the human and monkey forebrain (reviewed in (58).
ERβ on the other hand is primarily expressed in hippocampal formation, the entorhinal cortex, the thalamus, and claustrum (61). In contrast, ERβ mRNA expression was low in the hypothalamus and the amygdala, opposite to the expression of ERα in these areas (61). Because tamoxifen binding to ERα mostly resulted in activation tamoxifen probably acts is an agonist on the ERα rich brain area's describe above. However, Riggs *et al*. states that all SERMs studied this far only showed antagonistic effects of tamoxifen for hypothalamic centers regulating gonadotrophin secretion (42), demonstrating that the effect of tamoxifen cannot simply be predicted by the type of ER. Nevertheless, expression of the different types of ER's in the brain suggest SERM's, including tamoxifen, to induce effects in these brain areas, however the specific functions of the different ERs and the different effects on these receptor are not yet well understood. Besides, the function of ERs also depend on different environmental factors like co-regulators and different genes involved, which are not yet completely understood in accordance to cognition and tamoxifen.

Aim of the experiment

The aim of this study is to investigate the effect of tamoxifen with or without methotrexate on cognition. The effects of the aromatase inhibitor anastrozole on cognitive impairment were also investigated in this study, but this report will focus on the effect of tamoxifen and methotrexate only. Two lines of experiments were conducted. The first experiment, experiment A, was set up to investigate the effect of tamoxifen per se on cognitive behavior. The second experiment, experiment B, aimed to discover the effect of tamoxifen in combination with methotrexate on cognitive behavior. Moreover, morphological changes in hippocampal tissue due to treatment with tamoxifen and methotrexate were investigated with respect to the amount of spinophilin and synaptophysin.
Based on the interaction of tamoxifen with the ER tamoxifen is thought to mimic the neuroprotective effects. Nonetheless, patients do report memory impairment after breast cancer treatment with tamoxifen and methotrexate. Since many studies already showed negative effects of methotrexate on cognition, methotrexate is thought to be the main cause of cognitive impairment. Therefore methotrexate is expected to decrease the expected positive effects of tamoxifen on cognition.

Understanding of the effect cognitive function in rats after tamoxifen treatment can be tested by the use of various behavior tasks. In this experiment cognition was measured through two memory based tasks: the novel object recognition and the spatial object recognition. The latter shown to be a typical hippocampal task by studies in which lesions are inflicted in the hippocampus. These lesions

had no or little impact on the ability of recognizing objects, but did affect performances in recognizing relocated objects (62, 63). Since tamoxifen is expected to have a positive effect on the hippocampus, tamoxifen treated animals should perform better in the spatial task. Other studies show the hippocampus to play a significant role in the ability of the recognition of objects as well (64, 65 reviewed in (66)). This suggest that a positive effect on the hippocampus would also improve the performances on the object recognition.

But performance in object and spatial recognition tasks are not affected by memory and spatial orientation only, anxiety levels and exploratory behavior are also thought to effect performance in these tasks (67). Anxiety was therefore measured through performances on the elevated plus maze and the open field test. Performances on the elevated plus maze and the open field will show if tamoxifen mimics the effects of estrogen on anxiety behavior.

All these behavior tasks are chosen because they are known to be rather mild tasks with regard to stress. The amount of stress due to these tasks was not expected to induce significant differences in the brains, which enables all animals to perform all tasks and additionally the brain tissues could be used for western blotting to determine differences caused by treatment. Two proteins in hippocampal tissue were detected with the use of western blotting: spinophilin and synaptophysin.

Furthermore, an association between the acquisition of new memories and spine density in the CA1 hippocampal region in rats is shown in several studies (35). Hence, spinophilin was measured in hippocampal tissue, indicating the spine density in this brain area. Spinophilin is a protein phosphatase-1 and actin-binding protein localized adjacent to the post-synaptic membrane and plays a role in glutamatergic neurotransmission and dendritic spine morphology (68). Spines are small membranous protrusions that receive input from synapses of an axon and thereby are important for communication between neurons. They are located on the dendrites of pyramidal neurons and can be found on many neurons, but are particularly numerous on pyramidal cells of both the hippocampus and the PFC (35). However, the number of spines also fluctuates and is dependent on the state the animal is in (35). In female rats, spine density is found to be dependent the estrus cycle, a decrease of 30% in apical dendritic spine density was found in CA1 hippocampal pyramidal cells over the 24-hr period between the late proestrus and the late estrus phases. During the diestrus spine density went up again towards the highest spine density, which is found in proestrus (69). Besides, in ovariectomized rats the CA1 hippocampal pyramidal cells in the hippocampus show a dramatic decrease of approximately half of the dendritic spine density compared to those of intact female rats. Moreover, administration of two injections of estradiol benzoate (10 µg/0.1 mL in sesame oil) three days after ovariectomy, prevented this decrease in spine density in rats. The dendritic spine density in this group was not significantly different from that in intact group (70). Based on the increase in spine density found after administration of estrogen, tamoxifen is also expected to show an increase in spine density.

Additionally, synapse density in the rat hippocampus is measured through detection of the presynaptic marker: synaptophysin. Synaptophysin is a 38-kDa calcium-binding glycoprotein of presynaptic vesicles. In several studies it has shown to exhibit sufficient specificity and selectivity for prompt quantitation of synapses (71). Most synapses are located on an axon, though sometimes they can also be located on a dendrite or soma. Synapses are essential for communication between

neurons, they transmit signals from one neuronal cell to the target neuronal cell. A decline in synapses could therefore lead to an impaired communication between neuronal cells, which could subsequently lead to malfunction of the affected tissue.

Estrogen is known to increase synaptogenesis in brain areas that are known to be important for memory (72, 73). Sharma *et al*. shows that both 4 weeks daily administration of estrogen (0.1 mg/kg body weight) and the SERM tamoxifen (0.05 mg/kg body weight) increases synaptic plasticity in the rat hippocampus (74). Ovariectomy was found to reduce the amount of synaptophysin, but administration of estrogen or tamoxifen resulted in an upregulation of synaptophysin. Whereas the hippocampus is associated with memory and cognition (75), this upregulation of synaptophysin in the hippocampus supports the concept of possible beneficial effects of estrogen and tamoxifen on memory and cognition. Thus administration of tamoxifen is expected to increase synapse density.

Materials and Methods

Two lines of experiments are conducted. The first experiment done in adult female Wistar rats investigated the effect of hormonal therapy per se. The second experiment done in adult female Wildtype Groningen rats looked into the effect of hormonal therapy in combination with chemotherapy.

Experiment A: Wistar

General

48 Adult female Wistar Hannover rats (Harlan, Horst, The Netherlands, average bodyweight at the start of the experiment 282.8 g ±4.07 S.E.M.) were housed individually in clear Plexiglas cages (25 cm x 25 cm x 30 cm) on a layer of sawdust with a fixed 12:12 h light:dark cycle with lights on at 11.00 p.m. Food (standard lab chow diet) and water were available ad libitum except during behavioral testing. Body mass of the animals was measured from Monday till Friday and during the treatment they were also weighed on Saturday and Sunday. All procedures were approved by the local animal experimental welfare and care committee (DEC, Groningen, the Netherlands).

Timeline

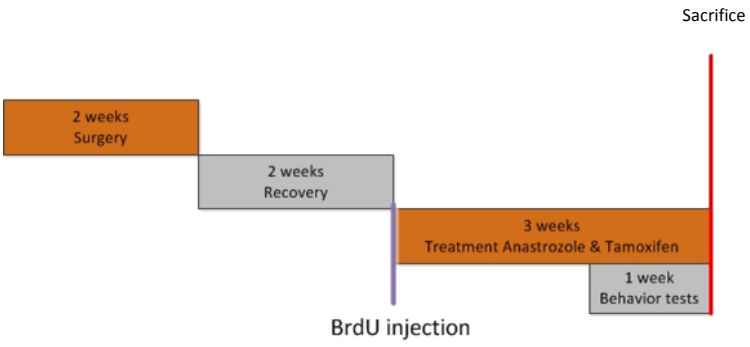


Figure 5 Timeline experiment A. In the first two weeks the animals were ovariectomized and a IP-cannula was placed. Hereafter the animals got two week of recovery. BrdU injection for immunocytochemical analysis was given just before the start of the three weeks of hormonal therapy. In the last week of administration with tamoxifen, anastrozole or vehicle, four behavior tests were performed; the elevated plus maze; the open field; novel object recognition; and spatial object recognition. The animals were sacrificed directly after the three weeks of hormonal therapy.

Experiment A started with two weeks of surgery, the animals were ovariectomized in order to induce a post-menopausal state. Also an intraperitoneally (IP) cannula was placed for administration of the medication. Surgery was followed by two weeks of recovery. Just before the start with hormonal therapy (anastrozole, tamoxifen or vehicle), all animals received a BrdU injection for immunocytochemical analysis, however this analysis was not done in this experiment due to time limitations. During the last week of hormonal treatment, behavior testing was performed. Four behavior tests were conducted: the elevated plus maze and the open field for measuring anxiety; and the novel object recognition and spatial object recognition for measuring attention and memory. 24 hours after hormonal administration the animals were sacrificed (Figure 5).

Operations

48 animals were ovariectomized under anesthesia with isoflurane (1L flow of oxygen and 2,5 mL flow of isoflurane). During this operation also an intraperitoneally (IP) cannula was placed for administration of the medication and the animals received 0,1 mL of finadyne (50 mg/mL). However

due to problems with the resistance of the animals, only 28 animals remained after surgery. Two weeks of recovery were given to the animals after surgery. 6 animals were not operated and served as a control for brain anatomy.

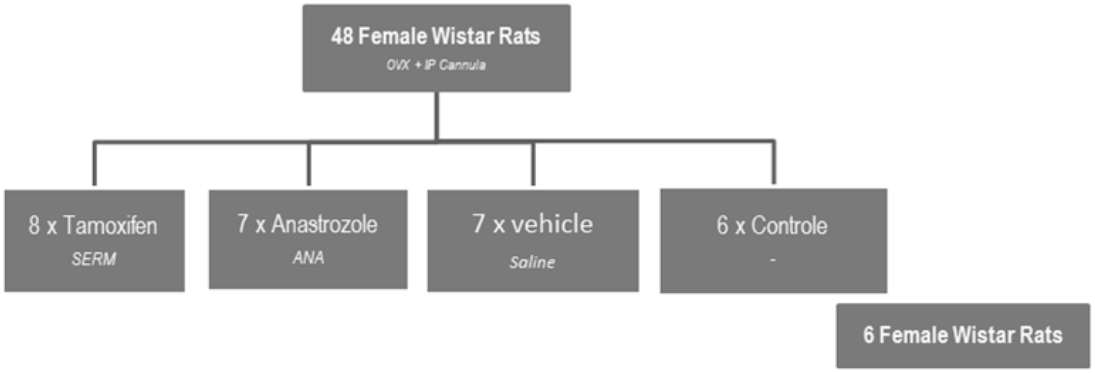


Figure 6 Treatment groups. 48 female Wistar rats were ovariectomized and an IP-cannula was placed. Due to low resistance of the animals, only 28 animals remained after surgery. These 28 animals were divided in four groups: 1) 8 animals received tamoxifen (1 mg/kg); 2) 7 animals received anastrozole (0.1 mg/kg); 3) 7 animals received vehicle; and 4) 6 animals did not receive any treatment. An additional group of 6 animals (non-OVX, non-treatment) was added to see the effect of OVX on the animals.

Treatment

Before treatment all the animals received an IP injection of BrdU (250 mg/kg) for immunocytochemistry at the end of the experiment. The animals were divided into 5 groups: 1) Six animals did not receive any treatment or injection due to problems with their cannula, they were used to see the effect of treatment and injection itself on the animals 2) Another six animals did not receive any treatment or injection to examine the brains of non-treated Wistar Hannover rats, since they were not yet used in this laboratory; 3) Seven animals received vehicle (10% cyclodextrine, 0,15 mL) daily for a period of 3 weeks; 4) Eight animals received tamoxifen (1 mg/kg) dissolved in 10% cyclodextrine daily for a period of 3 weeks; 5) Seven animals received anastrozole (0.1 mg/kg) dissolved in 10% cyclodextrine daily for a period of 3 weeks (Figure 6). After the three weeks of treatment, the animals were euthanized by cardiac perfusion with saline/paraformaldehyde under anesthesia with dry ice. The uteri, adrenals and thyroid glands were removed, trimmed free of fat and excess fluid was removed. Wet weights of the organs were measured. Brains were also removed, but no analyses on the brains is done yet.

Behavior testing

In the second week of treatment all the animals, except for the non-operated animals, performed several behavior tests: modified open field test (OP), the elevated plus maze (EPM), the novel and spatial object recognition (NOR and SOR).

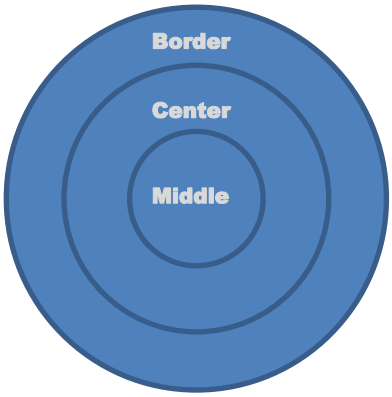


Figure 7 Open Field. The open field was divided in three zones: the center; middle ; and border.

Tabel 1 Timeline behavioral tests experiment A. The behavioral tests were conducted on consecutive days, noted as day 1, 2, 3 and 4. The elevated plus maze and the open field test were performed at day 1 and 2. The novel object recognition test and the spatial object recognition were both performed on day 4. The time of the day this test was perform can be found in the column 'time of day'.

Day	Time of day	Test
1.	11:00-16:00	Elevated plus maze
2.	11:00-16:00	Open Field
3.	15:00-18:00	Habituation in the field for the novel- and spatial object recognition. Two times 3 minutes per animal habituation.
4.	11:00-18:00	Novel object recognition and spatial object recognition

Modified open field test

All animals performed the modified open field test in a random order. The open field (diameter 122cm, height 48cm, light setting 3 = 16 lux, measured in the middle zone of the open field) was modified with an object (a black weight of 1 kg) in the center of the open field, in order to motivate the animals to move to the center of the open field. The animals were placed in the center of the open field and were tracked for 5 minutes by using Ethovision software. After 5 minutes, the rat droppings were counted and removed from the open field and the open field was cleaned with mild soapy water. All the animals were tested on the same day. Data were collected using Ethovision software. The open field was divided into three zones: the center, the middle and the border (Figure 7). The path of the animals was tracked and scored for the time spent these three different zones. Also the latency time for entering the zones was recorded. Furthermore the number of rat droppings was counted.

Elevated plus maze

All the animals performed the elevated plus maze in a random order. The elevated plus maze test was performed in a maze 50 cm above the ground with four arms in shape of a cross: two open arms and two closed arms with walls 40 cm of height. A 60 W lamp was placed at the side of a closed arm. The light intensity in the elevated plus maze was 40 - 65 lux measured in the open arm (Figure 8). The test animal was placed in the middle of the maze with the head faced in the right closed arm and recorded on video for 5 minutes. After these 5 minutes the rat was taken out of the maze and placed back in its home cage. The maze was cleaned with mild soap water before another rat was tested. Data were collected from video by using Eline software. Time spent in the open arms, closed arms and middle was measured. Also the number of crossings between the open arm, closed arm and middle was recorded.

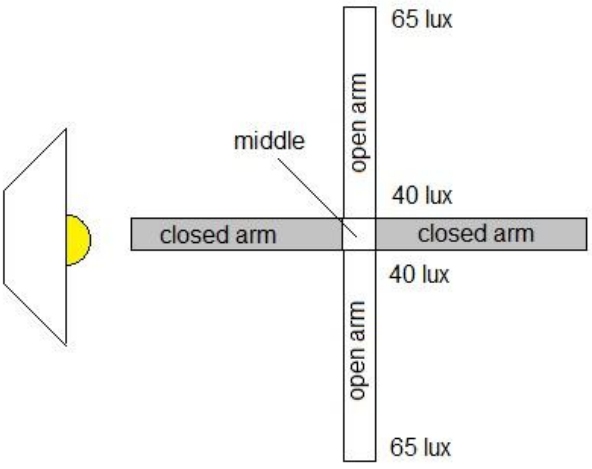


Figure 8 Top view of the elevated plus maze. A 60 W lamp placed at the side of a closed arm was used in the elevated plus maze. The light intensity was measured on the open arms: 40 lux near to the middle and 65 lux at the ends of the open arms.

Novel and spatial object recognition test

All the animals performed the novel and spatial object recognition (NOR and SOR) test in a random order. The NOR and SOR test was performed in a square field (72cmx72cmx40cm). A 60 W lamp was placed against one wall of the field. The light intensity in the field was 6 lux measured in the center of the field and 5 lux measured in the corners of the field (Figure 9). The NOR and SOR test consists of 4 trials of 3 minutes. The first trial was called the habituation phase, during this phase the animal can freely explore the field. After 3 minutes two object were added in two corners of the field, the acquisition phase. The animal can explore the objects for 3 minutes. After 3 minutes the objects were removed from the field and cleaned. One of the 'familiar' objects was placed back and a novel object replacing the other 'familiar' objects was placed in the field, this was called the first recollecting phase. Again the animal could explore the field and the objects for 3 minutes. In the last trial, called the second recollecting phase, the 'novel object' was replaced to another corner in the field and the animal should explore the field again for 3 minutes. After the last trial the field was cleaned and a new test animal was to perform the test. All the trials were recorded on video and scored, using Eline software, for 1. Exploring cage; 2. Exploring familiar object; 3. Exploring novel object; 4. Immobility and 5. Washing behavior.

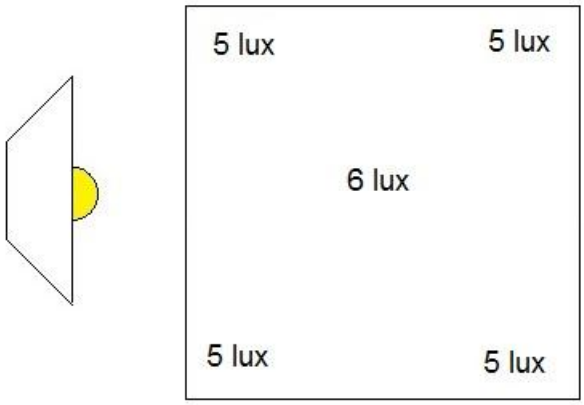


Figure 9 Top view of the novel and spatial object field. A 60 W lamp was placed against one wall of the field. The light intensity in the field was 5 lux measured in the center of the field and 4 lux measured in the corners of the field.

Statistics

Body weight were analyzed using two-way repeated measure ANOVA. Organ weight differences were analyzed using two-way ANOVA. Significant differences between treatment groups in the behavior tests were analyzed using two-way ANOVA. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.

Experiment B: Wildtype Groningen

General

48 Wildtype Groningen rats (Groningen, The Netherlands, average bodyweight at the start of the experiment 264,7 g ± 16,7 S.E.M.) were used in this experiment. 24 rats were housed individually in clear Plexiglas cages (25 cm x 25 cm x 30 cm) and the other half was housed individually in clear Plexiglas cages (55 × 33 × 20 cm) both on a layer of sawdust with a fixed 12:12 h light:dark cycle with lights on at 11.00 p.m. Food (standard lab chow diet) and water were available *ad libitum* except during behavioral testing. All procedures were approved by the local animal experimental welfare and care committee (DEC, Groningen, the Netherlands).

Timeline

Experiment B started with two weeks of surgery, the animals were ovariectomized in order to induce a post-menopausal state. Surgery was followed by two weeks of recovery. Either methotrexate (250 mg/kg) or saline was administered IP after recovery, directly followed by three weeks of hormonal therapy: anastrozole (0.2 mg/kg); tamoxifen (2 mg/kg); or vehicle. In the last week of hormonal treatment behavior testing was performed. Four behavior tests were conducted: the elevated plus maze and the open field for measuring anxiety; novel object recognition and spatial object recognition for measuring attention and memory. Directly after hormonal administration the animals were sacrificed. Hippocampal brain analysis were conducted using Western blotting. Relative amounts of spinophilin and synaptophysin were measured (Figure 10).

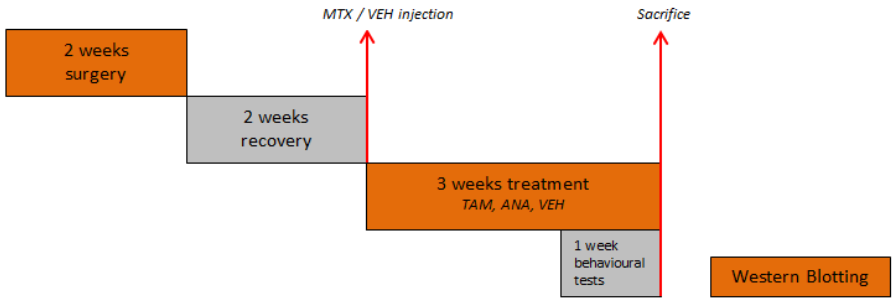


Figure 10 Timeline experiment B. In the first two weeks the animals were ovariectomized and a IP-cannula was placed. Hereafter, the animals got two week of recovery. Just before hormonal treatment, the animals were IP injected with either methotrexate (250 mg/kg) or saline. In the last week of administration with tamoxifen, anastrozole or vehicle, four behavior tests were performed; the elevated plus maze; the open field; novel object recognition; and spatial object recognition. The animals were sacrificed directly after the three weeks of hormonal therapy. Hippocampal tissue was used for analyses with western blotting. The relative amounts of spinophilin and synaptophysin was measured.

Operations

All the animals were ovariectomized under anesthesia with isoflurane (1L flow of oxygen and 2,5 mL flow of isoflurane) and received 0.1 mL of fynadyne(50 mg/mL). After surgery two weeks of recovery was given to the animals.

Treatment

The animals were divided into 6 groups: 1) 8 animals received an IP injection methotrexate (250 mg/kg) once and tamoxifen (2 mg/kg) dissolved in 10% cyclodextrine daily for a period of 3 weeks; 2) 8 animals received an IP injection saline once and tamoxifen (2 mg/kg) dissolved in 10% cyclodextrine daily for a period of 3 weeks; 3) 8 animals received an IP injection methotrexate(250 mg/kg) once and anastrozole (0.2 mg/kg) dissolved in 10% cyclodextrine daily for a period of 3 weeks; 4) 8 animals received an IP injection saline once and anastrozole (0.2 mg/kg) dissolved in 10% cyclodextrine daily for a period of 3 weeks; 5) 8 animals received an IP injection methotrexate (250 mg/kg) once and vehicle (3 mL) daily for a period of 3 weeks; 6) 8 animals received an IP injection saline once and vehicle (1 mL/kg) daily for a period of 3 weeks (Figure 11). Eighteen hours after administration of methotrexate (250 mg/kg) the animals received leucovorin in a 8% concentration of the MTX they received. 26, 42 and 50 hours after MTX administration, leucovorin was again administered at a concentration of 4% of the injected MTX. Administration of leucovorin after MTX is essential, because MTX administration without leucovorin is lethal. Leucovorin has folic-acid like vitamin activity. Dihydrofolate reductase is a key enzyme in the folic-acid metabolism, but the therapeutic efficiency of methotrexate is largely based on the inhibition of this enzyme. Since folic-acid

metabolism is essential in the body for the production of red- and white blood cells and the function of neurons, lack of folic-acid can be lethal, due to severe diarrhea and weight loss (11). Leucovorin does not require the action of dihydrofolate reductase, so the vitamin like function of leucovorin is unaffected by MTX (76). This makes leucovorin a essential 'rescue agent' in addition to chemotherapy. Besides, pilot studies showed that leucovorin itself does not have an effect on neurogenesis (11).

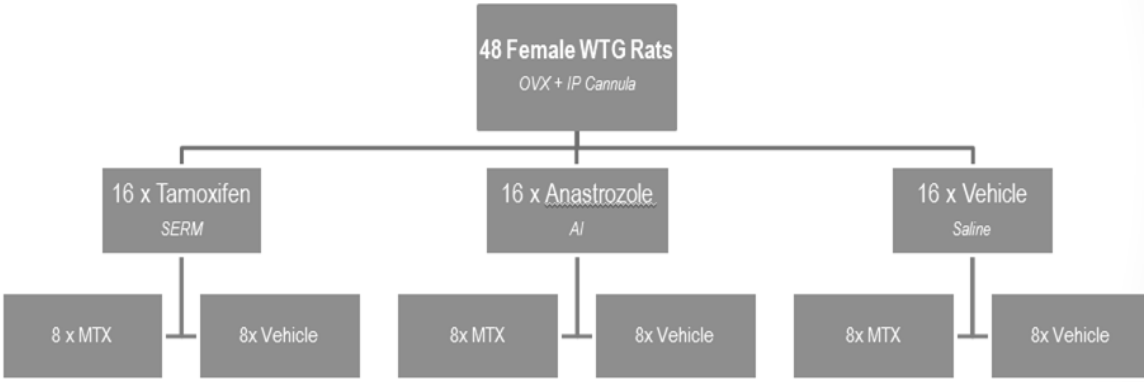


Figure 11 Treatment groups experiment B. 48 female Wildtype Groningen rats were ovariectomized and an IP-cannula was placed. The animals were divided in six groups: 1) 8 animals received tamoxifen (2mg/kg) and MTX (250mg/kg); 2) 8 animals received tamoxifen (2mg/kg) and saline; 3) 8 animals received anastrozole (0.2mg/kg) and MTX (250mg/kg); 4) 8 animals received anastrozole (0.2mg/kg) and saline; 5) 8 animals received vehicle and MTX (250mg/kg); and 6) 8 animals received vehicle and saline.

Behavior testing

In the second week of treatment all the animals performed several behavior tests: modified open field test (OP), the elevated plus maze (EPM), the novel object recognition (NOR) and the spatial object recognition (SOR).

Tabel 2 timeline behavioral tests. The behavioral tests were conducted on consecutive days, noted as day 1, 2, 3 and 4. Every day one behavior test was performed, written down in the column 'test'. The time of the day this test was perform can be found in the column 'time of day'.

Day	Time of day	Test
1.	11:00-14:00	Elevated plus maze
2.	11:00-16:00	Novel object recognition
3.	11:00-16:00	Spatial object recognition
4.	11:00-13:30	Open field

Modified open field test

All animals performed the modified open field test in a random order. The open field (diameter 122 cm, height 48 cm, light setting 3 = 16 lux, measured in the middle zone of the open field) was modified with an object (a black weight of 1 kg) in the center of the open field, in order to motivate the animals to move to the center of the open field. The animals were placed in the border of the open field and were tracked for 5 minutes by using Ethovision software. After 5 minutes, the rat droppings were counted and removed from the open field and the open field was cleaned with mild soapy water. All the animals were tested on the same day.

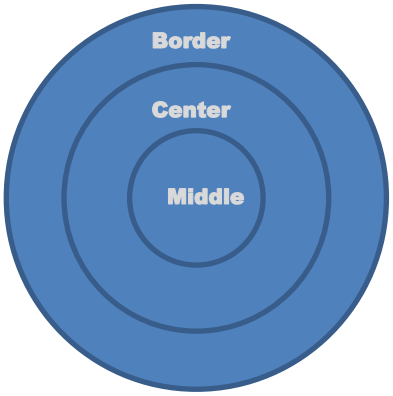


Figure 12 Open Field. The open field was divided in three zones: the center; middle ; and border.

Data were collected using Ethovision software. The open field was divided into three zones: the center, the middle and the border zone. The path of the animals was tracked and scored for the time spent these three different zones (Figure 12). Also the latency time for entering the zones was recorded. Furthermore the number of rat droppings was counted.

Elevated plus maze

All the animals performed the elevated plus maze in a random order. The elevated plus maze test was performed in a maze 50 cm above the ground with four arms in shape of a cross: two open arms and two closed arms with walls 40 cm of height. A 60 W lamp was placed at the side of a closed arm. The light intensity in the elevated plus maze was 40 - 65 lux measured in the open arm (Figure 13). The test animal was placed in the middle of the maze with the head faced in the right closed arm and video recorded for 5 minutes. After these 5 minutes the rat was taken out of the maze and put back in its home cage. The maze was cleaned with mild soap water before another rat was tested. Data were collected from video by using Eline software. Time spent in the open arms, closed arms and middle was measured. Also the number of crossings between the open arm, closed arm and middle was recorded.

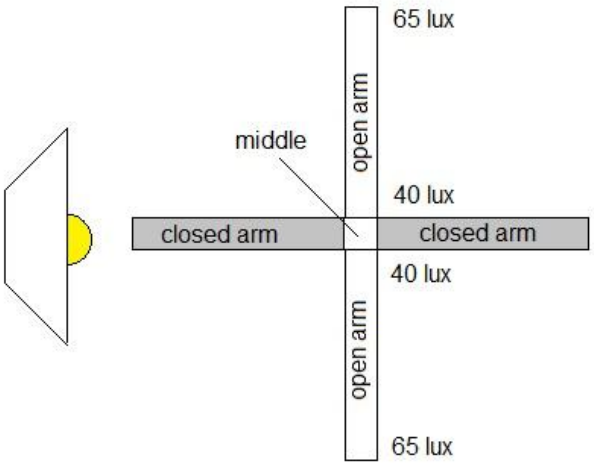


Figure 13 Top view of the elevated plus maze. A 60 W lamp placed at the side of a closed arm was used in the elevated plus maze. The light intensity was measured on the open arms: 40 lux near to the middle and 65 lux at the ends of the open arms.

Novel object recognition test

All the animals performed the novel object recognition (NOR) test in a random order. The NOR test was performed in a square field (72cmx72cmx40cm). A 60 W lamp was placed against one wall of the field. The light intensity in the field was 5 in the middle and 6 lux measured in the middle of the field and at the corners of the field. For orientation of the rats, the field was marked with graphic forms on the walls (Figure 14). The NOR test consists of 3 trials of 3 minutes. In the first trial is called the habituation phase, during this phase the animal could freely explore the field. After 3 minutes the animal was removed from the field and two object were places in two corners of the field, this is called the acquisition phase. The animal was placed back in the field. After another 3 minutes the animal and the objects were removed from the

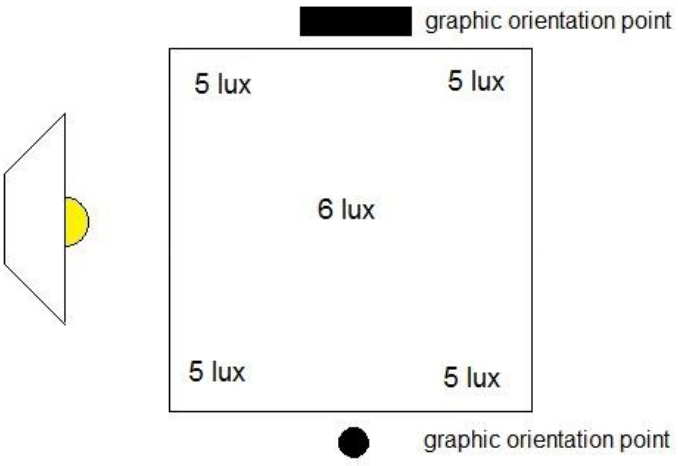


Figure 14 Top view of the novel and spatial object field. A 60 W lamp was placed against one wall of the field. The light intensity in the field was 5 lux measured in the center of the field and 4 lux measured in the corners of the field. Graphic orientation points are added to two sides of the open field, to enable the animals to orientate.

field. The 'familiar' objects were cleaned and one of the 'familiar' objects was placed back in the field together with a novel object replacing the other 'familiar' objects. The animal was again free to explore the field and the objects for 3 minutes, this is called the recollecting phase. After the last trial the field was cleaned and a new test animal was to perform the test (see also the attachment 'protocol Novel Object Recognition'). All the trials are recorded on video and scored, using Eline software, for 1. Exploring cage; 2. Exploring familiar object; 3. Exploring novel object; 4. Immobility and 5. Washing behavior (See attachment 'Protocol Novel & Spatial Object Recognition).

Spatial object recognition test

All the animals performed the spatial object recognition (SOR) test in a random order. The SOR test was performed in a square field (72cmx72cmx40cm). A 60 W lamp was placed against one wall of the field. The light intensity in the field was 5 in the middle and-6 lux measured in the middle of the field and at the corners of the field. For orientation of the rats, the field was marked with graphic forms on the walls (Figure 14). The SOR test consists of 3 trials of 3 minutes. In the first trial is called the habituation trial, the animal could freely explore the field. After 3 minutes the animal was removed from the field and two object were places in two corners of the field, called the acquisition phase. The animal was placed back in the field. After another 3 minutes the animal and the objects were removed from the field. The objects are cleaned. One of the objects was placed back on the same place and the other was moved to a different corner in the field, this is the recollecting phase. The animal could now again explore the field and the objects for 3 minutes. After the last trial the field is cleaned for a new test animal to perform the test (see also the attachment 'protocol Spatial Object Recognition'). All the trials are recorded on video and scored, using Eline software, for 1. Exploring cage; 2. Exploring same object; 3. Exploring moved object; 4. Immobility and 5. Washing behavior (See attachment 'Protocol Novel & Spatial Object Recognition).

Western blotting

After behavioral testing, western blots were performed to determine whether anti-estradiol treatment and MTX treatment altered spinophilin and synaptophysin levels in the hippocampi of female rats.

The rats were anesthetized on dry ice and were decapitated the day after the last behavioral tests took place. After decapitation the brains were quickly removed and both hippocampi were taken out. These hippocampi were then put in cups and were snap-frozen in liquid nitrogen. After this the cups were stored at -80°C until further use.

Tissue preparation

Because of time reasons, western blotting was only performed on the hippocampi of the first batch of animals. Tissue preparation was based on the protocol used by Brake *et al.* (77), with a few adjustments made. Hippocampal tissue was homogenized in ice-cold lysis buffer containing 0.32M sucrose, 2mM EDTA, 2 mM EGTA and 20 mM HEPES, along with protease inhibitors (trypsin inhibitor, 1 mg/mL; aprotinin, 7 mg/mL; pepstatin, 4 mg/mL). Tissues were further homogenized by 2 times 5 seconds sonification. Sonificated tissue was then centrifuged at 4˚C for 10 minutes at 800 g. Supernatants were collected (S1) and were again centrifuged at 4˚C at 21.000g for 30 minutes. Remaining pellets (P1) were not used. Thereafter, the supernatant of the centrifuged S1 (so S2) were removed and pellets (P2) were resuspended in 100mM PBS. The resuspended P2 pellets were again

centrifuged at 21.000g for 30 minutes. Supernatants (S3) were collected and pellets (P3) were again resuspended with 100mM PBS (pH 7.4) and stored at -80˚C.
Protein concentrations of the stored samples were determined the next day by using a Bradford assay. Protein concentration of the P3 of the two highest and lowest (hippocampal) weight samples were first determined, to determine the range of the amount of protein in the samples. For each of these samples three dilutions were made: 1) pure sample; 2) 5x diluted sample; 3) 10x diluted sample. This was done to see which dilution would fit best to get a protein density of about 1 mg/mL, since this is needed to get good results on the western blot. In this experiment it seemed best to use the pure samples. The pure P3 samples of each rat in batch 1 were then measured in a Bradford assay to determine the amount of protein in each sample. After this, each sample was standardized to contain 1.1 mg/mL protein tissue in a total amount of 200 mL per sample, by adding the needed amount of sample- and homogenization buffer to achieve this concentration. Furthermore, 40µl SDS was added and 4 equal aliquots of 50µl were made of each sample. Sample aliquots were stored at -80˚C until further use.

Protein assays

Protein assays were taken through gel electrophoresis. The proteins were separated by 10% gels (see protocol). After electrophoresis the proteins were transferred onto a PVDF membrane using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell followed by one hour of blocking at room temperature with 50% TBS-T + 50% Blocking buffer (see protocol) for actine and TBS-T containing 5% non-fat milk for spinophilin and synaptophysin. Thereafter, membranes were washed 3 times 5 minutes with TBS-T and then probed with 1:1.000.000 anti-actine; 1:1000 anti-spinophilin in TBS-T. The first anti-bodies were incubated overnight at 4°C. The next day, the membranes were washed again 3 times 5 minutes with TBS-T and then probed with the second anti-bodies: 1:5000 anti-mouse for actine and 1:5000 anti-rabbit for spinophilin. The second anti-bodies were incubated for 1 hour and then washed again 3 times 5 minutes with TBS-T. Now the membranes were taken to the ChemiDoc XRS+ system. Before taking the picture Pierce Detection Reagent 1 & 2 was added to the membrane 1:1. The membrane was clamped between two copy sheets before put into the ChemiDoc. Finally, the antibody-reactive bands were visualized using ChemiDoc XRS+ system. For synaptophysin the membranes used for measuring actine were reused by washing off the antibodies with stripping buffer. First the stock stripping was heated for about 5 minutes until clear. 20 mL of stripping buffer was pipetted out of the stock and was heated for 1.5 hour at 70°C in the hot water bath. The actine membrane was washed two times 15 minutes with 10 mL stripping buffer. After washing off the antibodies the membrane was again washed with 5 mL TBS buffer overnight. The next days the first (1:1000 anti-synaptophysin in TBS-T) and second anti-body (1:5000 anti-rabbit) could be added and incubated and pictured like ascribed above for spinophilin and actine.
The density of the amount of spinophilin, synaptophysin and actine on the blot was measured by ImageLab (Bio-Rad Laboratories Inc.). The amount of actine served as a control for the amount of protein present in the samples. The relative spinophilin and synaptophysin amounts were calculated by dividing their densities on the blot through the actine density in the corresponding sample (See also attachment ‘western blot’).

Statistics

Body weight were analyzed using two-way repeated measure ANOVA. Uterus weight differences were analyzed using two-way ANOVA. Significant differences between treatment groups in the behavior tests were analyzed using two-way ANOVA. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.

Results

Experiment A: Wistar

Body weights

Bodyweights were measured every day at the start of hormonal treatment. All rats show a slight increase in body weight, except for the animals treated with tamoxifen (1 mg/kg) (Figure 15). Animals treated with 1 mg/kg tamoxifen showed a significant ($p<0.001$) difference in weight gain over time. No significant difference is found in weight gain between the control, vehicle and anastrozole (0.1 mg/kg).

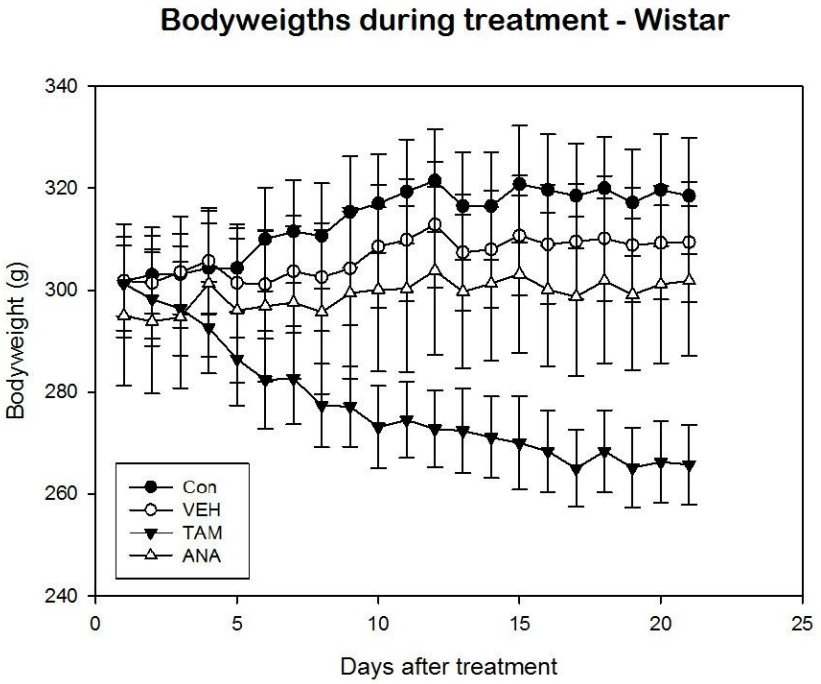


Figure 15 Bodyweights during treatment. Bodyweight change over time (in grams) in 21 days, starting at the first day of treatment with either vehicle (VEH) (n=7), tamoxifen (TAM) (n=8) or anastrozole (ANA) (n=7). Also is a control group (Con) (n=6) included, that did not receive any injections at all. Bars represent standard errors of the mean (S.E.M.). Rats receiving tamoxifen significantly lost weight over time ($p<0.001$), this in contrast to the other groups that gained weight.

Organ weights

Adrenal- and thyroid gland were weighted as measurement for stress induced in the animals, no significant difference was found (data not shown). Furthermore, uterus weights in animals treated with tamoxifen are significantly increased ($p<0.001$) (0,20g, ±S.E.M. 0,004) compared to animals treated with anastrozole (0,11g, ±S.E.M. 0,01) or vehicle (0,12g ±S.E.M. 0,01), demonstrating effectiveness of the treatment (Figure 16). Additionally, organ weights of the non-treatment control group did not differ significantly from the vehicle treated group (data not shown).

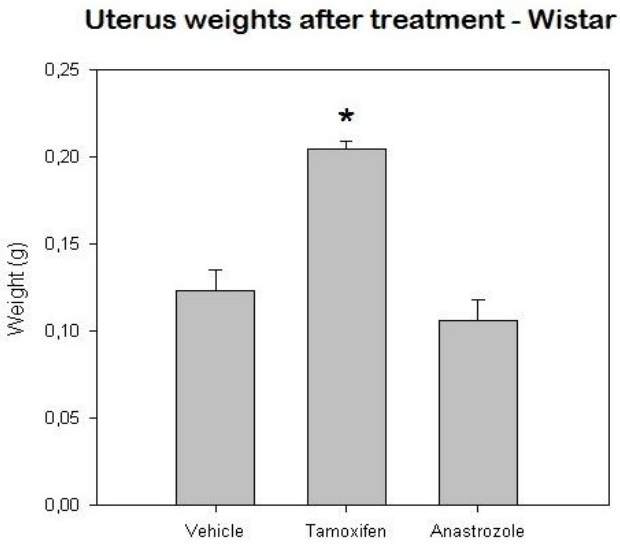


Figure 16 Uterus weights. Dry fat-free uterus weights after treatment with either vehicle (n=7), tamoxifen (n=8) or anastrozole (n=7), with the standard errors of the mean (S.E.M.). Uterus weights of tamoxifen treated animals are significantly higher ($p<0.001$).

Behavior testing

Four different behavior tests were conducted in the last week of hormonal treatment: the elevated plus maze; the open field; novel object recognition; and spatial object recognition.

Elevated plus maze

For anxiety measurement in the animals the elevated plus maze was conducted. Figure 17 shows the time animals spent in the open arm divided by the time they spent on the closed arm. It seems like animals treated with anastrozole spent less time in the open arm, however due to great variation this result was not significant.

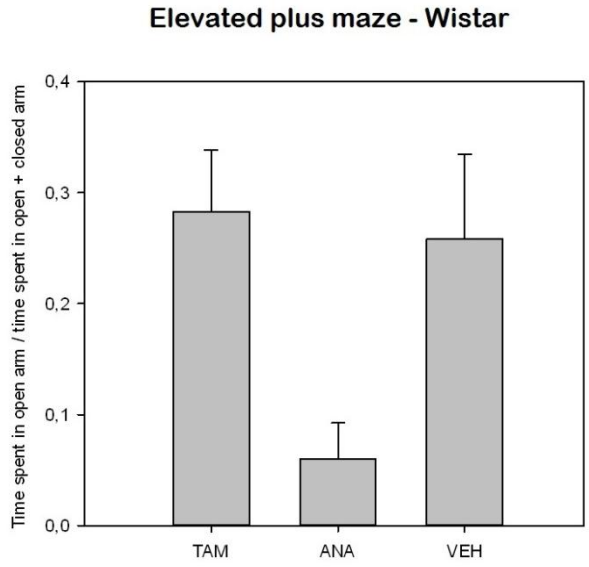


Figure 17 Elevated plus maze. Time the animals spent in the open arm of the elevated plus maze is shown relatively to the total time spent in the open and closed arms of the elevated plus maze. The time spent in the different arms was measured in animals either treated with tamoxifen (TAM) (n=8), anastrozole (ANA) (n=7) or vehicle (VEH) (n=7). Thought animals treated with anastrozole seem to spent less time in the open arm, no significant difference was found.

Modified open field test

Additionally, the modified open field was conducted to measure anxiety in the animals. The animals were introduced in the middle of the open field, the latency of the animals to leave this zone towards the other two zones: the center and the border, is depicted in Figure 18a. The treatment groups do not differ in their latency towards the different zones. In Figure 18b the total time the animals spent

in the tree different zones is shown. No effect of hormonal treatment is found in the time animals spent in different zones.

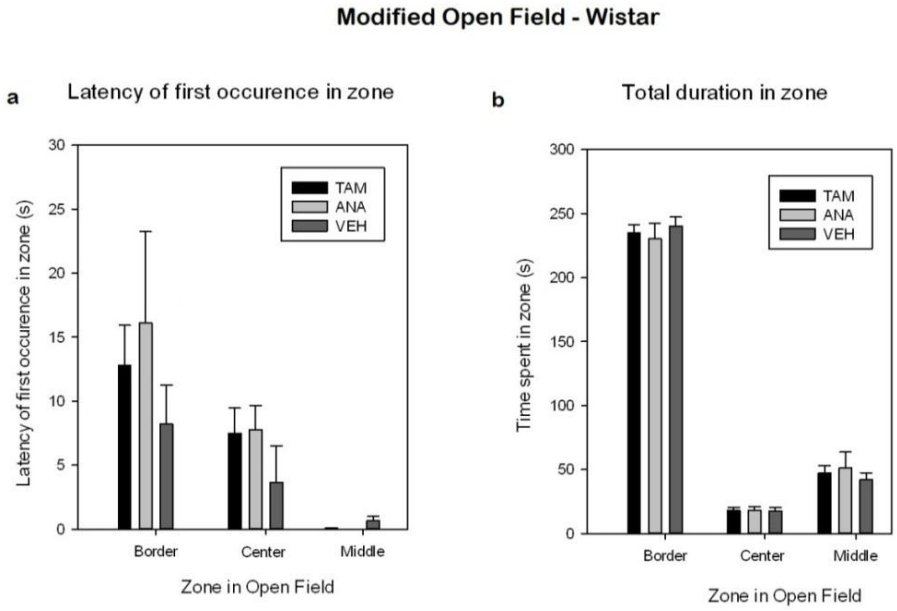


Figure 18 Open field. Results from the open field test, split up in the three different zones of the open field: border, center and middle. Measurement were taken from rats treated with either tamoxifen (TAM) (n=8), anastrozole (ANA) (n=7) or vehicle (VEH) (n=7). a) shows the latency of first occurrence (in seconds) in the three different zones. No differences were found between the groups. b) shows the total time (in seconds) the animals spent in the three different zones. Error bars represent standard error of the mean (S.E.M.). The groups did not differ significantly from each other.

Novel object recognition test

To measure memory and attention the novel object recognition test was conducted. Figure 19 shows the time animals spent on the novel object divided by the total they spent on both objects times 100. This demonstrates the ability of the animals to discriminate between the novel and the familiar object. A score higher than 50 means the animal spent more time on the novel object. All treatment groups spent about 80% at the novel objects. There was also no significant difference in total exploration, immobility or washing behavior between the groups (data not shown).

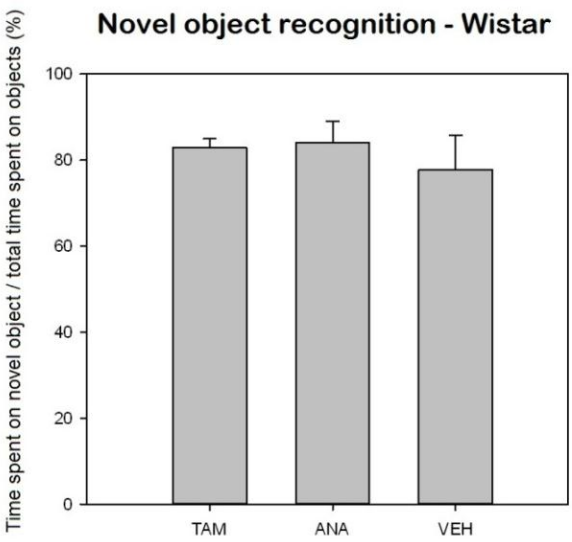


Figure 19 Nobel object recognition. Time the animals spent on the novel object is shown relatively to the total time the animals spent on both objects. The percentage time spent on the novel object was measured in animals either treated with tamoxifen (TAM) (n=8), anastrozole (ANA) (n=7) or vehicle (VEH) (n=7). No significant difference was found between the groups.

Spatial object recognition test

Finally, the spatial object recognition test was conducted to test memory and attention, particularly hippocampal memory was measured in this task. Figure 20 shows the time animals spent on the moved object divided by the total they spent on both objects times 100. This demonstrates the ability of the animals to discriminate between the moved and the familiar object. A score higher than 50 means the animal spent more time on the moved object. All treatment groups seem to spent at least 50% at the moved object. Tamoxifen treated animals seem to spent the most time on the moved object compared to the anastrozole and vehicle treated animals, however no significant difference was found. There was also no significant difference found in total exploration, immobility or washing behavior between the groups (data not shown).

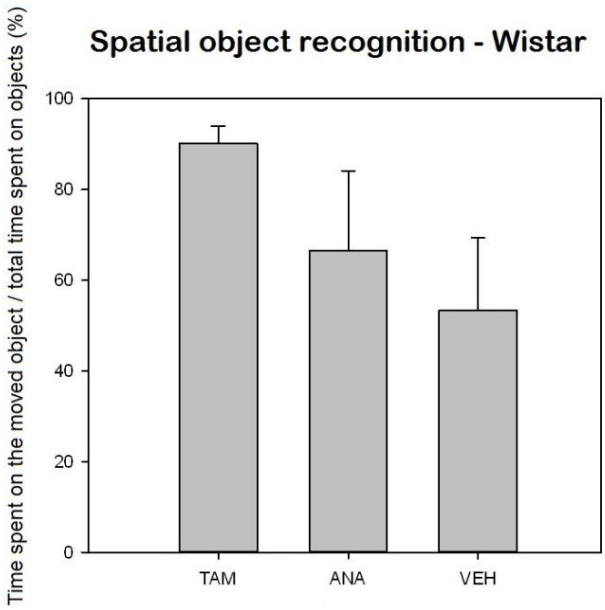


Figure 20 Spatial object recognition. Time the animals spent on the moved object is shown relatively to the total time the animals spent on both objects. The percentage time spent on the moved object was measured in animals either treated with tamoxifen (TAM) (n=8), anastrozole (ANA) (n=7) or vehicle (VEH) (n=7). No significant difference was found between the groups.

Experiment B: Wildtype Groningen

Body weights

Bodyweights were measured every day at the start of hormonal treatment. All rats show a slight increase in body weight, except for the animals treated with tamoxifen (2 mg/kg) (Figure 21). Animals treated with 2 mg/kg tamoxifen showed a significant ($p<0.001$) difference in weight gain over time (Figure 21). Treatment with methotrexate seemed to induce weight loss, however this effect in body weight was not significant (Figure 22). Neither was there an effect on bodyweight with hormonal treatment (data not shown).

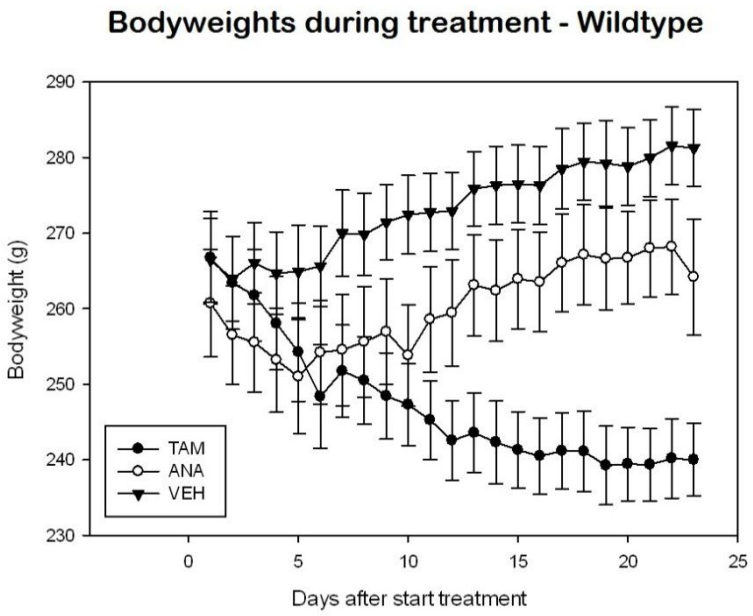


Figure 21 Bodyweights during treatment. Bodyweight change over time (in grams) in 23 days, starting at the first day of treatment with either vehicle (VEH) (n=16), tamoxifen (TAM) (n=16) or anastrozole (ANA) (n=15). Rats receiving tamoxifen significantly lost weight over time ($p<0.001$), this in contrast to the other groups which gained weight.

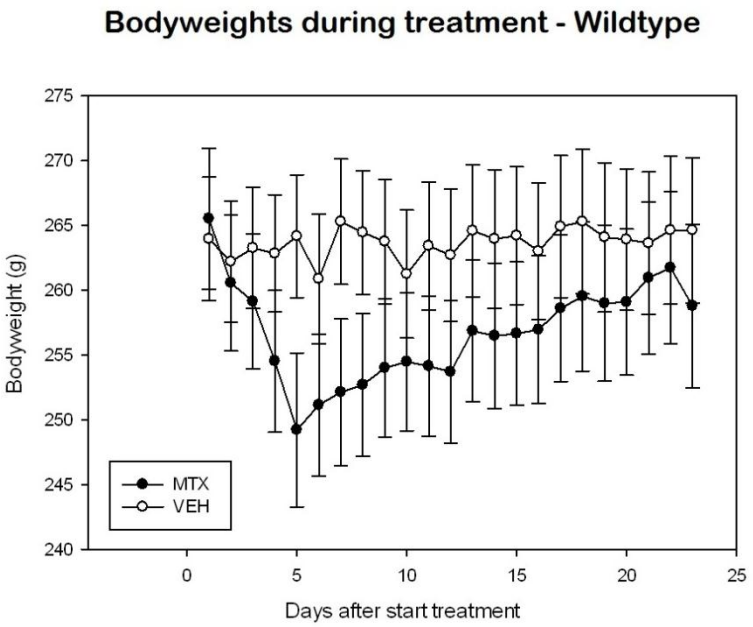


Figure 22 Bodyweights during treatment. Bodyweight change over time (in grams) in 23 days, starting at the first day of treatment with either methotrexate (MTX) (n=23) or vehicle (VEH) (n=24) Rats receiving MTX lost more weight over time compared to animals receiving vehicle, however this difference was not significant over time.

Uterus weights

Uterus weights were weighted as measurement for effectiveness of the hormonal treatment. In animals treated with tamoxifen are significantly increased ($p<0.001$) (0.12g, ±S.E.M. 0,005) compared

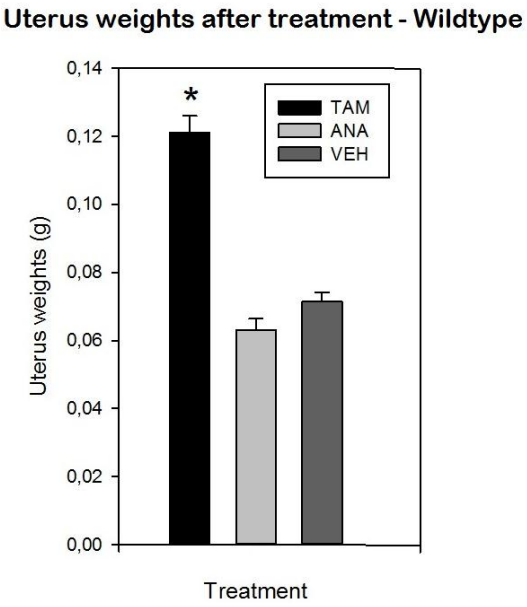


Figure 23 Uterus weights. Dry fat-free uterus weights after treatment with either vehicle (n=16), tamoxifen (n=16) or anastrozole (n=15). Uterus weights of tamoxifen treated animals are significantly higher ($p<0.001$).

to animals treated with anastrozole (0,063g, ±S.E.M. 0,003) or vehicle (0,072g ±S.E.M. 0,003), demonstrating tamoxifen did have an effect in the body (Figure 23).

Behavior testing

Four different behavior tests were conducted in the last week of hormonal treatment: the elevated plus maze; the open field; novel object recognition; and spatial object recognition.

Elevated plus maze

For anxiety measurement in the animals the elevated plus maze was conducted. Figure 24 shows the time animals spent in the open arm divided by the time they spent on the closed arm. The treatment groups do not differ in time they spent in the open arm. Additionally, no interaction effect of methotrexate on hormonal therapy was found (data not shown).

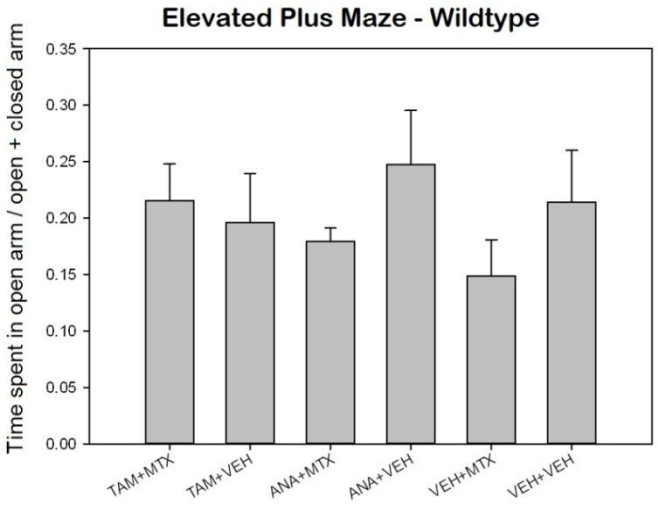


Figure 24 Elevated plus maze. Time the animals spent in the open arm of the elevated plus maze is shown relatively to the total time spent in the open and closed arms of the elevated plus maze. The time spent in the different arms was measured in animals either treated with tamoxifen and methotrexate (TAM+MTX) (n=8); tamoxifen and vehicle (TAM+VEH) (n=8); anastrozole and methotrexate (ANA+MTX) (n=7); anastrozole and vehicle (ANA+VEH); vehicle and methotrexate (VEH+MTX) (n=8); or vehicle and vehicle (VEH+VEH) (n=8). No significant difference between the groups was found.

Modified open field test

Additionally, the modified open field was conducted to measure anxiety in the animals. The animals were introduced in the border of the open field, the latency of the animals to leave this zone towards the other two zones: the center and the middle, is depicted in Figure 25a. The treatment groups do not differ in their latency towards the different zones. In Figure 25b the total time the animals spent in the tree different zones is shown. No effect of hormonal treatment is found in the time animals spent in different zones. Likewise, no effect was found when dividing the groups into animals receiving methotrexate and vehicle (data not shown). Nor was there an interaction effect of methotrexate on the hormonal treatment found with the open field test (data not shown).

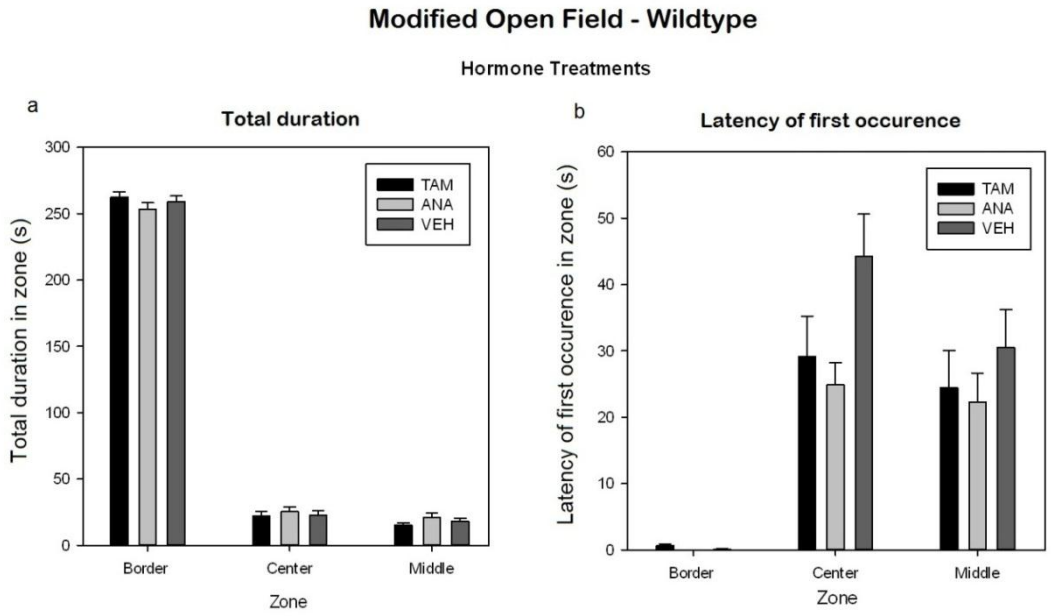


Figure 25 Open field. Results from the open field test, split up in the three different zones of the open field: border, center and middle. Measurement were taken from rats treated with either tamoxifen (TAM) (n=16), anastrozole (ANA) (n=15) or vehicle (VEH) (n=16). a) shows the latency of first occurrence (in seconds) in the three different zones. No differences were found between the groups. b) shows the total time (in seconds) the animals spent in the three different zones. No differences were found between the groups.

Novel object recognition test

To measure memory and attention the novel object recognition test was conducted. Figure 26 shows the time animals spent on the novel object divided by the total they spent on both objects times 100. This demonstrates the ability of the animals to discriminate between the novel and the familiar object. A score higher than 50 means the animal spent more time on the novel object. All treatment groups spent about 60% at the novel object, except for the group treated with tamoxifen and methotrexate, they seem to spent about 80% of the time at the novel object, but this difference was not significant. No significant difference in total exploration, immobility or washing behavior between the groups was found either (data not shown).

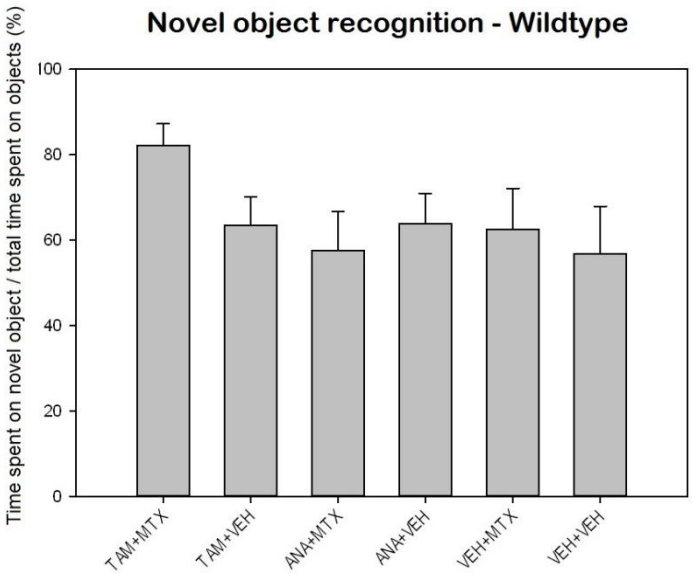


Figure 26 Novel object recognition. Time the animals spent on the novel object is shown relatively to the total time the animals spent on both objects. The percentage time spent on the novel object was measured in animals either treated with either tamoxifen and methotrexate (TAM+MTX) (n=8); tamoxifen and vehicle (TAM+VEH) (n=8); anastrozole and methotrexate (ANA+MTX) (n=7); anastrozole and vehicle (ANA+VEH); vehicle and methotrexate (VEH+MTX) (n=8); or vehicle and vehicle (VEH+VEH) (n=8). No significant difference between the groups was found.

Spatial object recognition test

Finally, the spatial object recognition test was conducted to test memory and attention, particularly hippocampal memory was measured in this task. Figure 27 shows the time animals spent on the moved object divided by the total they spent on both objects times 100. This demonstrates the ability of the animals to discriminate between the moved and the familiar object. A score higher than 50 means the animal spent more time on the moved object. The groups did not differ significantly from each other, even though it may seem that animals treated with anastrozole spent a bit less time to the moved object compared to the other groups. When comparing the groups based on hormonal therapy no significant difference was found either, nor when comparing the groups based on methotrexate administration (data not shown). Time spent in total exploration, immobility or washing behavior between the groups also did not show significant differences (data not shown).

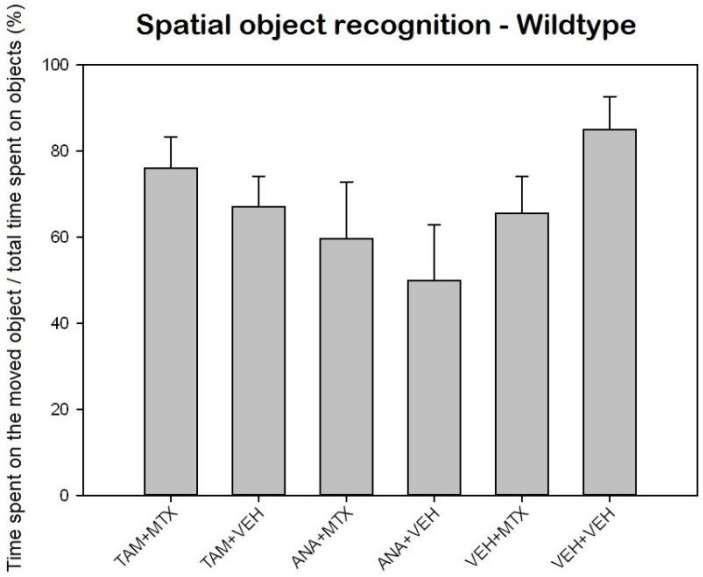


Figure 27 Spatial object recognition. Time the animals spent on the moved object is shown relatively to the total time the animals spent on both objects. The percentage time spent on the moved object was measured in animals either treated with either tamoxifen and methotrexate (TAM+MTX) (n=8); tamoxifen and vehicle (TAM+VEH) (n=8); anastrozole and methotrexate (ANA+MTX) (n=7); anastrozole and vehicle (ANA+VEH); vehicle and methotrexate (VEH+MTX) (n=8); or vehicle and vehicle (VEH+VEH) (n=8). No significant difference between the groups was found.

Western blotting

Directly after three weeks of hormonal treatment the animals were sacrificed and the brains were removed from the animals. Hippocampal tissue was used for analyses with Western blotting. Two protein were measured: spinophilin and synaptophysin.

Spinophilin

Spinophilin was measured in the animals and indicates the relative amount of spines in the hippocampal tissue. Figure 29 shows the values of spinophilin divided by the values of actin. In performing western blotting with spinophilin, two bands were detected. The first band was the biggest, probably accounting for spinophilin as a whole. The second band was less dense and probably represents degradation products of spinophilin. The results were split up into: all treatments; chemo treatment; or hormone treatment. When divided in ''all treatments'' no difference was found between the groups. Division by ''chemo treatment'' showed a slightly bigger amount of spinophilin in the first band for the methotrexate group, however due to large variation this was not significant. Finally, dividing by ''hormonal treatment'' suggests tamoxifen treated animals have a higher amount of spinophilin compared to anastrozole and vehicle treated animals, seen in both band 1 and band 2. Looking only at band 2 also suggests for a decrease in spinophilin in

anastrozole treated animals compared to vehicle treated animals. However, probably due to the small sample size (n=24) non of these differences were found significant. (See attachment 'western blot performance' for pictures)

Synaptophysin

Synaptophysin was measured in the animals and indicates the relative amount of synapses in the hippocampal tissue. Figure 29 shows the values of synaptophysin divided by the values of actin. The results were split up into: all treatments; chemo treatment; or hormone treatment. When divided in ''all treatments'' no difference was found between the groups, nor did division by ''chemo treatment'' or ''hormone treatment''. It may also be due to the small sample size (n=24) no significant differences were found. (See attachment 'western blot performance' for pictures)

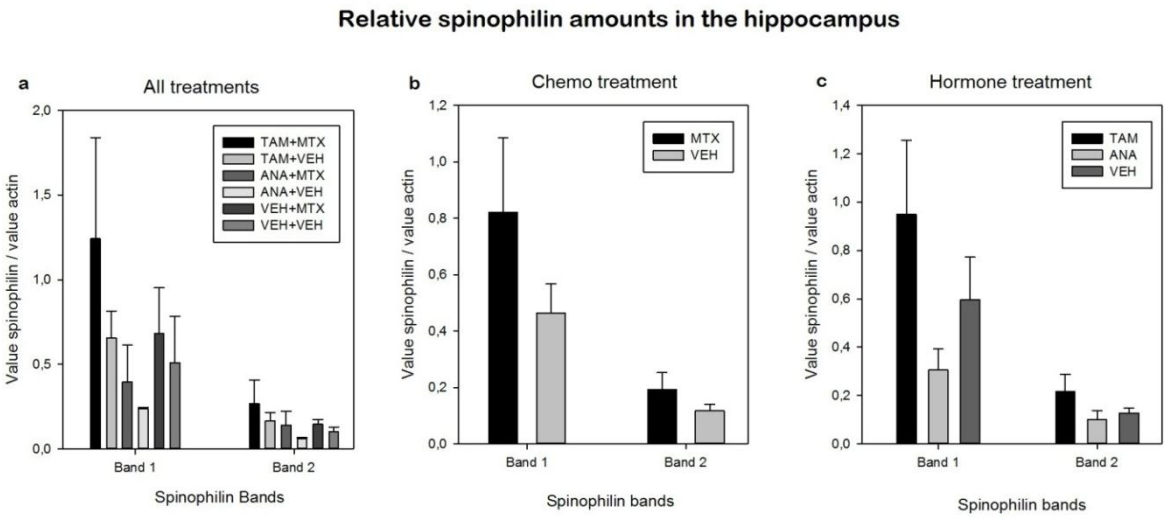


Figure 29 Relative amounts of spinophilin in the rat hippocampus. Spinophilin amounts are shown relatively to actine amounts. Two bands were found trough detection of spinophilin: band 1 has the highest molecular weight; band 2 is found at a smaller molecular weight. a) The amounts of spinophilin is shown, divided in animals treated with either tamoxifen and methotrexate (TAM+MTX) (n=4); tamoxifen and vehicle (TAM+VEH) (n=4); anastrozole and methotrexate (ANA+MTX) (n=4); anastrozole and vehicle (ANA+VEH) (n=4); vehicle and methotrexate (VEH+MTX) (n=3); or vehicle and vehicle (VEH+VEH) (n=4). No significant difference between the groups was found. b) The amounts of spinophilin is shown, divided in animals treated with either methotrexate (MTX) (n=11) or vehicle (n=12). Animals treated with methotrexate seem to show higher amount of spinophilin in the first band, but no significant difference between the groups was found. c) The amounts of spinophilin is shown, divided in animals treated with either tamoxifen (TAM) (n=8); anastrozole (n=8) or vehicle (n=7). Animals treated with tamoxifen seem to show the highest amount of spinophilin in both bands and anastrozole treated animals seem to have lower amounts of spinophilin seen in the first band. However, no significant difference between the groups was found.

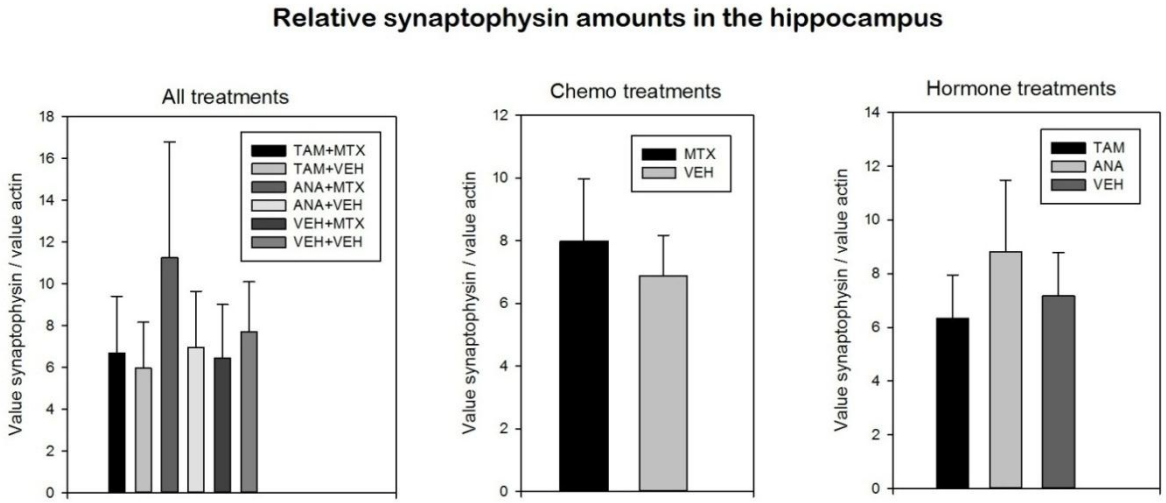


Figure 28 Relative amounts of synaptophysin in the rat hippocampus. Synaptophysin amounts are shown relatively to actine amounts. a) The amounts of synaptophysin is shown, divided in animals treated with either tamoxifen and methotrexate (TAM+MTX) (n=4); tamoxifen and vehicle (TAM+VEH) (n=4); anastrozole and methotrexate (ANA+MTX) (n=4); anastrozole and vehicle (ANA+VEH) (n=4); vehicle and methotrexate (VEH+MTX) (n=3); or vehicle and vehicle (VEH+VEH) (n=4). No significant difference between the groups was found. b) The amounts of synaptophysin is shown, divided in animals treated with either methotrexate (MTX) (n=11) or vehicle (n=12). No significant difference between the groups was found. c) The amounts of synaptophysin is shown, divided in animals treated with either tamoxifen (TAM) (n=8); anastrozole (n=8) or vehicle (n=7). No significant difference between the groups was found.

Conclusion and discussion

This study consisted of two different experiments: experiment A with Wistar rats; and experiment B with Wildtype Groningen rats. The effect of hormonal treatment with tamoxifen on anxiety and cognition in Wistar rats was examined in experiment A. Experiment B was designed to investigate the effect of tamoxifen in combination with methotrexate on anxiety and cognitive function. In both experiment A and B no significant effects on anxiety and cognition after treatment with tamoxifen and / or methotrexate could be found.

Experiment A: Wistar

In experiment A anxiety and cognitive effects after 3 weeks treatment with tamoxifen in female OVX Wistar rats were measured through four different behavior tests: the elevated plus maze; the open field; the novel object recognition; and the spatial object recognition. In all four tests no effect of tamoxifen treatment could be found.

In elevated plus maze the time the rats spent in the open arm was divided by the time they spent on the closed arm. The animals are thought to feel more save in the closed arms compared to the open arms, but rats are also thought to be curious and explorative. So an anxious animal is thought to spend more time in the closed arm compared to a less anxious animal. Animals treated with vehicle or animals treated with tamoxifen (1 mg/kg) both spent about the same relative time in the open arms. This suggest tamoxifen does not induce anxiety in female ovariectomized rats.

Also in the open field no effect of tamoxifen on anxiety was found. The time spent in each zone, respectively the center; the middle; and the border, and the latency of first occurrence into each zone was measured. In the open field the border of the open field is thought to be the most safest area for the rats, since it is confined by the wall. However, we placed an object in the center area to provoke the curiosity of the animals. So very anxious animals are thought to spend more time in the save border areas, while less anxious animals are thought the go more to the center area to explore the object. Also the latency times towards the central areas are expect to be higher in anxious animals compared to less anxious animals.
In this study the different treatment groups did in their time spent in the different zones or the latency towards these zones, indicating that anxiety levels did not differ between the treatment groups. Additionally, the number of droppings were counted as a measure of anxiety, but did not differ either. Together with the findings out of the elevated plus maze, this supports the idea that tamoxifen does not induce anxiety. This also implies variation in anxiety between the treatment groups could not have an effect on the cognitive tasks performed later on.

In literature, tamoxifen is found to block the anti-anxiety effects of ERβ selective anxiolytic compounds seen in both the open field and the elevated plus maze. Tamoxifen is thought to block anxiolytic behavior by antagonizing ERβ (78, 79). However, tamoxifen treated animals did not differ in anxiety compared to vehicle treated animals (78), meaning that only when an anxiolytic substance is administered first, tamoxifen can increase or normalize the level anxiety. In this study no ERβ anxiolytic substance was administered to animals beforehand, therefore the results are consistent with the literature, tamoxifen does not induce anxiety per se.

Cognitive function was measured through the novel object recognition and spatial object recognition. In the novel object recognition no significant difference was found in cognitive function. The amount of time spent on the novel object was divided by the total time spent on both objects times 100 was calculated. The animals were thought to correctly recognize the objects if more time was spent on the novel object, since they should already know the familiar object and therefore the novel object should be more interesting. So if cognition or memory is impaired in an animal, this animal is thought to perform worse on the novel object recognition and spent about the same time on novel as on the familiar object, since it does not recognize the familiar object.
Animals treated with tamoxifen did not differ from animals treated with vehicle in time they spent on the novel object. Both groups spent about 80% of their time on the novel object, indicating both groups were well able to discriminate between the novel and the familiar object. Most time was spent on the novel object, knowing that rats are more curious towards novel objects, it can be concluded that both groups did not have difficulty recognizing and remembering the familiar object. In other words, cognitive function measured by the novel object recognition was neither impaired in vehicle- nor in tamoxifen treated animals.

Likewise, in the spatial object recognition no effect on cognitive function was induced by tamoxifen. In this test the amount of time spent on the moved object divided by the total time spent on both objects times 100 was calculated. In this test the animal was thought to perform correctly if more time was spent on the moved object, since the familiar object was not interesting anymore. If an animal spends about the same amount of time to both objects, this indicates that the animal is not able to recognize the object being moved which may be due to cognitive impairment.
Tamoxifen treated animals seemed to spent more time on the moved object compared to vehicle treated animals. However, this difference was not significant, due to large variation. Besides, the animals spent most of their time to washing behavior instead of exploration of the objects, therefore the results may be distorted: a lot of animals did not spent more than 3% of the total time of 3 minutes on the objects and therefore were excluded. This resulted in a smaller sample size. So the sample size of this experiment was small and the variance was big, which makes it difficult to get significant results. Concluding, the spatial object recognition did not demonstrate an effect on cognition in tamoxifen treated animals versus vehicle treated animals.

Nevertheless, clinical studies suggest a role for tamoxifen in cognition. Unfortunately pre-clinical data about the effect of tamoxifen on cognition and memory in rats is scarcely available and show contradicting outcomes. Velázquez-Zamora *et al*. (80) found tamoxifen to have a positive effect in rats on learning in the Y-maze. However, this test is thought to measure working memory, while the novel object recognition and spatial object recognition the way conducted in this study is thought to measure short term memory. They additionally demonstrated tamoxifen to mimic estradiol effect on spine density in the prefrontal cortex. However, whereas estradiol specifically increased the numerical density of thin dendritic spines in this study, tamoxifen increased density of all spine types similarly. This small difference may be the reason why tamoxifen did not mimic estrogen effects on cognition in the present study.
However, negative effects of tamoxifen on cognition have also been proposed. A study of Sumner *et al*. (54) suggested the serotonergic system to be an important system in cognition and found tamoxifen to be a pure estradiol antagonist on the serotonergic mechanisms in the brain. Therefore tamoxifen was thought to block the positive effects of estradiol via the serotonergic system found in

the brain. Additionally, O'neil *et al*. (81) suggested tamoxifen to act in the opposite direction from estradiol's protective effects on memory. They found estrogen to stimulate neuronal outgrow in the cortex, hippocampal and basal forebrain neurons, but these neurotropic effects were not found after administration with tamoxifen. Hence O'Neil *et al*. concluded tamoxifen not to have the same memory protective effects as estradiol. Thus, studies do not show a clear picture of the effects of tamoxifen on cognition and memory. Hence the findings of the present study adds to the literature showing tamoxifen does not mimic estradiol in the effect on cognition and memory in rats.

Taken altogether, this demonstrates tamoxifen treatment does not affect cognition or anxiety. It should be noted that some imperfections run into this experiment. First of all, a lot of animals were lost in because of infections due to the OVX operations, therefore the number of animals was greatly reduced. Secondly, the administration of hormonal therapy went via a cannula, but in some animals this cannula got blocked so the treatment had to be administered IP. Furthermore, some cannula's were partially blocked, as a result the exact amount of medicine administered to the animals could not be measured. However, the uterus weights of tamoxifen treated animal were almost doubled compared to the uterus weights of vehicle treated animals, suggesting tamoxifen did have an effect in the body. In post-menopausal women tamoxifen is also found to double the endometrial thickness (81- 83). Also studies done in rats show increased uterus weights after treatment with tamoxifen (84-86). Moreover, significant body weight reduction was demonstrated in the present study, which is also reported as an effect of tamoxifen in other studies (83, 87, 88). This weight loss suggests an estrogen-like role for tamoxifen in body weight regulation. Estrogen is found to cause weight loss in ovariectomized rats on an high fat diet by altering adipocyte size (89, 90). Thus increased uterus weights and decreased bodyweights in tamoxifen treated animals indicate tamoxifen definitely did have an effect on the animals, nonetheless the precise amounts administered cannot be verified.

Finally, the novel- and spatial object recognition might better be conducted divided over two days, instead of directly behind each other on one day. During the fourth trial the animals showed a lot of washing behavior during the spatial object recognition task and almost no attention was paid to the objects anymore. Therefore, the results from this experiment should be looked at with suspicion. By dividing the two tests over two days the animals have to perform only three trials a day, instead of four, which might result in more attention spent towards the objects during the last trial. Moreover, these behavior tests may not be the best suitable for testing the reported cognitive impairments seen in clinical studies, while the most reported impairments were found in verbal memory and processing speed (19, 21, 22, 55, 91-98). It remains difficult to test verbal memory and processing speed in an animal model, but a more demanding learning task may induce more clear differences between treatment groups.

<u>Experiment B: Wildtype Groningen</u>

In experiment B the effect of hormonal treatment with tamoxifen in combination with methotrexate in Wildtype Groningen rats was investigated. No effects on anxiety and cognitive behavior were found after administration of tamoxifen either with or without methotrexate. Nor were morphological effects on spine density and synapse density in hippocampal tissue found after treatment with tamoxifen and or methotrexate.

Firstly, anxiety behavior measured in the elevated plus maze was not affected by tamoxifen either with or without methotrexate. In this test time spent in the open arm was divided by the time the rats spent on the closed arm. The animals are thought to feel more save in the closed arms compared to the open arms, but rats are also thought to be curious and explorative. So an anxious animal is thought to spent more time in the closed arm compared to a less anxious animal. Time spent in the different arms did not differ between the different treatment groups; neither tamoxifen nor methotrexate has an impact on anxiety in the elevated plus maze.

Secondly, no effect of tamoxifen with or without methotrexate on anxiety was seen in the open field. The time spent in each zone, respectively: the center; the middle; and the border, and the latency of first occurrence into each zone were measured. In the open field the border of the open field is thought to be the most safest area for the rats, since it is confined by the wall. However, we placed an object in the center area to provoke the curiosity of the animals. So very anxious animals are thought to spent more time in the save border areas, while less anxious animals are thought the go more to the center area to explore the object. Also the latency times towards the central areas are expect to be higher in anxious animals compared to less anxious animals.
Both time spent and latency towards the different zones did not differ between different treatment groups, indicating there was no difference in anxiety levels between the groups. Additionally, the number of droppings were counted as a measure of anxiety, but these did not differ either between the different treatment groups. So, also in the elevated plus maze neither tamoxifen nor methotrexate had an impact on anxiety.
As discussed above, in previous research tamoxifen is not shown to have an obvious effect on anxiety per se, this is again supported by the results of experiment B. The effects of methotrexate on anxiety on the other hand are not yet investigated in animals. Some clinical studies investigated the effect of methotrexate towards the quality of life, including anxiety, but demonstrate contradicting effects; a clinical study by Kornblith *et al.* looked at the quality of life in patients receiving capecitabine compared to standard chemotherapy, including methotrexate. They found a three times higher percentage of clinically important anxiety and depression in patients treated with methotrexate compare to patients treated with capecitabine (99). Another study by Groenvold *et al.* did not find a difference in anxiety between BC patients treated with ovarian ablation and patients treated with methotrexate (100). Besides, anxiety in BC patient can also be due to expectations of the treatment and its side effects. Hence, it could be that anxiety seen in patients treated with methotrexate is more based on expectations, since rats are not likely to have expectations this will not be measured in rats.

Furthermore, no effect of tamoxifen and or methotrexate on cognition was measured in the novel object recognition test. The amount of time spent on the novel object divided by the total time spent on both objects times 100 was calculated. The animals were thought to correctly recognize the objects if more time was spent on the novel object, since they should already know the familiar object and therefore the novel object should be more interesting. So if cognition or memory is impaired in an animal, this animal is thought to perform worse on the novel object recognition and spent about the same time on novel as on the familiar object, since it does not recognize the familiar object.
All animals spent about 60% of the time on the novel object. Only the animals treated with tamoxifen and methotrexate spent a bit more time on average on the objects, about 80%, which could indicate

tamoxifen to have a slightly beneficial effect on cognition. Nevertheless, since this difference was not significant no difference between the treatment groups or interaction effect with methotrexate could be concluded. Thus, treatment with tamoxifen or methotrexate did not affect cognition in the novel object recognition.

Also in the spatial object recognition no effect of treatment on cognition was found. For this test the amount of time spent on the moved object was divided by the total time spent on both objects times 100 was calculated. In this test the animal was thought to perform correctly if more time was spent on the moved object, since the familiar object was not interesting anymore. If an animal spends about the same amount of time to both objects, this indicates that the animal is not able to recognize the object being moved which may be due to cognitive impairment.
All treatment groups spent about 70% of the time on the novel object, showing all treatment groups were able to recognize the moved object, which indicates no cognitive impairment was induces in any treatment group.

To conclude, nor tamoxifen nor treatment with methotrexate had an effect on cognition in OVX Wildtype Groningen rats. As mentioned above in experiment A, only a few animal about the effect of tamoxifen on cognition studies are published and the results of these studies are not corresponding. The same goes for methotrexate, clinical studies suggests a negative effect of methotrexate on cognition, however pre-clinical research is sparsely available. Nonetheless, the few pre-clinical mainly show a negative effect of methotrexate on cognition. Seigers *et al.* (11) found a dose-dependent negative effect of methotrexate on hippocampal cell proliferation and cognitive behavior in rats and impaired spatial memory in the morris water maze in animals treated with methotrexate. On top of that, animals treated with methotrexate were also found to perform worse in the object recognition test. This was confirmed in a study of Fardell *et al.*, where methotrexate treated animals performed worse in the novel object recognition (101). However, in line with the findings of the present study, Gandal *et al.* did not find an effect of methotrexate on novel object recognition performance. This inconsistency between the different studies may be due to procedural differences; in the present study rats were administered a single high dose of methotrexate at 250 mg/kg IP, whereas in the study of Seigers *et al.* methotrexate (250 mg/kg) was given intravenous. Intravenous injection may cause a higher toxicity effect, because the high dose of methotrexate is directly introduced into the bloodstream. Nevertheless, in the study of Fardell *et al.* they also found a negative effect of methotrexate on performance in the novel object recognition test with a single high dose of methotrexate at 250 mg/kg IP. Another procedural difference was the latency time between the acquisition phase and the recollecting phase; in the present study this latency time was only 1 minute, while both Seigers *et al.* and Fardell *et al.* used a latency time of 1 hour. This latency time may be necessary to detect impairment in the memory and learning process in the hippocampus and may explain the lack of significant differences found in the present study.

Finally, western blot analysis on spinophilin and synaptophysin in hippocampal tissue did not show any effect of tamoxifen or methotrexate on the spine- or synapse density. Spine density was measured by detection of spinophilin. No significant differences between the different treatment groups were found, which suggests neither tamoxifen nor methotrexate had an effect on spine density in the rat hippocampus. Noteworthy, tamoxifen treated animals seemed to show higher levels of spinophilin compared to vehicle treated animals, indicating higher spine density for

tamoxifen treated animals. Since estrogens are known to increase spine density in hippocampal tissue (102), this would suggest for an estrogen-like effect of tamoxifen in rat hippocampal tissue. This is also supported be the previous described increase in spine density induced by tamoxifen (54). However, no previous literature shows the effect of tamoxifen or methotrexate on spinophilin particularly to confirm or argue the findings in this study. Moreover, due to a small sample size no significant effect were found, but results so far are promising and are calling for sequential research with bigger sample sizes.

Pre-synaptic density was measured by detecting synaptophysin. No difference was seen between the different treatment groups. Concluding, neither tamoxifen nor methotrexate had an effect on pre-synaptic density in hippocampal tissue in rats.

No literature describes the effects of methotrexate on synaptophysin and only one study investigated synaptophysin levels after tamoxifen treatment. Tamoxifen (0.05 mg/kg) was demonstrated to increase synaptophysin levels after ovariectomy at the same rate estrogen did (74). This outcome supports the suggestion that tamoxifen mimics the effects of estrogen on the brain. However, the present study was not able to show this effect, so maybe the estrogen-like effect of tamoxifen on the brain is not that evident. The different outcomes between the two studies may also be due to the way of administration. In the study of Sharma *et al.* the animals were injected with 0.05 mg/kg tamoxifen subcutaneously daily for a period of four weeks, while in the present study the animals were injected IP with 2 mg/kg daily for a period of three weeks. It could be that subcutaneous injections are more effective, or that a longer period of administration is necessary to see effects of tamoxifen. However, in the present study a much higher amount of tamoxifen was used, which is expected to induce more extreme effects. Yet, the small sample size used in the present study for western blot analyses could also explain the lack of significant differences between the groups. Hence, more research with bigger sample sizes are needed to clarify the effects of tamoxifen and / or methotrexate on the synapse density measured by synaptophysin. Concluding from this study neither tamoxifen nor methotrexate affects synaptophysin levels in hippocampal tissue.

Furthermore, the age of the animals may also have an impact on the brain and behavior of the animals after administration with either tamoxifen or methotrexate. Several studies show different effects of estrogen replacement over age. 17beta-estradiol is found to regulate the blood-brain barrier differently in young versus older animals; estrogen replacement decreased the barrier permeability in the hippocampus of younger females, but increased the barrier permeability in older acyclic female rats (103). Since the animals used in this study were fairly young, a decreased permeability of the blood-brain barrier may cause a tempered impact of tamoxifen and methotrexate on the brain. Moreover, Selvamani *et al.* demonstrated larger cortical and striatal infarcts in reproductive senescent female rats after estrogen replacement, while estrogen treatment in mature adults showed a reduced cortical infarct size compared to their age matched controls (104). Since tamoxifen acts via the estrogen receptors, this may also indicate different age effects of tamoxifen on the brain. Besides, a critical period is proposed for the neuroprotective role of estrogen replacement therapy; proposing that estrogen replacement therapy should be initiated soon after the menopause in order to be neuroprotective. Estrogen replacement therapy is even suggested to act negatively on cognition when giving to older women, after this critical period (reviewed in (40,

105). In the present study the rats ovariectomized first had a longer period of low estrogen levels, it may be that these animals already overreached the critical period so tamoxifen had no or only negative effects on their cognition. To discover whether this is true for the present study, additional analyses should be done on the rats operated first compared to later operated rats and the critical period in rats should be determined.

Taken altogether, this study indicates that tamoxifen with or without methotrexate does not affect cognitive behavior in rats. Western blot analysis with spinophilin and synaptophysin demonstrated no effect of tamoxifen with or without methotrexate on spine- or synapse density in the rat hippocampus. These findings suggest tamoxifen has no adverse nor beneficial effects on cognition, also when combined with methotrexate.

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Attachments

Protocol Novel & Spatial Object Recognition

Voor de novel en spatial object recognition gaan we twee dagen testen. De eerste dag doen we de novel object recognition en de tweede dag de spatial object recognition.

Novel object recognition

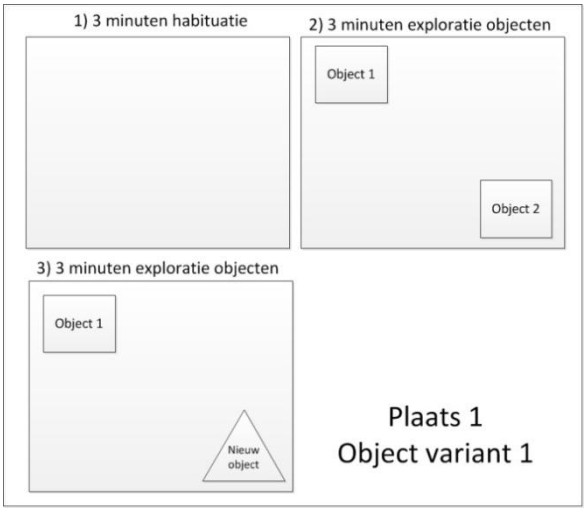
De dag voor de novel object recognition laten we de ratten 1x 3 minuten aan de bak habitueren.

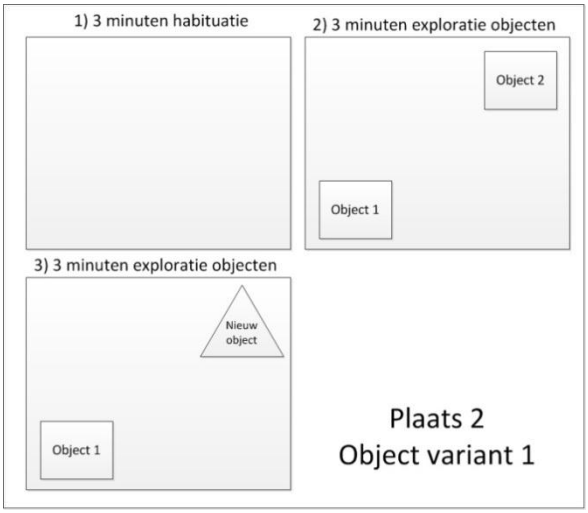
Op de dag van de novel object recognition wordt gestart met nogmaals 3 minuten habituatie. Daarna wordt de rat uit de bak gehaald, met een sopje wordt de bak schoongemaakt en twee identieke (lego) objecten worden in de bak gezet. Vervolgens wordt de rat in het midden van de bak gezet. Na 3 minuten worden de rat en de objecten weer uit de bak gehaald en worden de objecten en bak schoongemaakt. Vervolgens wordt één object vervangen door een nieuw object en wordt ook het bekende object weer, op dezelfde plaats, in de bak teruggeplaatst. De rat wordt ook weer in de bak geplaatst en na 3 minuten er weer uit gehaald.

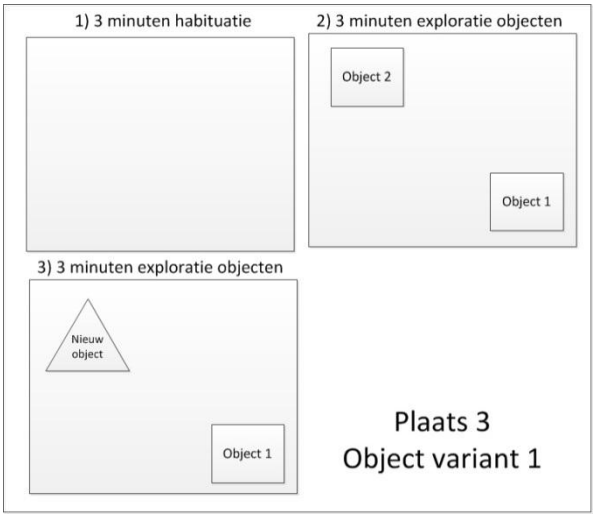
Er wordt gecontroleerd voor zowel het object als de plaats van het object. Het controleren voor het object gebeurt door twee verschillende varianten van object keuze te gebruiken. Het controleren voor plaats gebeurt door het nieuwe object in elk van de vier hoeken te plaatsen zodat er geen effecten kunnen worden gemeten door een eventuele voorkeurshoek.

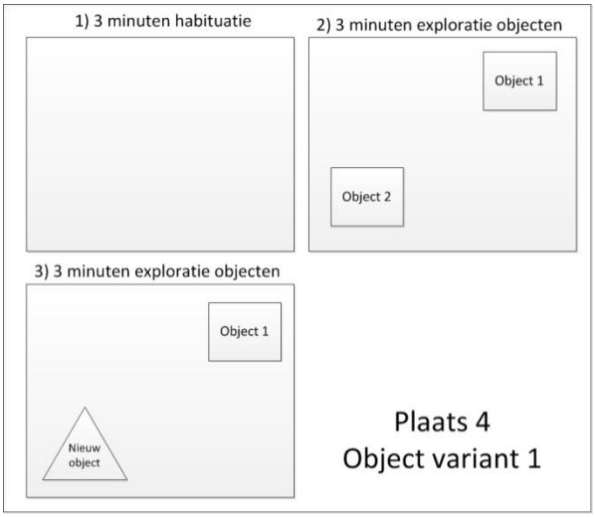
Zie illustraties hieronder voor een meer visuele uitleg.

OBJECT VARIANT 1

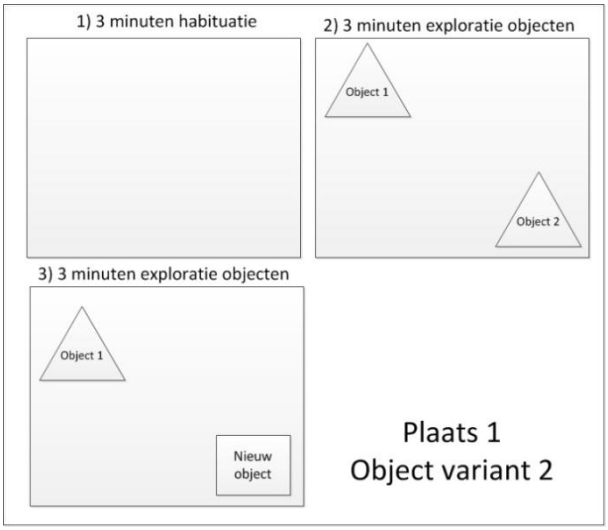


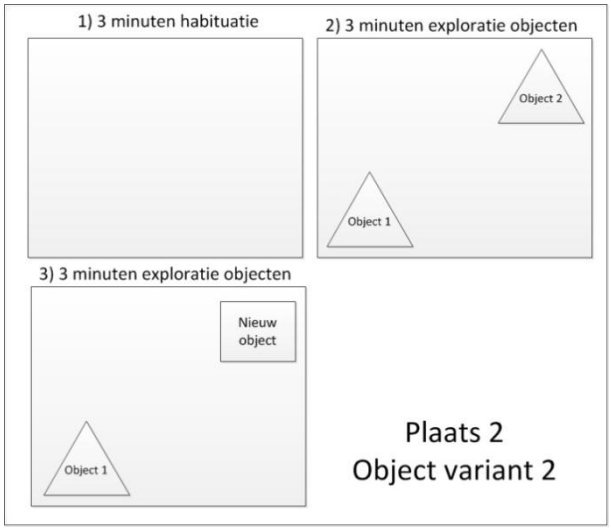


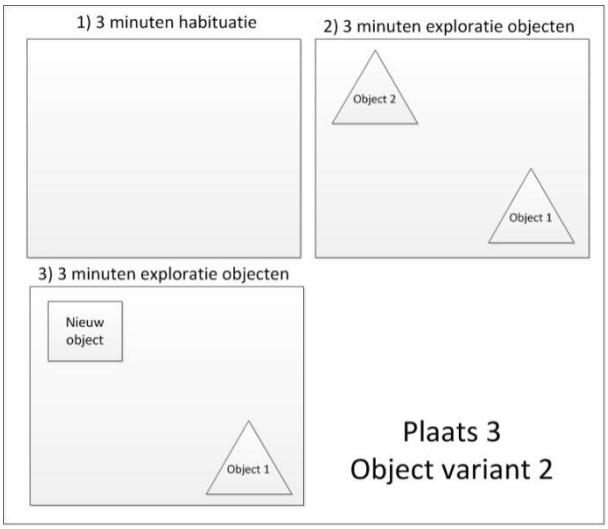


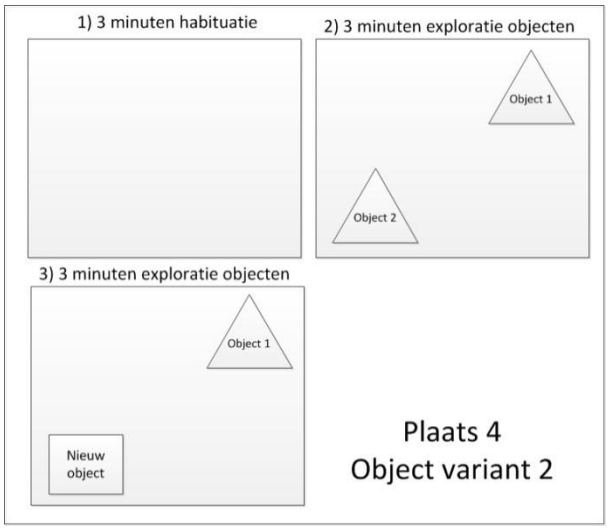


OBJECT VARIANT 2







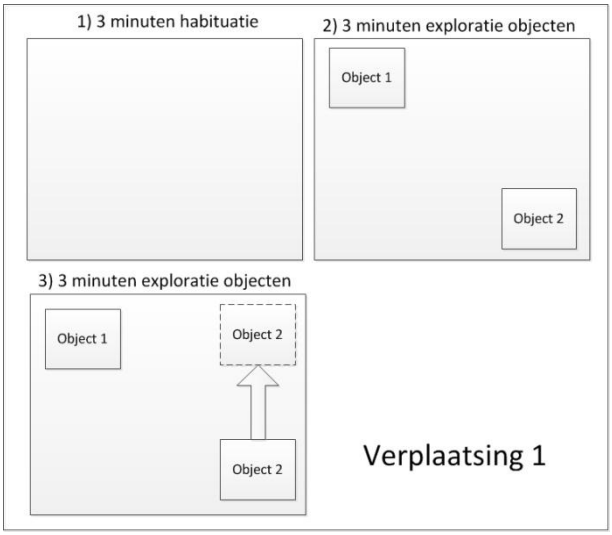


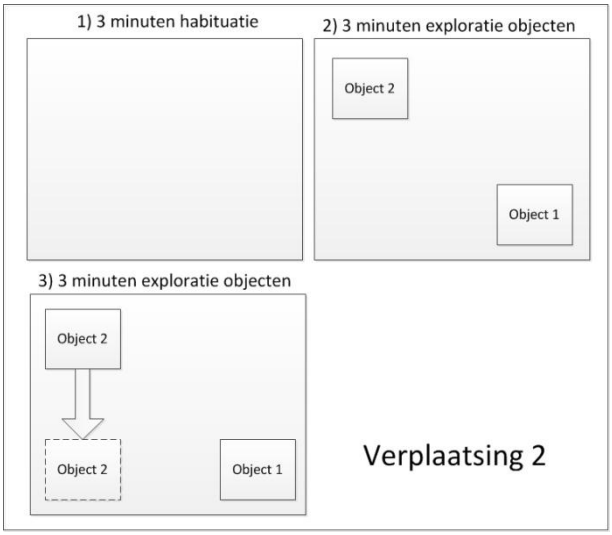
Spatial object recognition

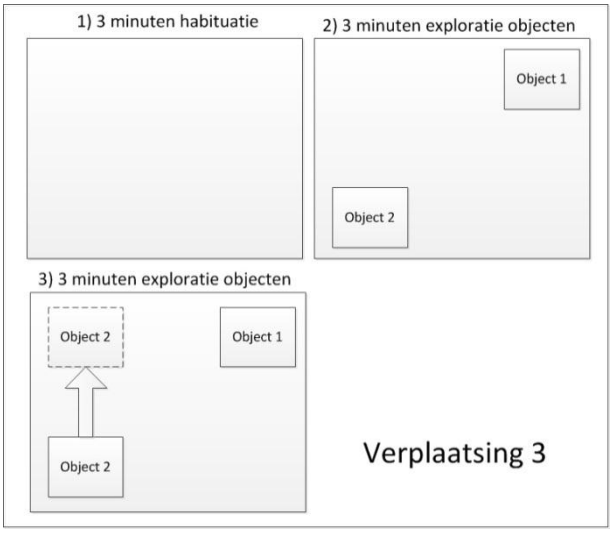
Op de dag van de spatial object recognition wordt gestart met nogmaals 3 minuten habituatie. Daarna wordt de rat uit de bak gehaald, met een sopje wordt de bak schoongemaakt en twee identieke (lego) objecten worden in de bak gezet. Vervolgens wordt de rat in het midden van de bak gezet. Na 3 minuten worden de rat en de objecten weer uit de bak gehaald en worden de objecten en bak schoongemaakt. Vervolgens wordt één object verplaatst en wordt ook het bekende object weer, op dezelfde plaats, in de bak teruggeplaatst. De rat wordt ook weer in de bak geplaatst en na 3 minuten er weer uit gehaald.

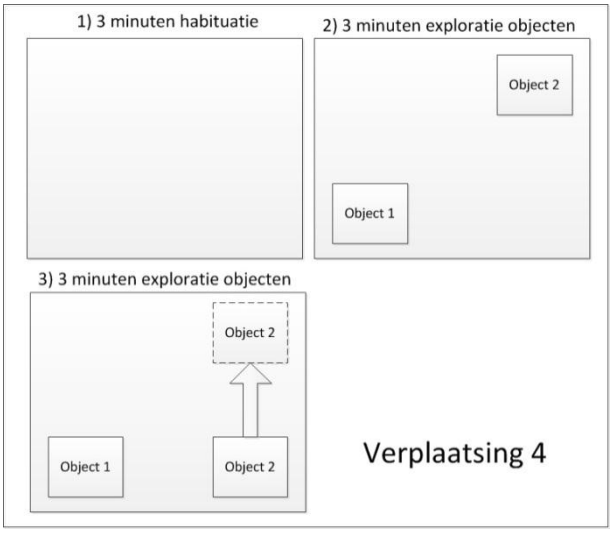
Het controleren voor plaats gebeurt door het verplaatste object in elk van de vier hoeken te plaatsen zodat er geen effecten kunnen worden gemeten door een eventuele voorkeurshoek.

Zie illustraties hieronder voor een meer visuele uitleg.

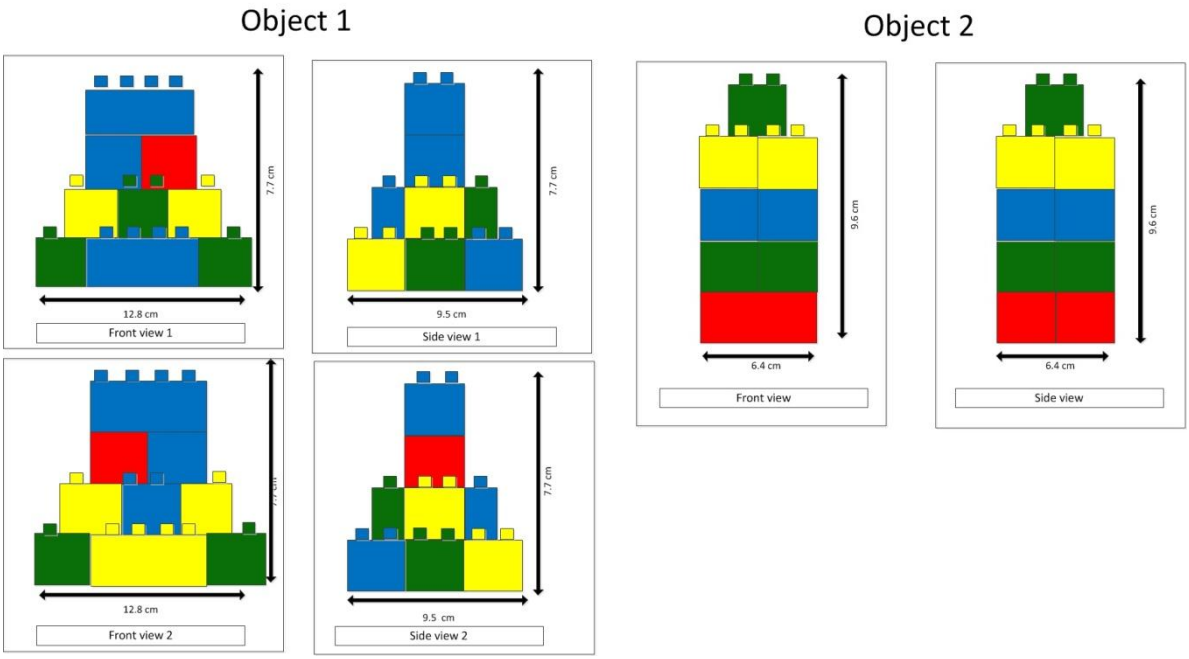








Objects used for object- and spatial object recognition



Western blot

Marjolein Centen

Preparing gels

The separation gel

All the chemicals are put together in this order:

- UP water 14.4 mL
- 40% acrylamide mix 7.5 mL
- 1.5 M Tris (pH 8.8) 7.5 mL
- 10% SDS 300 µL
- TEMED 30 µL
- 10% APS 300 µL

The solution is shaken slightly before poring it into the holder. The space between the two glass plates is filled with the solution just until the green holder line. Some SDS is sprayed on top of the gels, so a protective layer is formed. After the gel was completely polymerized (this can be seen when the remaining gel in the tube is polymerized), SDS is poured out of the system and remainder SDS removed with some filter paper (make sure not to touch the gel surface with the paper.).

Stacking gel

All the chemical are put together is this order:

- UP water 13.08 mL
- 40% acrylamide mix 2.25 mL
- 0.5 M Tris (pH 6.8) 2.25 mL
- 10% SDS 180 µL
- TEMED 18 µL
- 10% APS 180 µL

The solution is shaken slightly before poring it on top of the separation gel in the holder. The holder is filled just until the top with the solution and the cleaned combs are put between the two glasses. After about 10 minutes the stacking gel is polymerized.

Preparing the gels for running

Gels are removed from the holders and put in the 'running system', with the small plates facing each other. Some running buffer (1x) is poured in the system to fill the inner chamber and remove the combs gently.

Loading the gel
Load the gel with the samples.

After loading the gels the lid is put on the system and connected to the power supply.

- The gels are ran at 50 volts until the samples reached the running gel and formed a clear sharp front, the marker can already be visible.
- Now the voltage is raised to 110 Volts. The gel is ready until the blue color front is not visible anymore (approximately 1,5 hours).

Transferring to membranes

PVDF membranes are cut (approx. 8x8cm), 1 per gel (use gloves and pick up by the edges!) and the Whatman filters (7x8.5cm), 2 per gel.

After running the gels are removed from the running systems and cut in two, so 1 half gel with actine and the other half of the gel with spinophilin.

Blotting
Now the gel can be transferred to PVDF-membranes (PVDF membranes are more sensitive than nitrocellulose membranes).

The two glass plates holding the gel are removed from their holders. The glass plates are put on the table with the large plate down. Hold the large plate down while the small plate is lifted by the spacers.
With the small glass plate the stacking gel is separated from the separation gel and a corner of the gel is cut off.
The PVDF-membrane is **first** shortly sopped (activated) in methanol (60 sec) and then for at least ± 5 min in Towbin buffer. Whatman filters sopped in blotting buffer.

PEGASUS BLOTTING CHAMBER:

- o 1 Whatman filter
- o Gel
- o PVDF membrane
- o 1 Whatman filter

Avoid sliding!

The blotting procedure takes place in the cold room (4°C) (- =black to + =red).

V-constant, 70V, 90min/3 gel in cold room

Troubleshooting:
Be sure that the membrane is completely soaked with buffer. Due to the methanol (which is necessary to activate the membrane) the membranes do not easily get soaked with buffer. So shake the membranes through the buffer vigorously! If the membranes are not soaked completely, you will get a high background.

Prepare blocking buffer (I-block and non-fat milk) and store it at 5°C.

Incubation of 1st anti-body

Put the blotted PVDF-membranes in a 15mL tube (protein side facing to the inner part of the tube!) and add 8 mL blocking buffer for 1 hour at 5°C on the roller. Use for actine 8 mL I-block for blocking and for spinophilin use 8 mL non-fat milk (5%).

In the meanwhile prepare the first antibody mixture approximately 10 minutes before use:

	Anti-body	**Dilution**	**In 6 mL**	**In 10 mL**	***Particulars**	**Solution**
Actine	Mouse anti- actine	1:1.000.000	6 µL*	10 µL	First: Put 1µL of antibody in 1 mL TBS-T to get 1:1000 dilution	1 part TBS-T 1 part I-block
Spinophilin	Rabbit anti-spinophilin	1:1000	6 µL	10 µL		5% BSA

Incubate the blots with the 1st antibody overnight on the roller in the cold room (5°C).

Incubation of 2nd anti-body

Wash the blot 3x5 minutes in 8 mL TBS-T 1x pH 7.6.
After washing add the 2nd antibody

	Anti-body	**Dilution**	**In 6 mL**	**In 10 mL**	**Solution**
Actine	Anti-mouse	1:5000	1,2 µL	2 µL	1 part TBS-T 1 part I-block
Spinophilin	Anti-rabbit	1:5000	1,2 µL	2 µL	Non-fat milk

After 1 hour of incubation, wash the blots again: 3x5 minutes in 8 mL TBS-T 1x pH 7.6.

Making photographs in ChemiDoc XRS+ system

For one membrane: mix in one eppi 200 mL Pierce Detection Reagent 1 and 200 mL Pierce Detection Reagent 2. Put the membrane on a laser film and add the mixture over the membrane. Cover the membrane with another laser film and make sure that there are no air bubbles.

Put the membrane in the Molecular Imager ChemiDoc XRS+ System with the protein side facing up.
No correct the position of the membrane and set the program to the right protocol:

Our pilot: start at 5 seconds till 300 seconds with 60 images.
Make sure there is no filter and that the program is set for chemiblot.

Buffers

SHOULD NOT CONTAIN SODIUM AZIDE (INHIBITOR OF HRP)

TBS (10x) (1 liter)
24.2 g TRIS
80 g NaCl
fill up to 1 liter H2O UP
pH 7.6
Store at room temperature (RT)

Blocking buffer (500 ml)
1 g I-Block (TROPIX)
50 ml 10x TBS
Fill up to 500 ml with H2O UP
Heat to roughly 55°C (no particles should be visible anymore)
Cool down to 5°C
Add 0.5 ml Tween20
Store at 5°C

Blocking buffer 5% non-fat-milk
5gr non-fat-milk powder
Dissolve in 50 mL TBS-T
Fill up to 100 mL with TBS-T
Filter the solution with filter paper
Store at 5°C

Washing buffer (1 liter)/ TBS-tween
100 ml 10x TBS (See above)
Fill up to 1 liter with H2O UP
1 ml Tween20 (leave the pipet tip in the bottle)
Store at 5°C.

Towbin buffer (1 liter) /Blotting buffer
3 g Tris
14.4 g Glycine
2 ml 10% SDS
200 ml Methanol
Fill it up to 1000 ml with H2O UP
pH 8.6

Running buffer (5x) (1 liter)
94 g glycin
15.1 g Tris
Add 50 ml 10% SDS
10% SDS (100ml)
Dissolve 10g SDS in 100 ml.
Store at RT
Fill up to 1000 ml with UP H2O
Store at 5°C

APS (1ml)
Dissolve 100 mg in 1 ml H2O UP
Store at 5°C (can be used up to 1 week)

1.5 M Tris (100 ml)
Dissolve 18.17 g Tris in
85 ml H2O UP
Set pH to 8.8 (let the pH stabilize over 25 minutes roughly!)
Fill up to 100 ml with H¬2O UP
Store at 5°C

0.5M Tris (100ml)
Dissolve 6.06 g Tris (not Tris HCl) in
85 ml H2O UP
Set pH to 6.8 (let the pH stabilize over 25 minutes roughly!)
Fill up to 100 ml H2O UP
Store at 5°C.

Sample buffer (5x) for 100ml

50 % Glycerine	50 g
312,5 mM Tris/HCl pH 6.8	3,72g
10 % SDS	10 g
25 % β-Mercaptoethanol	25 g
0,1 % Brome phenol blue	0,1 g

Fill up to 100 ml with UP H2O and store at 5°C

5% BSA
5gr BSA
Dissolve in 50 mL TBS-T
Fill up to 100 mL with TBS-T
Filter the solution with a filter syringe
Store at 5°C

Western blot performances

Pilot

In the pilot we loaded the gells as follows:

Gel 1. (10 locks)

1	2	3	4	5	6	7	8	9	10
7 µL marker	10µL Rat 3 P3	20µL Rat 3 P3	10µL Rat 7 P3	20µL Rat 7 P3	10µL Rat 19 P3	20µL Rat 19 P3	10µL Rat 20 S3	20µL Rat 20 S3	7 µL marker

Gel 2. (10 locks)

1	2	3	4	5	6	7	8	9	10
20 µL Rat 7 P3	7 µL marker	10 µL Rat 7 P3	20 µL Rat 3 P3	10 µL Rat 3 P3	20 µL Rat 20 S3	10 µL Rat 20 S3	20 µL Rat 19 S3	10 µL Rat 19 S3	7 µL marker

We chose to test 2 amounts of sample: 10µL and 20µL. Based on this pilot we continued with the amount of 20µL of sample. We tested mostly P3 (pallet) fractions, but also some S3 (supernatant) fraction to see whether they would also show a band, since the S fraction may be used in future studies.

This resulted in the following blots:

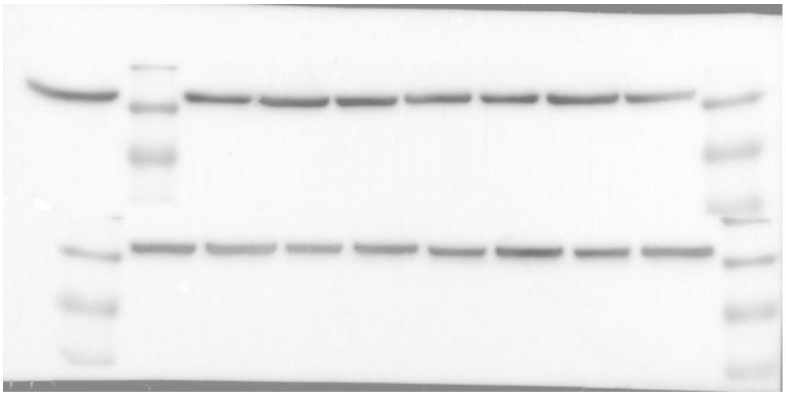


Figure 30 Pilot Actine and ladder. The upper picture shows the actine bands on gel1 and the lower picture shows the actine bands on gel2.

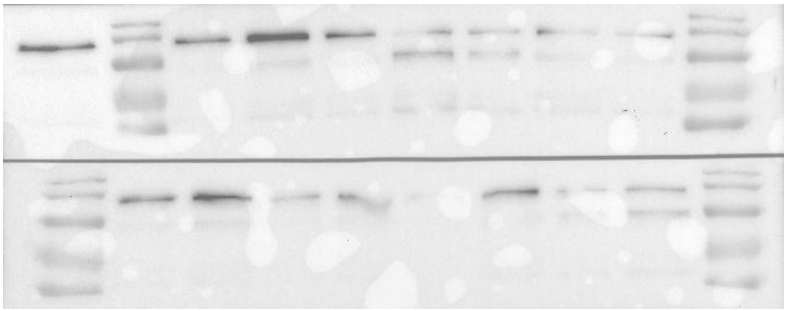


Figure 31 Pilot spinophilin and ladder. The upper picture shows the spinophilin bands on gel1 and the lower picture shows the spinophilin bands on gel2.

In the next two western blot we loaded the gells as follows:

Gel 1. (10 locks)

1	2	3	4	5	6	7	8	9	10
Marker	P3 1 TAM-MTX	P3 3 TAM-VEH	P3 5 ANA-MTX	P3 8 ANA-VEH	P3 17 VEH-MTX	P3 20 VEH-VEH	P3 10 TAM-MTX	P3 22 VEH-MTX	Marker

Gel 2. (10 locks)

1	2	3	4	5	6	7	8	9	10
P3 2 TAM-MTX	Marker	P3 6 ANA-MTX	P3 11 TAM-VEH	P3 15 ANA-VEH	P3 18 VEH-MTX	P3 23 VEH-VEH	P3 16 -> luchtbel! ANA-VEH	P3 24 VEH-VEH	Marker

Gel 3. (10 locks)

1	2	3	4	5	6	7	8	9	10
P3 4 TAM-VEH	Marker	P3 7 ANA-VEH	P3 9 TAM-MTX	P3 13 ANA-MTX	P3 19 VEH-VEH	P3 21 VEH-MTX	P3 12 TAM-VEH	Marker	P3 16 ANA-MTX

But the results of these western blots were not good, since we made a mistake in activating the PVDF membrane: we put the membrane first in towbin buffer and than activated it in methanol. We should had first activate it in methanol (just before putting it on the gel) and thereafter put it in towbin buffer.

The third attemps succeeded with the gells loaded as follows:

Gel 1. (10 locks)

1	2	3	4	5	6	7	8	9	10
Sample buffer (ivm luchtbel	Marker	P3 3 TAM-VEH	P3 5 ANA-MTX	P3 8 ANA-VEH	P3 17 VEH-MTX	P3 20 VEH-VEH	P3 10 TAM-MTX	P3 22 VEH-MTX	Marker

Gel 2. (10 locks)

1	2	3	4	5	6	7	8	9	10
P3 2 TAM-MTX	P3 6 ANA-MTX	Marker	P3 11 TAM-VEH	P3 15 ANA-VEH	P3 18 VEH-MTX	P3 23 VEH-VEH	P3 16 -> luchtbel! ANA-VEH	P3 24 VEH-VEH	Marker

Gel 3. (10 locks)

1	2	3	4	5	6	7	8	9	10
P3 4 TAM-VEH	Marker	P3 7 ANA-VEH	P3 9 TAM-MTX	P3 13 ANA-MTX	P3 19 VEH-VEH	P3 21 VEH-MTX	P3 12 TAM-VEH	Marker	P3 1 ANA-MTX

This resulted in the following blots:

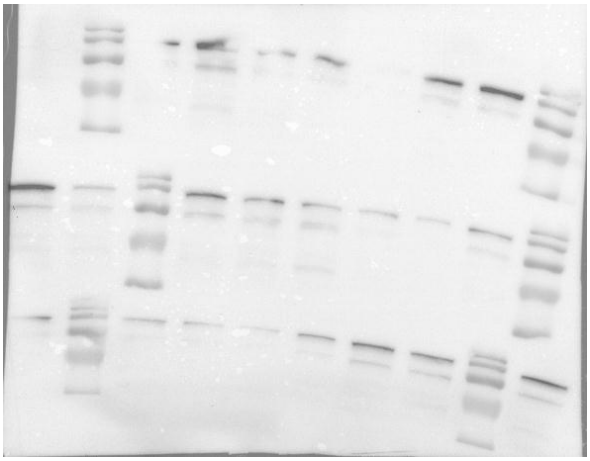


Figure 32 Spinophilin and ladder. The upper picture shows the spinophilin bands of gel1; the middle picture shows the spinophilin bands of gel3; and the lower picture shows the spinophilin bands of gel2.

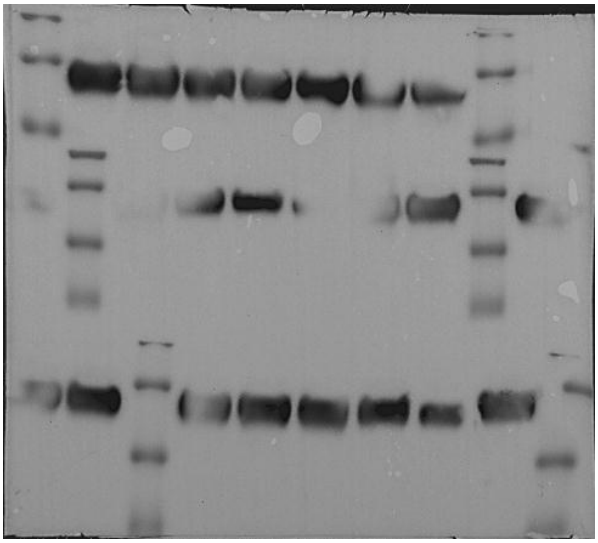


Figure 33 Synaptophysin and ladder. The upper picture shows the synaptophysin bands of gel1; the middle picture shows the synaptophysin bands of gel3; and the lower picture shows the synaptophysin bands of gel2.

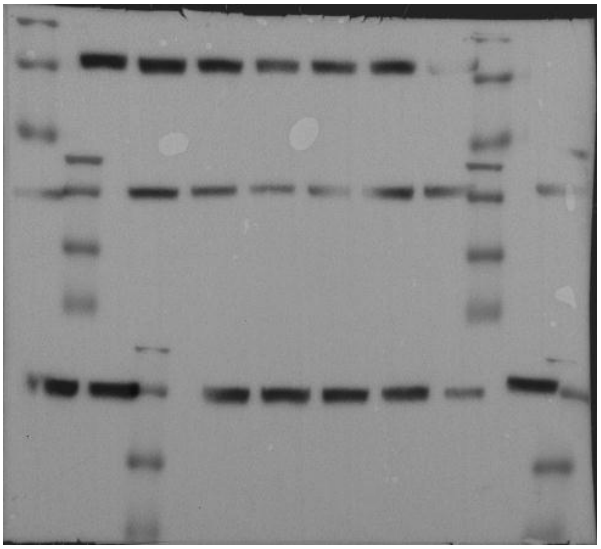


Figure 34 Actine and ladder. The upper picture shows the actine bands of gel1; the middle picture shows the actine bands of gel3; and the lower picture shows the actine bands of gel2.