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Postoperative cognitive dysfunction (POCD) after cardiac and major non-cardiac surgery.

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Abstract

Introduction Postoperative cognitive dysfunction (POCD) is predominantly seen in the elderly and is defined as persistent cognitive decline following surgery in one or more cognitive domains. The most important risk factor for developing POCD seems to be the severity of surgery, with a high rate of occurrence after cardiac surgery. The exact mechanisms underlying POCD are still unclear. But lately, the focus has shifted towards the inflammation hypothesis. The aim of this study is to investigate cognitive performance, mood and changes at protein level in the brains of rats after different types of surgery.

Methods To test this we divided male Wistar rats in 6 different groups; control, anesthesia, jugular vein catheterization, abdominal surgery, induced myocardial infarction and thorax surgery. One week after their surgery the animals were subjected to several behavioral and cognitive tests; sucrose preference test, open field, novel object/location recognition and Morris water maze. After the tests the animals were sacrificed and their brains and hearts were removed for further analysis.

Results We saw only a significant difference in the sucrose preference test, in which the animals that received a jugular vein cannula drank significantly less sucrose water compared to the control group. For all the other tests we saw no significant differences.

Conclusion & Discussion The results from the sucrose preference test indicate that the animals that received a cannula experience depressive like behavior. Since the other tests showed no significant differences, it seems that the surgeries do not affect other forms of behavior nor do they affect short or long term memory. A possible explanation for our lack of significant results is that our surgeries were not severe enough. It is also possible that the animals did not develop POCD, although their surgeries were severe enough, as in humans not every elderly patient that undergoes a severe surgery develops POCD. A third explanation involves the age of the animals, our animals were quite young, whereas POCD is mainly seen in the elderly. Finally, the number people reporting cognitive dysfunction declines over time. So, it might be that we tested the animals too long after the surgeries and that they had already recovered from their POCD.

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Introduction

Postoperative cognitive dysfunction (POCD) is predominantly seen in the elderly and is defined as persistent cognitive decline following surgery in one or more cognitive domains (Hovens et al., 2012). The affected domains include information processing, executive function, memory and attention (Cibelli et al., 2010; Krenk et al., 2010). A long term observational study among 701 Danish patients who suffered from POCD found that POCD had a very high impact on everyday life and that patients experienced a reduced quality of life (Steinmetz et al., 2009). The patients had a higher mortality rate than the age-matched control group. In addition, people suffering from POCD were more likely to leave the labour market at a younger age.

Risk factors

In 1955, Bedford was the first to report cognitive dysfunction in elderly patients who underwent surgery with general anesthesia (Bedford, 1955; Newman et al., 2007). After this initial report research focused on POCD after cardiac surgery. It was thought that POCD occurred more frequently in cardiac surgery due to possible hypoxic or ischemic episodes during this kind of surgery. Also, the depth of anesthesia necessary for cardiac surgery was thought to contribute to the development of POCD (Barrientos et al., 2012). Recently, it has become clear that POCD occurs after other forms of surgery as well (Wan et al., 2007).

The severity of cardiac surgery is seen as the main reason why there is an increased risk of developing POCD after cardiac surgery compared to non-cardiac surgeries. Besides the severity of surgery several other risk factors have been identified. They include advanced age, poor education, pre-existing cognitive impairments and genetic pre-dispositions (Cao et al., 2010; Hovens et al., 2012).

As mentioned above, the use of anesthetics and analgesia was also thought to be a risk factor, while it is known that they can produce long-lasting cognitive dysfunction under certain circumstances (Krenk et al., 2010). Research done in cell culture and animal

models shows that exposure to anesthetics increases the expression of amyloid- β , which is involved in the pathogenesis of Alzheimer's disease, and the apoptotic marker caspase 3 (Hudson and Hemmings, 2011). Opioids, which are used as an analgesic, can cause a decrease in adenosine and acetylcholine levels. Both adenosine and acetylcholine regulate memory and attention, and especially a decrease of acetylcholine levels is linked to cognitive dysfunction (Krenk et al., 2010). However, there is not much known about the exact role of anesthetics and analgesia in POCD. In spite of the known cognitive dysfunction after use of anesthetics and analgesia in some circumstances, the incidence of POCD is similar after surgery including regional or general anesthesia (Cibelli et al., 2010). Thus it seems unlikely that the use of anesthetics and analgesia alone can cause POCD.

The inflammation hypothesis

Even though there are many risk factors known to contribute to POCD, the exact mechanisms underlying POCD are still unclear. Recently, the focus of researchers has shifted towards the inflammation hypothesis (Wan et al., 2007). After surgery there is an increase in pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α as a consequence of tissue damage (Hu et al., 2010). These peripheral cytokines are capable of crossing the blood-brain barrier, which is easier in the elderly since with increasing age the blood-brain barrier becomes more permeable. Increased levels of pro-inflammatory cytokines, especially in the hippocampus, results in impairments in long-term potentiation (LTP) and lower performance scores in hippocampal-dependent cognitive tests (Cao et al., 2010). Therefore neuroinflammation might well be the reason for developing POCD.

Research done by Cibelli and colleagues (2010) supports this hypothesis. They found that minocycline, which is a non-specific inhibitor of inflammation, protected animals that had undergone surgery from cognitive dysfunction. In addition minocycline prevented the postoperative activation of

microglia in these animals and prevented the release of pro-inflammatory cytokines.

Inflammation in cardiac surgery vs.

non-cardiac surgery

When we compare the inflammatory reaction of patients who underwent cardiac surgery with the inflammatory reaction of patients who underwent major non-cardiac surgeries, we see increased levels of IL-1 β and IL-6 in both groups (Frangogiannis et al., 2002; Spies et al., 2002; Bujak and Frangogiannis, 2009; Fidalgo et al., 2011). In addition, there are increased levels of TNF- α , IL-10 and C-reactive protein (CRP) after cardiac surgery (Frangogiannis et al., 2002; Ramlawi et al., 2006; Matusik et al., 2012).

High levels of IL-1 β are known to cause deficits in learning and memory tasks that are hippocampus dependent, but not in hippocampus-independent tasks (Huang and Sheng, 2010). This is probably due to inhibiting LTP in several regions of the hippocampus, including CA1, CA3 and the dentate gyrus.

Research done in mice shows that IL-6 is an important regulator of neurogenesis which is important in learning and memory (McAfoose and Baune, 2009). Mice that over expressed IL-6 showed a reduction of neurogenesis in the dentate gyrus. In addition, it is known that IL-6 can influence synaptic plasticity by inhibiting LTP in the hippocampus.

The increased levels of TNF- α lead to the inducement of NF- κ B (Frangogiannis et al., 2002). This protein belongs to a family of

transcription factors that regulates the expression of genes with many different functions, including controlling the inflammation response, cell adhesion and growth control. In addition it is thought that TNF- α , through the activation of NF- κ B, influences synaptic plasticity of the hippocampus (Albensi BC and Mattson MP, 2000). Especially by inducing long-term depression of synaptic transmission in the CA1 region of the hippocampus.

Contrary to IL-1 β and IL-6, which are pro-inflammatory cytokines, IL-10 is an anti-inflammatory cytokine. When IL-10 activates its receptors a signaling pathway is activated that down regulates the production of IL-1 β and IL-6 (Dantzer R, 2004). In this way, IL-10 can possibly prevent or decrease the effects of IL-1 β and IL-6.

Since POCD is mainly seen in severe surgeries, the aim of this study is to investigate cognitive performance and changes at protein level in the brains of rats after different types of surgery. In addition, we will investigate if rats that underwent a surgery involving induced myocardial infarction show more depressive like behavior. Since it is known that depression is commonly seen in patients that have undergone cardiac surgery as a result of myocardial infarctions or other cardiovascular diseases (Echols and O'Connor, 2010). This will lead to more insight in the disease process of POCD and may aid in finding a treatment.

Materials and Methods

Animals

43 male Wistar rats are kept under standard housing conditions (temperature 20+/- 2°C, humidity 50 +/- 10), on a reversed 12:12 hours light-dark schedule (lights off 9:00 – 21:00) and with free access to food and water. The cages are cleaned weekly. At arrival the animals had a mean weight of 260grams.

Design

From the day of arrival until the day of sacrifice, the animals are weighed every morning. The animals are habituated to sucrose water 2, 3 and 4 days before the intervention (**FIG. 1**). In addition, baseline performance in the Novel Location Recognition task is tested 3 days before surgery. One week after arrival the animals receive surgery. They are randomly assigned to six experimental groups; (i) control group, (ii) anesthesia only, (iii) jugular vein cannula, (iv) abdominal surgery, (v) sham thorax surgery and (vi) induced myocardial infarction. After the intervention they have one week to recover before they are subjected to different behavioural tests during the dark phase.

Surgery

All surgeries are performed under sevoflurane anesthesia and a local sedation with Marcaine. *Control group* – The animals receive no surgery, anesthetics or analgesia.

Anesthesia only – The animals assigned to this group are kept under sevoflurane anesthesia for 60 minutes after which the anesthetic is removed.

Abdominal surgery – This is achieved by clamping the upper mesenteric artery for 30 minutes. After 30 minutes the clamp is removed and reperfusion is restored.

Sham thorax surgery – The thorax is opened for 45 minutes after which the thorax is closed again.

Induced myocardial infarction – This is achieved by a ligation of the left anterior descending coronary artery for 45 minutes. After this time, the ligation is removed and reperfusion is restored.

Jugular vein cannulation – The animals in this group undergo a surgery during which a cannula is placed in the jugular vein.

The animals in the abdominal surgery and induced myocardial infarction groups receive a one-time doses of Temgesic (0.01mg/kg s.c.) 1 hour after reperfusion. The animals in the anesthesia only, sham thorax surgery and jugular vein cannulation groups receive a one-time doses of Temgesic (0.01mg/kg s.c.) 1 hour after they have been locally sedated with Marcaine.

Once the anesthetic is removed and the animals have regained consciousness they are placed back in their home cage for further recovery.

Blood Sampling

After the animals are anesthetized with sevoflurane and before surgery starts, a blood sample is obtained from the tail vein. The samples are stored on ice in the refrigerator and after all the samples are obtained they are centrifuged at 2600g for 10 minutes. The plasma is collected and stored at -80°C until

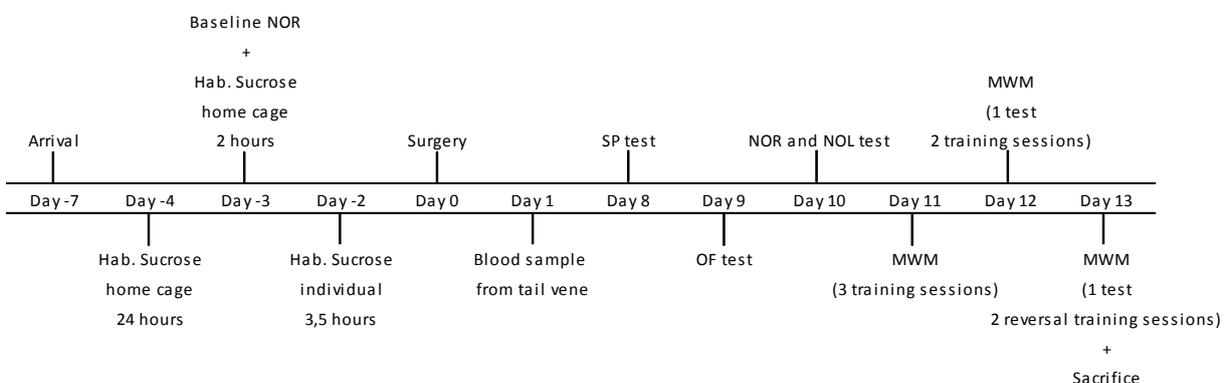


Figure 1. Time schedule of the experimental set-up. Hab. = habituation, NOL = Novel Object Location, NOR = Novel Object Recognition, MWM = Morris Water Maze, SP = Sucrose Preference.

further assays are performed.

24 hours after surgery all animals are anesthetized with sevoflurane again, including the animals that are assigned to the control group, and a second blood sample is obtained and processed as described above.

Sucrose Preference

To test if the animals are depressed we perform the sucrose preference test. 4 days before surgery the animals are habituated to 1% sucrose water in their home cage for 24 hours. Besides their usual water bottles, an additional water bottle containing 200 ml 1% sucrose water is present. 3 days before surgery the usual water bottles are removed and replaced with one water bottle containing 200ml tap water and one bottle containing 200ml 1% sucrose water for 2 hours. 2 days before surgery the animals are housed individually for 3,5 hours and they will have free access to one water bottle containing 200ml tap water and one bottles containing 200ml 1% sucrose water. These results are used to determine their baseline levels for sucrose preference.

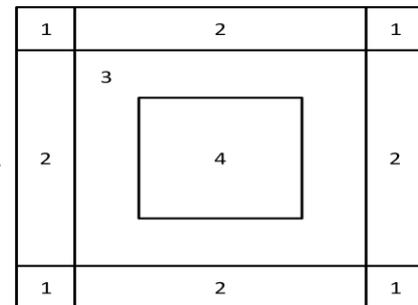
At the day of testing, 8 days after surgery, the animals are housed individually and they have free access to one water bottle containing 200ml tap water and one bottle containing 200ml 1% sucrose water. Before the bottles are placed in the cages the bottles are weighed. After 7 hours the bottles are removed and weighed again and the animals are returned to their home cages. Sucrose preference is calculated as follows: $\text{weight difference of 1\% sucrose water} / (\text{weight difference of 1\% sucrose water} + \text{weight difference of tap water}) * 100\%$.

Open field

To test exploration and anxiety in an unknown environment, the animals are tested in a square open field of 100*100*40cm 9 days after surgery. The arena is divided in four zones (**FIG. 2**). The animal is placed individually in the centre of the arena and its behavior is recorded for 5 minutes with Ethovision 3.0 (Noldus Information Technology, Wageningen, the Netherlands). We measure the time the animals spend in

the different zones of the field and the distance they move. After each test the arena is cleaned with 70% ethanol to remove smell cues.

Figure 2. Schematic overview of the Open Field arena. Zone 3 and 4 are also called Zone Center.

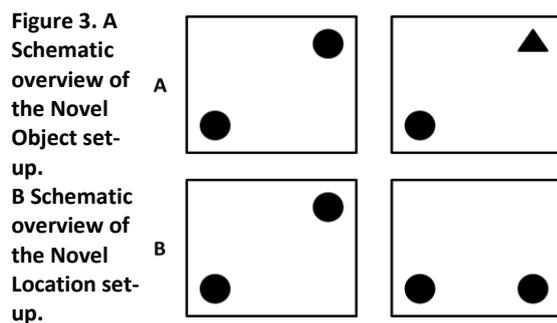


Novel Object and Location Recognition

To test interest and attention, the animals are subjected to the novel object and location recognition test. 3 days before surgery the animals are habituated to the test box (40*40*40cm, with two PVC and two Perspex sides) and baseline levels of novel location recognition are measured. The novel location recognition test consists of two phases; first the animal is allowed to explore two identical objects (transparent bottles filled with water) during three minutes. Then both objects are cleaned with 70% ethanol. One object is placed back at one of the previous locations, while the other object is placed back at a novel location. The animal is again allowed to explore both objects during three minutes. Behavior is recorded with a video camera and analyzed with Eline. Baseline levels are calculated as follows: $\text{percentage of time spent at the new location} / (\text{time spent on the new location} + \text{time spent on the previous location}) * 100\%$. After each test all objects and the test box are cleaned with 70% ethanol.

10 days after surgery the animals perform the novel object recognition test (**FIG. 3A**). This test also consists of two phases; first the animal is allowed to explore two identical objects (either two LEGO towers or two spray bottles filled with water) during three minutes. Then both objects are cleaned with 70% ethanol. One object is placed back at one of the previous locations, while at the other previous location a novel object (either a

LEGO tower or spray bottle filled with water, depending on the object used in the first phase) is placed. The animal is again allowed to explore both objects during three minutes. Behavior is recorded and analyzed as described above. After all animals are tested they perform the novel location recognition test again as described above (**FIG. 3B**). Instead of the transparent bottles filled with water, which are also used to determine baseline levels, the LEGO towers and spray bottles filled with water are used.



Morris Water Maze

To test learning abilities and short and long term memory, the animals are subjected to the Morris water maze (MWM). The MWM consists of a round pool of 140 cm diameter filled with water of 26 +/- 1°C. The pool is divided in 4 quadrants. In one of the quadrants a platform is placed, 1 cm below the water surface, invisible to the animal.

At 11 days after surgery all animals are trained during three training sessions in the MWM, with a one hour interval between the sessions. A training session consists of three consecutive trials. In each trial the animal is placed one time in each quadrant of the maze that does not contain the platform in random order and allowed to search for the platform. The trial stops 10 seconds after the animal finds the platform. When the animal does not find the platform in 1 minute it is gently guided towards the platform and left there for 10 seconds. After each training session the animal is dried with a towel and placed back in its home cage.

At 12 days after surgery the animals are tested in the MWM as follows: the platform is removed and the animal is randomly placed in one of the quadrants of the maze and

behavior is recorded for 1 minute using Ethovision. After all animals are tested, they receive one re-training trial followed by two training sessions.

At 13 days after surgery the animals are tested as described above. After all animals are tested they receive two reversal training sessions. These training sessions are performed as the training sessions described above, except for the location of the platform which is opposite to the previous location.

Sacrifice

90 minutes after the last MWM reversal training session the animal is sacrificed. The animal is first anesthetized by an i.p pentobarbital injection, after which a heart puncture is performed and a blood sample is collected. The blood samples are stored on ice in the refrigerator. After all the blood samples are obtained they are centrifuged at 2600g for 10 minutes. The animal is sacrificed by transcardial perfusion with saline and 4% PFA. The brains of all animals are collected for further analysis, as well as the hearts of the animals that received an induced myocardial infarct and part of the bowel of animals that received abdominal surgery. Brains and hearths are stored in 4% PFA for 5 days following sacrifice. To fixate them, they are placed in a sucrose solution and after 24 hours they are frozen with liquid nitrogen and stored in a freezer with a temperature of -80°C. The hearths are used to measure the size of the infarction and the bowels are stored for later analysis.

Immunohistochemistry

We performed DCX and IBA-1 stainings on brain slices of previously tested rats (see Appendix A and B for the exact protocol). These animals were used in an experiment to see how POCD develops over time after surgery. The animals were subjected to the same tests, either 1,2 or 3 weeks after surgery, and sacrificed in the same way as described above. Before analysis the brains are sliced into 30µm sections.

DCX staining - We analyzed the staining by making photo's which were used to count the DCX-positive cells and to measure the size of

the hippocampus. We corrected the amount of counted DCX-positive cells for hippocampus size.

IBA-1 staining – We analyzed this staining by making photos of the hippocampus, as well. During the process of making photo's a circle is drawn on the picture. In the program Image Pro-Plus 6.0 (Media Cybernetics, Maryland, USA) we used this circle to calculate first the surface area of all the cells in the circle and second to calculate only the surface area of the cell bodies in the circle. Microglia activation is calculated as: (surface area of the cell bodies / surface area of the entire cells) * 100%.

Data Analysis

Data are presented as group averages with standard error of the mean. Data are analyzed with one-way ANOVA and Tukey post hoc analysis to compare the different treatment groups or by two-way ANOVA followed by a Bonferroni post hoc analysis. Statistical analysis was performed using GraphPad Prism5.00 Software for Windows (La Jolla, California, USA). Statistical significance was determined at alpha is < 0.05.

Results

Body Weight

Mean body weight of each group at day 0 (day of surgery) was set as 100%. All the other measurements of body weight are depicted as percentages of the weight at day 0 (**FIG. 4**). Analysis by a one-way ANOVA and a Tukey post hoc analysis showed that there was a significant difference ($F_{5,78}=4,438$, $p=0,0013$) in weight between the control group and the animals that received an abdominal surgery or an induced myocardial infarction.

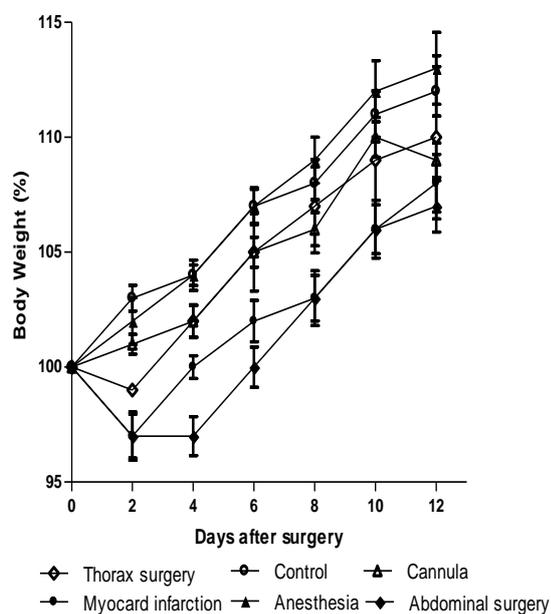


Figure 4. Mean body weight of each group as a percentage of the weight at day 0 (day of surgery).

Sucrose Preference

Sucrose preference is tested eight days after surgery. If there is no preference for either sucrose water or tap water the group mean should score 50%. Everything above 50% indicates a preference for sucrose water and everything under 50% indicates a preference for tap water. A one-way ANOVA showed that there was a significant difference ($F_{5,42}=8,755$, $p<0,0001$). When we performed a Tukey post-hoc analysis, this showed that only the animals who underwent the jugular vein cannulation differed significantly from all the other groups ($p<0,05$) (**FIG.5**). Between none of the other groups was a significant difference.

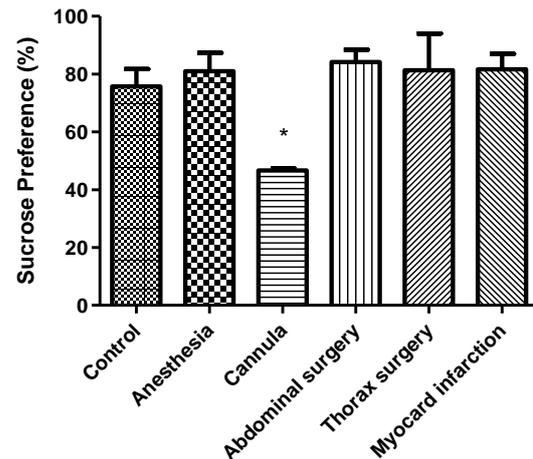


Figure 5. Sucrose preference as calculated with the following formula: weight difference of 1% sucrose water / (weight difference of 1% sucrose water + weight difference of tap water) * 100%. Bars with an asterisk are significantly different from the control group.

Open Field

Analysis by an one-way ANOVA indicated a significant difference ($F_{5,36}=2,761$, $p=0,0328$) between the mean time spent in the center of the open field (**FIG. 6A, B**). But when we performed a Tukey post-hoc analysis there was no significant difference between the groups.

We also analyzed the total distance the animals moved during the test by a one-way ANOVA (**FIG. 6C**). This showed that there was no significant difference between the groups.

Novel Object and Location

Recognition

Analysis of baseline levels of exploration by a two-way ANOVA followed by a Bonferroni post hoc analysis indicated there were no significant differences between the groups (data not shown).

Analysis of the test results by a two-way ANOVA and a Bonferroni post hoc test indicated that there was no difference within the groups on time spent exploring the objects during the habituation phase and the phase in which the novel object or novel location was introduced (**FIG. 7A, 7B**). Nor was there a difference between the groups on time spent exploring the novel object or novel location during both phases. We also calculated the time spent on exploring both objects as a

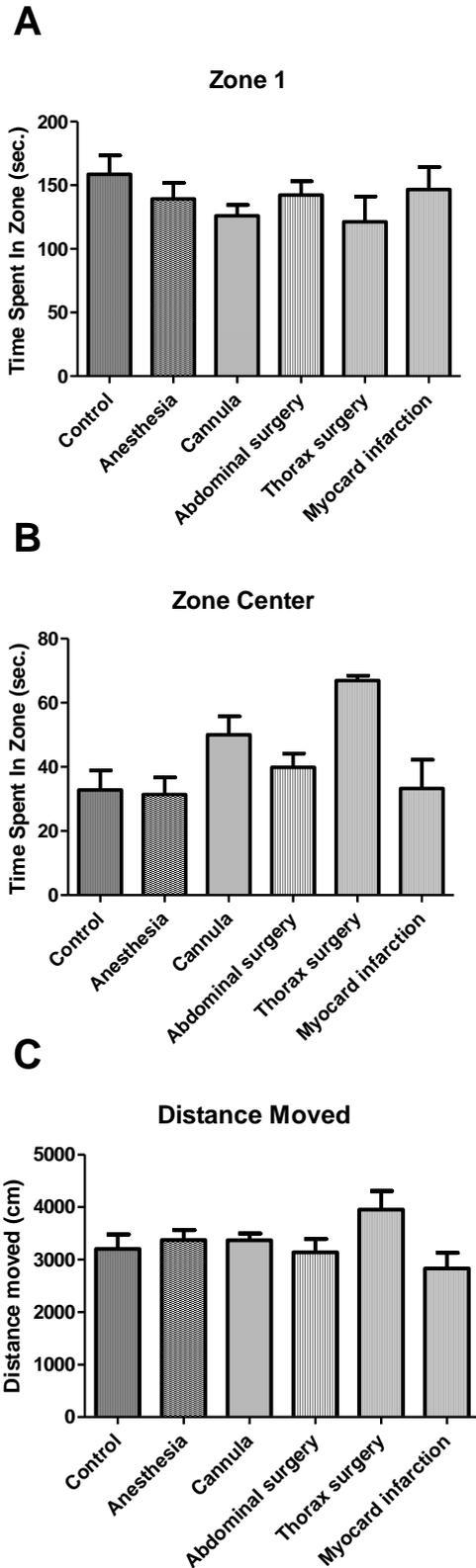


Figure 6. Open Field. A Time spent in zone 1. B Time spent in zone Center. C Distance moved (cm) during the test. There is no significant difference in the time spent in each zone of the open field, nor in the distance moved. For a schematic overview of the Open Field arena see figure 2.

percentage of the time that the animals spent on overall exploration. Overall exploration time was calculated as the time spent on exploring both objects or locations and the time spent on exploring the cage. Analysis by two-way ANOVA and a Bonferroni post hoc test showed no significant differences (Appendix C).

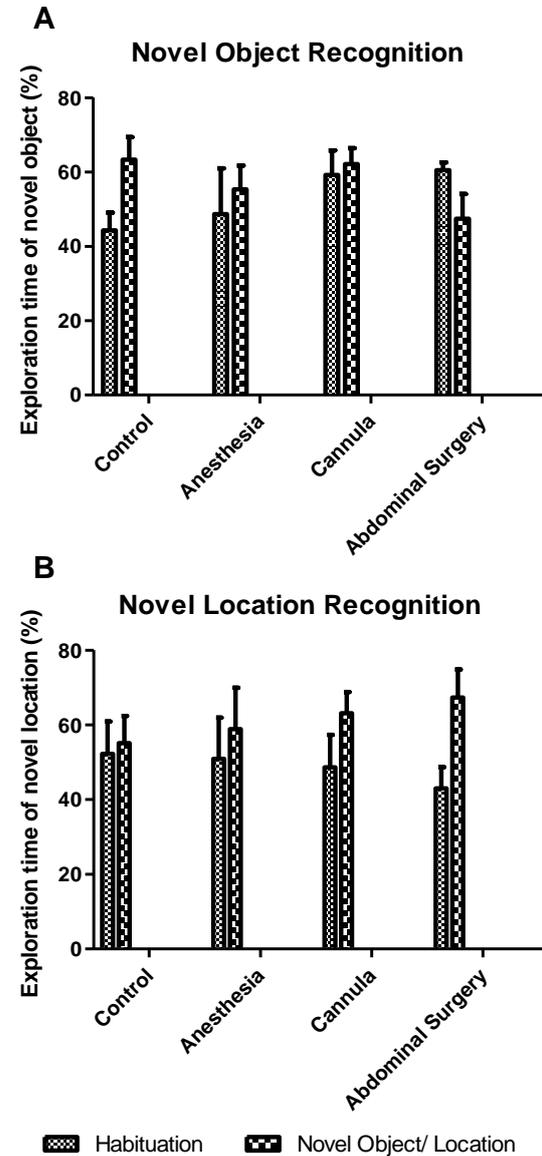


Figure 7. A Time spent exploring the new object as a percentage of the time spent on exploring both objects. B Time spent on exploring the object in the novel location as a percentage of the time spent on exploring both objects.

Morris Water Maze

Analysis of the latencies during the training sessions (the time it took the animals to find the platform) by a two-way ANOVA and

followed by a Bonferroni post hoc analysis, in which we compared all the groups to the control group, showed no significant differences (FIG. 8A).

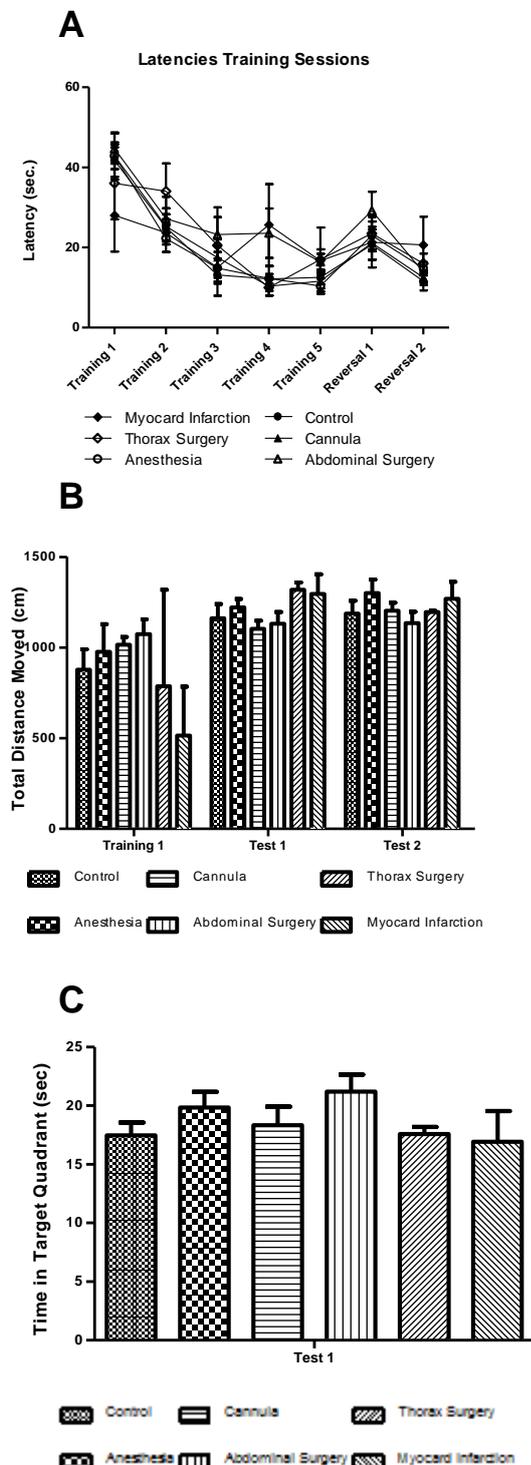


Figure 8. Morris Water Maze. A Average of the time (sec.) in which the animals found they platform during the training trails. B Average of the total distance moved (cm) per group during the first training trail and the tests on days 2 and 3. C Average time (sec) per group spent in the target quadrant the first test at day 2.

During the first training session and the tests on days 2 and 3 we recorded the performance of the animals and used this to calculate the total distance they had moved (FIG. 8B) and their velocity (Appendix C). When we compared the data from the groups that had undergone surgery with the control group by means of a two-way ANOVA there was no significant difference.

We measured the time spent in the target quadrant, the quadrant where the platform is located, as well. Analysis by means of a two-way ANOVA showed that there was no significant difference between the groups in time spent in the target quadrant during the first test (FIG. 8C) nor was there a significant difference between the groups during the second test (data not shown).

DCX staining

We analyzed the data with a one-way ANOVA which showed a significant difference ($F_{4,50}=3,102, p=0,0234$) (FIG. 9). Then we used a Tukey post hoc analysis and this showed that there was a significant difference between the control group and the group that had undergone surgery and was tested after one week ($p<0,05$).

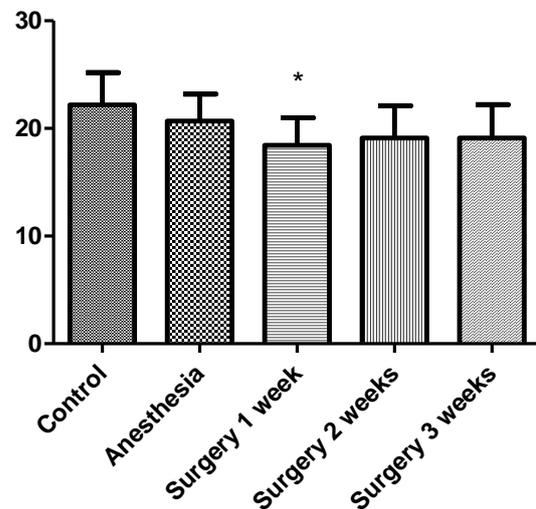


Figure 9. DCX staining of brain coupes obtained from a previous experiment. The amount of counted DCX-positive cells was corrected for hippocampus size. The asterisk indicates a significant difference compared to the control group ($p<0,05$).

IBA-1 staining

We analyzed the data for the three regions of the hippocampus (CA1, CA3 and dentate gyrus inner blade) per region with a one-way ANOVA and a Tukey post hoc analysis. This showed a significant decline in microglia activation for the CA1 region in animals tested after 3 weeks compared to animals tested after 1 week ($F_{3,38}=3,687$, $p=0,0201$) (**FIG. 10**). There was also a significant decrease of microglia activation for the DGIB in animals tested after 3 weeks ($F_{3,37}=8,963$, $p=0,0001$) compared to control animals ($p<0,05$), animals tested after 1 week ($p<0,0001$) and animals tested after 2 weeks ($p<0,05$). There were no significant differences for the CA3 region.

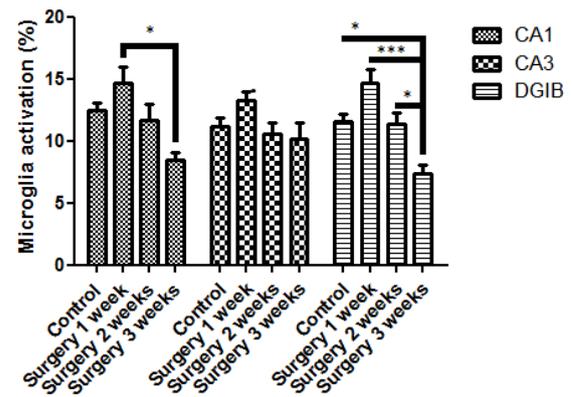


Figure 10. IBA-1 staining of brain coupes obtained from a previous experiment. The asterix indicates a significant difference (* $p<0,05$; *** $p<0,001$).

Conclusion and Discussion

POCD is mainly seen in the elderly after severe surgery, and affects mood and cognition.

To test the effect of the different surgeries on the mood of the animals, we performed a sucrose preference test and an open field test. The sucrose preference test is used as a model for anhedonia which is seen in depression (Willner et al., 1987). When animals are depressed their preference for sucrose water will be lower compared to the control group. As seen in Figure 5, only the animals that received a jugular vein cannula differed significantly from the control group. The other interventions did not seem to have an effect on the animals' preference for sucrose water. The high preference for sucrose in the induced myocardial infarction group is contradicting with what is described in literature and what we expected, since other articles show a decreased preference for sucrose water in animals that had a myocardial infarction (Wann et al., 2009). There is not much known about the effect of an abdominal surgery or a jugular vein cannula on sucrose preference, but we hypothesized that this group would also show a reduction in sucrose preference since these surgeries can be described as severe.

The open field test is used to measure differences in explorative behavior and anxiety (Prut and Belzung, 2003). However, whereas the sucrose preference test indicates that the animals that received a jugular vein cannula show more depressive like behavior, in the open field test there were no significant differences between the groups when we compared time spent in the corners nor when we compared time spent in the center of the open field arena (FIG. 6). The animals that received a jugular vein cannula seem to spent more time in the center of the open field, even though this is not significant. Since being in the center of the open field means that you are exposed to possible predators, this may be a sign of increased depressive like behavior in this group. Our results for this test are also contradicting with the existing literature, which reports less time spent in the center and more time spent in the corners of the open field for rats with a myocardial infarction

(Schoemaker and Smits, 1994), again there is little known about the effects of abdominal surgery and a jugular vein cannula.

The final tests that we performed to test changes in mood were the novel object recognition test and novel location recognition test. Usually these tests are performed to assess hippocampal and non-hippocampal memory (Barker and Warburton, 2011), but we used these tests to measure interest and attention as well. In these tests we did not see any significant differences between the groups, indicating that the surgeries had no effect on interest and attention (FIG. 7). However, there seems to be a trend in the novel location recognition test that suggests that the animals that received a jugular vein cannula or an abdominal surgery spent more time at the novel location. This is contradictive to what we expected since we hypothesized that the animals receiving a more severe surgery, like the jugular vein cannula or abdominal surgery, would show more depressive like behavior and as a consequence less explorative behavior.

To test the effect of the different surgeries on the cognition of the animals, they were subjected to the Morris water maze and to novel object and location recognition tests, which were described in the section above. While the novel object and location recognition tests are used to assess short term memory, the Morris water maze is used to investigate spatial learning and long term memory (D'Hooge and De Deyn, 2001). As can be seen in Figure 8, there are no differences in the Morris water maze between the groups' learning curves or time spent in the target quadrant, used as a measure for long term memory. This indicates that none of the interventions affected the abilities of the animals to learn this task nor did they affect long term memory. These results are partly contradicting with the available literature, while in the literature it is described that animals that underwent abdominal surgery perform worse on the Morris water maze test than the controls (Cao et al., 2010). On the other hand, there is an article by Wann and colleagues (2009) which reports no

differences between rats that underwent myocardial infarction and control rats, as is also seen in our experiment.

To summarize, only the animals that received a jugular vein cannula showed depressive like behavior in the sucrose preference test. In the open field test there were no significant differences between the groups, but there is a trend that shows that the animals that received a jugular vein cannula spent more time in the center of the open field arena. There were also no significant differences in the novel object and location recognition tests. Nor did we observe any significant differences between the groups in the Morris water maze test.

However, in a previous experiment that we performed in which the rats were tested in a similar manner 1, 2 or 3 weeks after their abdominal surgery with a cannulation we did see differences in mood and cognition compared to the control group. In addition, other researchers report cognitive decline and POCD in rats as well (Wang et al., 2012; Kong et al., 2013). Though it has to be noted that they used older rats for their research.

There are several explanations why we did not find POCD in our study. First, it might be that in the current experiment the surgeries were not severe enough. In this experiment the animals received either an abdominal surgery, an induced myocardial infarction or a jugular vein cannula. While in the previous experiment the animals received both an abdominal surgery and a jugular vein cannula. Second, POCD is mainly seen in the elderly after severe surgery. But not every elderly person that undergoes a severe surgery develops POCD (Wan et al., 2007). Therefore, it is possible that our animals did not develop POCD even though their surgeries could be qualified as severe, especially since we used young animals in this study.

Additionally, the number of people that report cognitive dysfunction after surgery declines over time (Steinmetz et al., 2009; Cao et al., 2010). It is possible that this also happened in our animals and that we would have seen differences in mood and cognition between the different groups if we had tested them sooner after their surgeries. This is confirmed

by an article from Cao *et al.* (2010) in which they saw only a learning impairment on postsurgical day 1 in adult rats (3-6 months) and on postsurgical days 1 till 3 in aged rats (20-24 months).

We also performed immunohistochemical stainings on material obtained from the previous experiment mentioned earlier.

When we performed an IBA-1 staining on the brain material from these animals we saw a decrease of microglia activation in the CA1 and DGIB of the hippocampus of animals that were tested 3 weeks after surgery compared to animals that were tested 1 week after surgery (FIG. 10). In addition, in the DGIB this reduction of microglia activation was also significant compared to control animals and animals that were tested 2 weeks after surgery. A possible explanation for this reduction of microglia activity might be that there is a depletion of inflammatory factor resources as a result of ongoing inflammation (Block and Hong, 2005; Degos et al., 2013; Hellwig et al., 2013).

We also performed a DCX staining on brain coupes from these animals. We saw a significant decrease in DCX positive cells in animals that were tested 1 week after surgery. The DCX staining is used to stain for new neurons, so this indicates that the animals that were tested one week after surgery had significantly less newly formed neurons compared to the control group (FIG. 9). This might be a consequence of the inflammatory reaction that occurs after surgery. As it is known that inflammation causes a decrease in neurogenesis (Song and Wang, 2011). It is thought that this decrease is associated with depressive like behavior and cognitive impairment.

Considering we did not see any changes in mood or cognition in the current experiment while we did see changes in the previous experiment, we think it might be worthwhile to include two additional experimental groups in the current experiment; abdominal surgery with a jugular vein cannula and induced myocardial infarction with a jugular vein cannula.

References

- Albensi BC, Mattson MP. (2000) Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse*; 35(2):151-159.
- Barker GR, Warburton EC. (2011) When is the hippocampus involved in recognition memory? *The Journal of neuroscience*; 31(29):10721-10731.
- Barrientos RM, Hein AM, *et al.* (2012) Intracisternal interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. *The Journal of neuroscience*; 32(42):14641-14648.
- Bedford PD. (1955) Adverse cerebral effects of anaesthesia on old people. *Lancet*; 269(6884):259-263.
- Block ML, Hong JS. (2005) Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Progress in Neurobiology*; 76(2):77-98.
- Bujak M, Frangogiannis NG. (2009) The role of IL-1 in the pathogenesis of heart disease. *Archivum Immunologiae et Therapiae Experimentalis*; 57(3):165-176.
- Cao XZ, Ma H, *et al.* (2010) Postoperative cognitive deficits and neuroinflammation in the hippocampus triggered by surgical trauma are exacerbated in aged rats. *Progress in neuro-psychopharmacology & biological psychiatry*; 34(8):1426-1432 .
- Cibelli M, Fidalgo AR, *et al.* (2010) Role of Interleukin-1b in Postoperative Cognitive Dysfunction. *Annals of Neurology*; 68(3):360–368.
- Dantzer R. (2004) Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *European journal of pharmacology*; 500(1-3):399-411.
- Degos V, Vacas S, *et al.* (2013) Depletion of bone marrow-derived macrophages perturbs the innate immune response to surgery and reduces postoperative memory dysfunction. *Anesthesiology*; 118(3):527-536.
- D’Hooge R, De Deyn PP. (2001) Applications of the Morris water maze in the study of learning and memory. *Brain Research Reviews*; 36(1):60-90.
- Echols MR, O’Connor CM. (2010) Depression after myocardial infarction. *Current heart failure reports*; 7(4):185-193.
- Fidalgo AR, Cibelli M, *et al.* (2011) Systemic inflammation enhances surgery-induced cognitive dysfunction in mice. *Neuroscience Letters*; 498(1):63-66.
- Frangogiannis NG, Smith CW, *et al.* (2002) The inflammatory response in myocardial infarction. *Cardiovascular research*; 53(1):31-47.
- Hellwig S, Heinrich A, *et al.* (2013) The brain's best friend: microglial neurotoxicity revisited. *Frontiers in Cellular Neuroscience*; 7(71):1-11.

- Hovens IB, Schoemaker RG, *et al.* (2012) Thinking through postoperative cognitive dysfunction: How to bridge the gap between clinical and pre-clinical perspectives. *Brain, Behavior and Immunity*; 26(7):1169–1179.
- Hu Z, Ou Y, *et al.* (2010) Inflammation: a bridge between postoperative cognitive dysfunction and Alzheimer's disease. *Medical hypotheses*; 74(4):722-724.
- Huang ZB, Sheng GQ. (2010) Interleukin-1 β with learning and memory. *Neuroscience Bulletin*; 26(6):455-468.
- Hudson AE, Hemmings HC Jr. (2011) Are anaesthetics toxic to the brain? *British Journal of Anaesthesia*; 107(1): 30-37.
- Kong F, Chen S, *et al.* (2013) Minocycline attenuates cognitive impairment induced by isoflurane anesthesia in aged rats. *PLoS ONE*; 8(4):e61385-e61385.
- Krenk L, Rasmussen LS, *et al.* (2010) New insights into the pathophysiology of postoperative cognitive dysfunction. *Acta anaesthesiologica Scandinavica*; 54(8):951-956.
- Matusik P, Guzik B, *et al.* (2012) Do we know enough about the immune pathogenesis of acute coronary syndromes to improve clinical practice? *Thrombosis and haemostasis*; 108(3):443-456.
- McAfoose J, Baune BT. (2009) Evidence for a cytokine model of cognitive function. *Neuroscience & biobehavioral reviews*; 33(3):355-366.
- Newman S, Stygall J, *et al.* (2007) Postoperative Cognitive Dysfunction after Noncardiac Surgery. A Systematic Review. *Anesthesiology*; 106:572-590.
- Prut L, Belzung C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European Journal of Pharmacology*; 463(1–3):3-33.
- Ramlawi B, Rudolph JL, *et al.* (2006) C-Reactive protein and inflammatory response associated to neurocognitive decline following cardiac surgery. *Surgery*; 140(2):221-226.
- Schoemaker RG, Smits JF. (1994) Behavioral changes following chronic myocardial infarction in rats. *Physiology & behavior*; 56(3):585-589.
- Silbert B, Evered L, *et al.* (2011) Cognitive decline in the elderly: is anaesthesia implicated? *Bailliere's best practice & research. Clinical anaesthesiology*; 25(3):379-393.
- Smith JA, Das A, *et al.* (2012) Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain research bulletin*; 87(1):10-20.
- Song C, Wang H. (2011) Cytokines mediated inflammation and decreased neurogenesis in animal models of depression. *Progress in neuro-psychopharmacology & biological psychiatry*; 35(3):760-768.
- Spies CD, Kern H, *et al.* (2002) Myocardial ischemia and cytokine response are associated with subsequent onset of infections after noncardiac surgery. *Anesthesia and analgesia*; 95(1):9-18.

Steinmetz J, Christensen KB, *et al.* (2009) Long-term consequences of postoperative cognitive dysfunction. *Anesthesiology*; 110(3):548-555.

Terrando N, Monaco C, *et al.* (2010) Tumor necrosis factor-alpha triggers a cytokine cascade yielding postoperative cognitive decline. *PNAS*; 107(47):20518-20522.

Wann BP, Bah TM, *et al.* (2009) Behavioural signs of depression and apoptosis in the limbic system following myocardial infarction: effects of sertraline. *Journal of psychopharmacology*; 23(4):451-459.

Wan Y, Xu J, *et al.* (2007) Postoperative Impairment of Cognitive Function in Rats. A possible Role for Cytokine-mediated Inflammation in the Hippocampus. *Anesthesiology*; 106(3):436-43.

Wang H, Xu Z, *et al.* (2012) Changes of learning and memory in aged rats after isoflurane inhalational anaesthesia correlated with hippocampal acetylcholine level. *Annales françaises d'anesthésie et de réanimation*; 31(3):e61-e66.

Willner P, Towell A, *et al.* (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology*; 93(3):358-364.

Appendix A – staining DCX

Aim:

- DCX stains newly formed neurons; this may be a marker of changes after surgery that is still visible on the long term.

Antibodies: 1st AB Double cortin (c-18) sc8066 lotJ0109 goat Santa Cruz
2nd AB Rabbit anti goat 305-065-603 lot.... Jackson

Protocol:

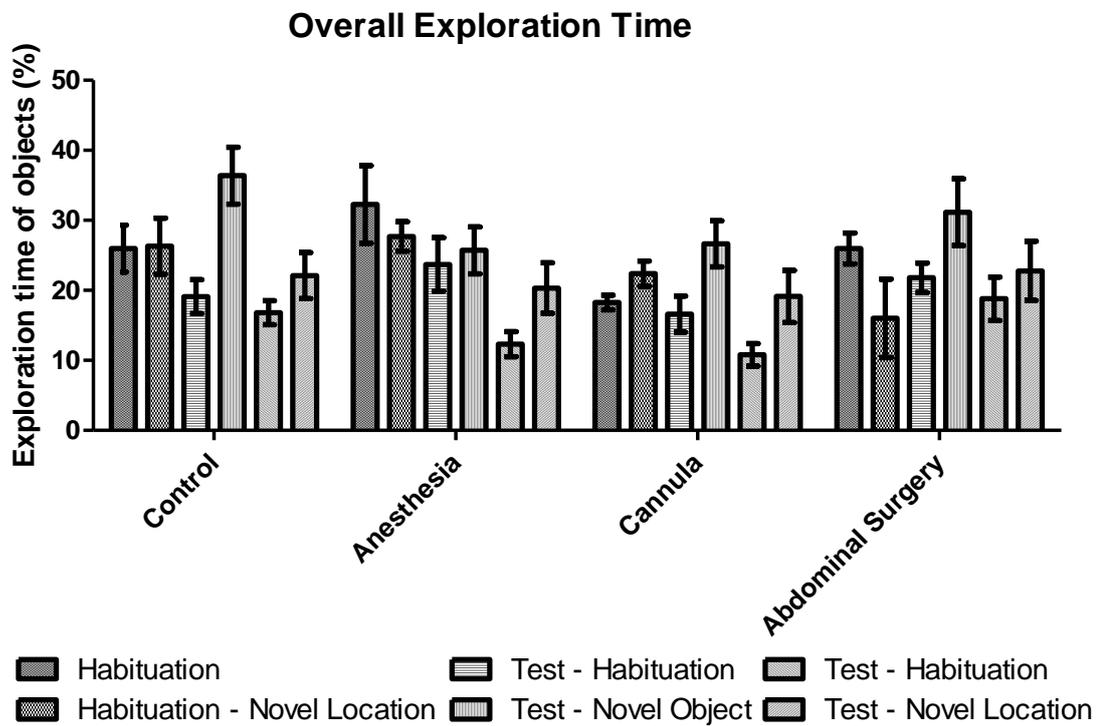
- Day 1**
- rinse 2x with 0.01M PBS
 - incubate 20 min with 3% H₂O₂
 - rinse 4x with 0.01M PBS
 - incubate 1 hour with 5% normal rabbit serum in 0.01M PBS
 - incubate with 1:1000 DCX 1st AB in 0.01M PBS, 0.1% Triton, 1% BSA, 1% normal rabbit serum
 - 3 hours at 37°C
 - overnight at room temperature
- Day 2**
- incubate 3 nights in cold room
- Day 5**
- rinse 5x with 0.01M PBS
 - incubate with 1:500 2nd AB in 0.01M PBS, 0.1% Triton, 1% BSA
 - overnight in cold room
- Day 6**
- rinse 5x with 0.01M PBS
 - incubate with ABC 1:500 in 0.01M PBS
 - 2 hours at room temperature
 - rinse
 - 3x with 0.01M PBS
 - 2 hours at room temperature
 - overnight in cold room
- Day 7**
- add DAB solution 3ml/cup (1 tablet for 15ml in 20ml H₂O, 3 grains of nickel/ 10ml)
 - activate with 100µl 0.1% H₂O₂ in H₂O
 - DAB -> reaction time depends on staining
 - rinse
 - 3x quickly with 0.01M PBS
 - depending on staining/ background ration overnight in cold room or not

Appendix B – staining IBA-1

- Day 1**
- rinse 3x 5 min with 0.01M PBS
 - incubate 30 min with 0.3% H₂O₂ in 0.01M PBS
 - rinse 3x 5 min with 0.01M PBS
 - sections are incubated with rabbit anti-IBA 1 (1:2500 Wako Chemicals, 019-19741) in 2% BSA in 0.01M PBS containing 0.1% TritonX for 72 hours on 4°C.
- Day 4**
- rinse 3x 5 min with 0.01M PBS
 - incubate 2 hours with biotin. conjugated secondary goat anti rabbit antibody 1:500, room temperature
 - rinse 6x 5 min with 0.01M PBS
 - DAB reaction 1 tablet/ 15ml solved in 30ml AD, add 100µl 0.1% H₂O₂
 - reaction was stopped after 2.5-4 min
 - rinse 3x 5 min with 0.01M PBS

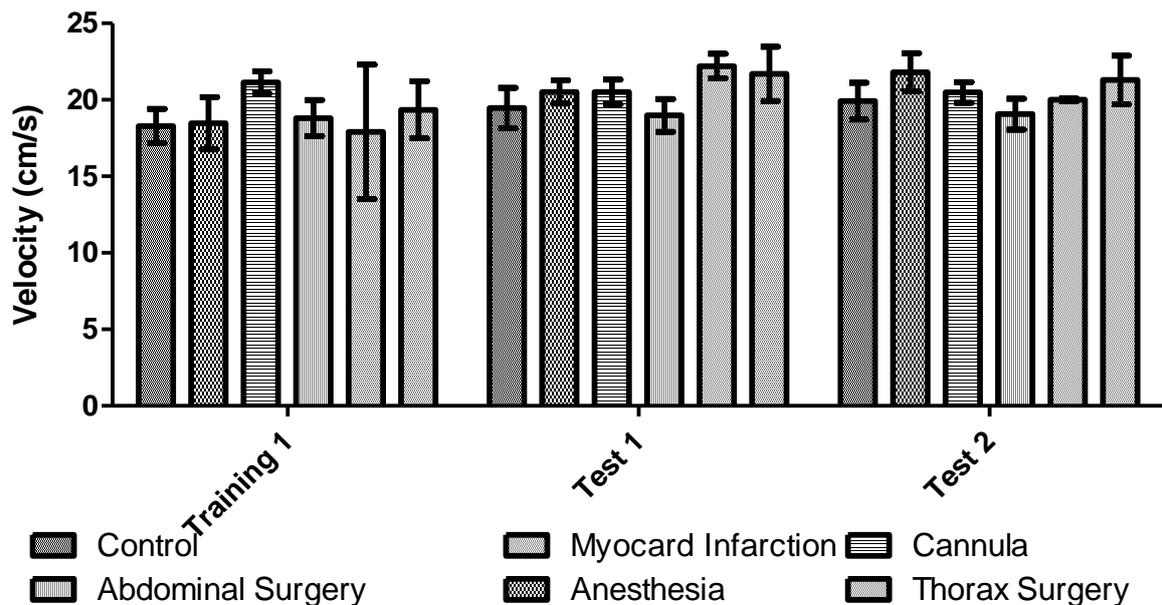
Appendix C – additional data

ADDITIONAL DATA FOR THE NOVEL OBJECT/LOCATION RECOGNITION TEST



Time spent on exploring both objects as a percentage of the time spent on overall exploration (exploration of objects and the cage).

ADDITIONAL DATA FOR THE MORRIS WATER MAZE TEST



Average velocity (cm/s) per group during the first training and the tests on day 2 and day 3. There were no significant differences.