7

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DEC datum goedkeuring#	Type aanvraag [2]	VROM/GGONR[3]	LNV/CBDNR[4]
15-08-2011	Nieuw	IG 10-023	
Hoofdproject	CARIM		
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Titel van het onderzoek:

Is NOX4 implicated in detrimental ROS production during ischemic stroke in rats and if so, is this target relevant in stroke therapy?

startdatum 05-09-2011 einddatum [9] 04-09-2015 *Duur van de proef*[10]: *17 days*

	Naam	Tel (+ Tel privé enkel VO, VVO en VM)	E-mailadres	Bevoegd-heid[5]	Cap. groep /afdeling
1.Verantwoordelijk onderzoeker (VO)				Art.9	
2. Vervanger VO (VVO)				(Art.9 application submitted)	
3. Verantwoorde-lijk medewerker (VM) GGO[7]				Art.9	
4. overige uitvoerenden				Art. 12	
5. PI and hoofd Farmacologie					

Diergroep	1	2	3	4	5	6
ctrl/exp/sham	Exp set 1	Exp set 2	Exp set 3	Exp set 3	Exp set 4	Exp set 5
Diersoort	02	02	02	02	02	02
Stam	WKY	WKY	WKY	WKY	WKY	WKY
Construct / mutatie ?	WT	WT	WT	WT	WT	WT
Herkomst (leverancier) *	Harlan	Harlan	Harlan	Harlan	Harlan	Harlan
Aantal	20	10	10	12	46	161
Geslacht	M	M	M	M	M	M
Dieren immuuncompetent ?	ja	ja	ja	ja	ja	ja
Leeftijd/gewicht	10-12 weeks	10-12 weeks	10-12 weeks	10-12 weeks	10-12 weeks	10-12 weeks
Doel van de proef *	29	29	29	31	31	31
Belang van de proef *	1	1	1	1	1	1
Toxicologisch onderzoek *	1	1	1	1	1	1
Bijzondere technieken *	1	1	1	1	1	1
Anesthesie *	4	4	4	4	4	4
Pijnbestrijding *	4	4	4	4	4	4
Mate ongerief *	05	02	04	04	05	05
Diergroep	1	1	1	1	1	1

* VHI-coderingen zie bijlage

TITEL: ***Is NOX4 implicated in detrimental ROS production during ischemic stroke in rats and if so, is this target relevant in stroke therapy?***

Verantwoording

1. Doel van de proef.

Oxidative stress is thought to play an important role in cerebral ischemia/reperfusion. Formation of reactive oxygen species (ROS) is particularly prominent in the stage when -following intervention- the tissue perfusion is restored (reperfusion). Thus, ROS are major determinants for tissue damage upon ischemia-reperfusion. So far, therapies with antioxidants, relying on reducing the amount of produced ROS, yielded disappointing results. A different approach however would be not to reduce the levels of already produced ROS but to interfere with triggers of ROS formation. NADPH oxidases (NOX) are the only enzyme whose sole function is ROS production and their activity is even higher in cerebral vessels than in systemic vessels. For this reason, they are thought to play a major role in stroke. There is now preliminary evidence that most relevant NOX isoforms in stroke are NOX2 and NOX4. NADPH oxidases may present a future therapeutic target and their inhibition might induce clinically relevant neuroprotection.

recently published that deletion of the NOX4 gene in mice greatly reduced ischemia-reperfusion damage of brain tissue after stroke. An important step towards clinical translation is to reproduce our previous findings in another species. As NOX4 knockout models (KO) in rats are not available, we want to knock down the NOX4 gene by intracerebroventricular (i.c.v.) injection of siRNA-carrying lentivirus. We first want to establish the new techniques required herefore. As we are not experienced in performing middle cerebral artery occlusion (MCAO) in rats, we will first need some extra **training rats to practice the transient MCAO (exp set 1).** [redacted] Moreover, we also have **to establish the intracerebroventricular injection (exp set 2)** and then to find out the appropriate **dose of siRNA-lentivirus that will be injected in rats to accurately knock down NOX4 expression (exp set 3).** Only after these 3 preliminary exp sets, we can proceed with the proper investigation of the impact of NOX4 knockdown in ischemic stroke. Therein **lesion size will be assessed in NOX4 knock-down rats (NOX4 KD) and in control rats 1 day after tMCAO (exp set 4).** Furthermore, in this same paper of neuroprotection was achieved after post-stroke administration of a novel NOX inhibitor to normal (wildtype) mice, whereas the infarct size could not further be decreased in NOX4 KO mice. This not only indicates that NOX4 plays an important role in stroke pathophysiology, but also that NOX inhibitors may have substantial therapeutic merit. Therefore, we want to screen different high potential NOX inhibitors. Based on clinical relevance we **will inject our drugs intrathecally (i.t.), but we first have to practice this type of injection ([redacted]).** Once the i.t. injections are routine, we want to **assess the neuroprotective effect of different specific NOX inhibitors in rats after ischemic stroke (exp set 5).** All animal handling that involves lentivirus injection will be performed in a MLIII lab (exp set 3,4+5).

2. Maatschappelijke relevantie en/of wetenschappelijk belang

Stroke is one of the most frequent pathologies in the industrialized world and its occurrence will even further increase in the future due to aging of the population. One fifth of affected people will die and even if they survive, brain damage can be so important that they have to cope with various disabilities for the rest of their life. Although prevalence of stroke is continuously rising, neuroprotective treatments that can be administered post stroke are still

scarce goods. The only FDA-approved medication, the tissue plasminogen activator (tPA), can only be administrated in 15% of the cases because of numerous contra-indications. Proposed experiments will help to have a better knowledge about the underlying mechanisms of neuronal injury during stroke and to open new therapeutic perspectives to reduce such damage.

3. Alternatieven

We aim to establish the pathophysiological role of NOX4 in ischemia-reperfusion damage in the brain and also to find out whether pharmacological inhibition of NOX can reduce such damage. Given the complex interplay between organs and regulatory systems in determining the neuronal response to ischemia-reperfusion, there are no alternatives to animal experiments. Since we aim to test the feasibility of a new approach that in future can be translated into a clinically applicable concept, pre-clinical experiments are desirable. However, the efficiency of the drugs that will be used in our study (exp set 5) to specifically inhibit NADPH oxidases has been established on cell-based and cell-free in vitro assays [1-5].

4. Ethische afweging

While the experiments on the one hand will help to find potential new therapeutic strategies to tackle stroke damage and thus improve the life expectancy and quality of life in man, they on the other hand will inevitably lead to discomfort in animals. However, this discomfort will be kept to a minimum by working according to the carefully constructed SOPs that have been screened by the DEC. In addition, the number of animals that will be used for the experiments is the minimum number that is required to generate solid (hence useful) findings.

Wetenschap

5. Wetenschappelijke onderbouwing

Although prevalence of stroke is continuously rising, neuroprotective treatments are still scarce goods. Therefore, it is important to have a better knowledge about the underlying mechanisms of neuronal injury during stroke. If cerebral blood supply is temporarily reduced by transient occlusion of the main feeding blood vessels, oxidative stress occurs, resulting in extensive brain damage. Nevertheless, despite the well established role of reactive oxygen species (ROS) in the pathophysiology of major ischemic diseases, so far the therapeutic success of tackling the produced ROS by using antioxidants has been disappointing [6]. The rapidly acting ROS may already have damaged cells near or at the site where they have been produced before the antioxidants are able to inactivate them. In addition, some antioxidants themselves are converted by ROS into reactive molecules and may hence contribute to tissue damage. A potentially more meritorious approach to tackle ROS-mediated tissue damage is to prevent their formation by blocking the trigger of excessive ROS production. NADPH oxidases (NOX) is a family of enzymes known to produce ROS and to be involved in a number of important pathological and physiological events [7] [8]. In the course of the last years, different isoforms of this enzyme have been discovered. Many isoform-specific knockout models have been generated to elucidate the role of each single NOX homologue. There is now preliminary evidence that most relevant forms in stroke are NOX2 and NOX4. Concerning the NOX4 isoform, the research group of [redacted] at UM), recently discovered that in mice, in which the NOX4 gene was knocked out, ischemic stroke produced far less brain tissue damage than in normal (wildtype) mice [redacted]. In addition, applying a NOX inhibitor to mice also reduced stroke-induced brain damage, even if the inhibitor was applied 2 or 12 hours after ischemia. This indicates that NOX4 plays a crucial role in ischemia-reperfusion damage in the brain and that NOX inhibition opens challenging new therapeutic perspectives. An important step before moving towards clinical studies would be to repeat our previous findings in a second species. As

knock-out model are too expensive in rats, we want to knock-down the NOX4 gene in rats with siRNA and compare the post-stroke lesion size to control rats. Since NADPH oxidases may present a future therapeutic target, we want to test the efficiency of specific NOX inhibitors in stroke such as (and a promising compound from the Nox inhibition might induce clinically relevant neuroprotection.

6. Wetenschappelijke beoordeling
This specific project has been approved by (

Proefdier

7. Proefdier keuze
7a. Soort, stam / herkomst / eindbestemming
For all experiment sets, wildtype WKY rats from Harlan will be used. We decided to use this strain because we are also planning future experiments in SHR rats and WKY rats are widely used as normotensive control for the SHR rats. Furthermore, in a recent review on stroke models, authors recommended the use of WKY for stroke studies [13].

7b. Sexe

For the preliminary pilots only male rats will be used. Since we are testing the feasibility of a potential treatment option for cerebral ischemia-reperfusion damage that may be translated to a clinical application in the future, it is essential that both male and female animals will be used. Thus, we will need to screen for sex differences if the first pilot is successful. In this case we will hand in an addendum later on.

7.c. **Aantallen**

In stroke experiments, our primary read-out will be the infarct size of the brain after ischemia-reperfusion. Using a power analysis according to the formula $n=2x\ s^2x\ (Z\square\square+Z\beta)^2/D^2$(L. Sachs, Angewandte Statistik, Springer, 1983, Berlijn, Springer Verlag), a s= 30 % and a minimal statistically assessable treatment effect on infarct size (D) of 30 % , this implies that at a power of 80 % the minimum number of animals is $n= 15.7\ s^2/D^2 = 15.7$ per group. As we have never applied the MCAO surgery in rats, we can only estimate the animal loss based on the literature. Previous publications on transient MCAO performed in rats reported 30% mortality after 60 minutes of MCAO and 23h of reperfusion [14]. Taking into account an animal loss (due to acute mortality of the infarction) of 30%, this implies a total animal number of $15.7/0.7 = 22.4 \approx 23$ rats.
Thus, as explained above, the total number of animals per group will be 23.
If in exp set 4 and 6 the first starting experiment was successful, we should also repeat the findings in females and adult animals in future experiments. According to published stroke guidelines, a permanent stroke model and sham-operated rats should also be included in each experimental setup. But for this we will add an addendum to this DEC if in a first experiment (transient ischemic stroke performed in young male rats) we see a significant difference in stroke size.

Experiment set 1:
Transient MCAO and i.c. injection have to be established in rats. For this operation (, art. 12) we calculate 10 mice per person. Thus we will need **20 male WKY rats (10-12 weeks).**

Experiment set 2:
I.c.v. injection that is needed for the injection of the siRNA-carrying lentivirus has also to be trained by : **20 male WKY rats (10-12 weeks).**

Experiment set 3:
To determine the correct dose of the siRNA-carrying lentivirus that has to be injected i.c.v., we will try 4 different doses. Each dose we would like to test in 3 animals: **12 male WKY rats (10-12 weeks).**

Experiment set 4:
Starting experiment: As explained above, 23 male WKY rats (10-12 weeks) will be needed for the NOX4-siRNA group and 23 male rats for the control group injected with a scrambled siRNA.
The total amount of rats needed for this experiment will be 46.
Of course, if the starting experiment shows negative results after 20 rats, we will not perform experiments with the remaining 26 rats.

Experiment set 5:
Number of groups: 7
- WT vehicle group
- 4 groups, where WT rats are treated with one of the 4 NOX inhibitors
- NOX4siRNA vehicle group
- NOX4siRNA group injected with \

Starting experiment: Each group will contain 23 male WKY rats (10-12 weeks).
As we have 7 groups, the total amount of rats needed for this experiment will be 161 rats.
Of course, if the starting experiment shows negative results after 10 rats/group, we will not perform experiments with the remaining 91 rats.

→ TOTAL: 259 rats

Dierproef

8. Experiment
Here, only the aim of the planned experiments is summarized. Flow charts and more detailed setups of the experiments are given in the addendum.
Aim experiment set 1: Establish transient MCAO in rats and practice the intrathecal drug infection.
Aim experiment set 2: Establish intracerebroventricular (i.c.v.) cannula insertion.
Aim experiment set 3: Determine the appropriate dose of siRNA-expressing lentivirus that will be injected i.c.v. to sufficiently knock-down the NOX4 gene in rats.
Aim experiment set 4: To find out whether the findings that we previously published for mice are applicable to rats as well [9], i.e. can the concept of inhibiting/shutting down NOX4 for treating stroke be applied to a wider range of species than mice alone (and thus be more easily translated to humans). As there is no KO model available in rats, the NOX4 gene will be knocked down by siRNA and than stroke size will be compared to control WKY rats.
Aim experiment set 5: To evaluate the therapeutic efficiency of specific NOX inhibitors

') in WKY rats after stroke.
Infarct volume will be compared to normal WKY rats or to NOX4 KD rats.

9. Experimentele condities

9a. Anesthesie

In all experiments anesthesia (and analgesia) will be applied according to the SOPs. All anesthetics seem to have protective effect in cerebral ischemia/reperfusion, whether this is caused by hypothermia, reduced blood flow or other interactions is not always clear. We will opt for isoflurane as anesthetic because a recent review recommended it for stroke studies [13] At least we will avoid anesthetics with a marked intrinsic neuroprotective effect such as ketamine. Since the control rats will also be anesthetized the same way, the impact of isoflurane on our results will be less important.

In experiment sets 1, 4 and 5: At day 0, middle cerebral artery occlusion (MCAO) is performed (SOP_MCAO). After closing the wound animals were allowed to recover to assess their neurological function (Bederson score and grip test). For transient MCAO, the wound will be re-opened again 1h after occlusion and the occluding filament will be withdrawn to allow reperfusion. Both operations (occlusion and reperfusion) will be conducted under isoflurane anesthesia (initiating at 3-4 %, maintenance at 1.5-2.5 %). Finally, at day 1 (immediately after a second evaluation of the neurological function), rats will be sacrificed by pentobarbitone injection (60 mg/kg BW i.p.) and blood withdrawal.

In experiment set 2, 3, 4 and partly in set 5, a cannula will be installed in the right lateral cerebral ventricle of rats (SOP_ICV) under isoflurane anesthesia (initiating at 3-4 %, maintenance at 1.5-2.5 %). For intrathecal injection as used in exp set 1, 4 and 5, animals will also be anesthetized prior to the injection of the drug. The animals used for training (exp set 1,2,3+5) will be sacrificed by injecting a pentbobarbitone overdose (200 mg/kg BW i.p.).

9b. Pijnbestrijding

Analgesia will be performed according to the SOPs. In general, the use of buprenorphine is preferred over NSAIDs because the anti-inflammatory effects of the latter could interfere with our experimental results. On day 0, buprenorphine (0.1 mg/kg BW) will be applied subcutaneously just before surgery (MCAO) in experiment sets 1, 4 and 6 and then again in the evening and the next morning after the surgery. At that dose, buprenorphine gives pain relief for 8-12 hours. After 24 hours animals will be sacrificed.

In exp set 2, 3, 4 and 5, a local analgesic (lidocaine) will be applied on the periost prior to i.c.v. cannula insertion. After lentivirus injection and cannula removal, buprenorphine (0.1 mg/kg BW s.c.) will also be applied during 24 hours (3 times) post-intervention, at least for the animals that are not sacrificed immediately after intervention. If the intrathecal injection will only take place after the MCAO surgery, rats already got painkillers that are effective for the following 12h and thus do not need an additional analgesic (exp set 5).

9c. Euthanasie en Humane eindpunten

We will monitor body temperature to detect possible infections and also because hyperthermia is a common complication in stroke (if the hypothalamus is damaged). Unfortunately neurological signs of discomfort cannot be avoided because they are intrinsic to our stroke study. But at least we try to limit the discomfort temporally, as the rats are sacrificed 24 hours after MCAO operation. Furthermore, we will place some macerated food pellets and water inside of the cage to simplify the feeding. Should any other sign of an additional disease be observed (i.e. inflammation), the Art. 14 functionaris will be consulted and if necessary the animals will be sacrificed by a pentobarbitone overdose (200 mg/kg BW i.p.). Animals that do not undergo MCAO will be regularly inspected and monitored for other signs of illness and discomfort (e.g. passive behaviour also at approaching, lack of grooming,

> 15 % weight loss, attitude, skin/fur condition).

Zorg

10a. Ongerief

Most severe discomfort in experiment sets 1,4 and 5 [redacted] consists in transient cerebral ischemia by middle cerebral artery occlusion under isoflurane anesthesia (discomfort score 05). To induce transient stroke, the wound has to be re-opened under isoflurane 1h after recovery to withdraw the occluding filament (discomfort score 05). It is important to let the animals regain consciousness in between to observe their behavior. Before reperfusion and 1 day after stroke induction, we will assess the neurological outcome and motor function by Bederson score (discomfort score 02) and grip test (discomfort score 02) respectively. [redacted]

In exp set 2-5 [redacted], an i.c.v. injection will be performed to inject the siRNA-carrying lentivirus under isoflurane (discomfort score 05). Half of the animals of exp set 2 [redacted] will be anesthetized and then sacrificed immediately after intervention by pentobarbitone overdose (200 mg/kg BW i.p.). When mice do not have to wake up after intervention, the discomfort is reduced to a score 02. The remaining animals of exp set 2 [redacted] have to regain consciousness because we want to check their health status after intervention.

To administer the different drugs in set 1 and 5 [redacted], an intrathecal injection (discomfort score 03-04) under isoflurane anesthesia will be necessary.

The rats will either be immediately sacrificed by pentobarbitone overdose (200 mg/kg BW i.p.) or 24h post-stroke by pentobarbitone (60 mg/kg BW i.p.) and blood withdrawal (discomfort score 02).

Thus for experiment set 1, 4 and 5 ([redacted] the total discomfort is serious because of the MCAO operation (score 05). [redacted]

Intervention	Time period	Frequency	Discomfort
s.c. injection of bruprenorphine	1 min	3x for MCAO [redacted], 3x for i.c.v. injection [redacted] and 6x when i.c.v. injection and MCAO [redacted]	03
MCAO	15 min	1x [redacted]	05
Filament removal	10 min	1x [redacted]	05
Bederson score	10 min	2x [redacted]	02
Grip test	10 min	2x [redacted]	02
[redacted]	[redacted]	[redacted]	[redacted]
i.c.v. placement of the cannula, lentivirus injection and removal of the cannula	1 h	1x [redacted]	04
[redacted]	[redacted]	[redacted]	[redacted]
i.t. injection of the NOX	15 min	[redacted]	03-04

inhibitor			
Pentobarbitone i.p. and blood withdrawal	10 min	1x [redacted]	02
Pentobarbitone overdose i.p.	1 min	1x [redacted]	02

10b. Welzijnsevaluatie

Rats will be regularly inspected for signs of illness or discomfort. In our experience, stroke may lead to an acute (within 12-24 hours) drop-out of 10-25 % of the animals due to sudden death. After recovery, signs of discomfort such as clock-wise circling are also observed. While its condition is deteriorating, the animal will be sacrificed as specified under point 9 of this DEC protocol. Should other signs of illness, discomfort or disease occur, the Artikel 14 functionaris will be contacted.

11. Verzorging en huisvesting

[redacted] Surgery and 1 day of recovery (in a special heated room) of the animals will take place in the animal lab of [redacted] Thereafter animals will be sacrificed

12. Deskundigheid

All animal-handling procedures will be conducted by experienced artikel 12 [redacted] or by [redacted] (waiting for artikel 9 approval) under supervision of artikel 12 co-worker. [redacted]

13. Standard Operation Procedures (SOP)

For the procedures/animal handlings (including anesthesia and analgesia) we would like to refer to the enclosed SOPs that have been approved by CPV.
SOP_MCAO (transient and permanent MCAO in rat and mice), SOP_functional outcome (Bederson score and grip test), SOP_ICV (intracerebroventricular injection), SOP _IT (intrathecal injection).

Relevante literatuur

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4. Tegtmeier F, Walter U, Schinzel R et al. Compounds containing a N-heteroaryl moiety linked to fused ring moieties for the inhibition of NAD(P)H oxidases and platelet activation. European Patent 1 598 354 A1 (2005).
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Radic Biol Med, 43(3), 332-347 (2007).
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12. Vendrov AE, Madamanchi NR, Niu XL, et al. NADPH oxidases regulate CD44 and hyaluronic acid expression in thrombin-treated vascular smooth muscle cells and in atherosclerosis. J Biol Chem, 285(34), 26545-57 (2002).
13. Howells DW, Porritt MJ, Rewell SSJ, et al. Different strokes for different folks: the rich diversity of animal models of focal cerebral ischemia. Journal of Cerebral Blood Flow & Metabolism, 30, 1412–1431 (2010).
14. Bigdeli MR. Prereconditioning with prolonged normobaric hyperoxia induces ischemic tolerance partly by upregulation of antioxidant enzymes in rat brain tissue. Brain Research,1260, 47-54 (2009).

Experiment set 4

Aim:
To find out whether the findings that we previously published for mice are applicable to rats as well [9], i.e. can the concept of inhibiting/shutting down NOX4 for treating stroke be applied to a wider range of species than mice alone (and thus be more easily translated to humans). As there is no KO model available in rats, the NOX4 gene will be knocked down (NOX4KD) by siRNA and than stroke size will be compared to control WKY rats.

Parameters to be assessed in vivo:

- Functional neuronal outcome by Bederson score
- Motor function and coordination by Grip test.

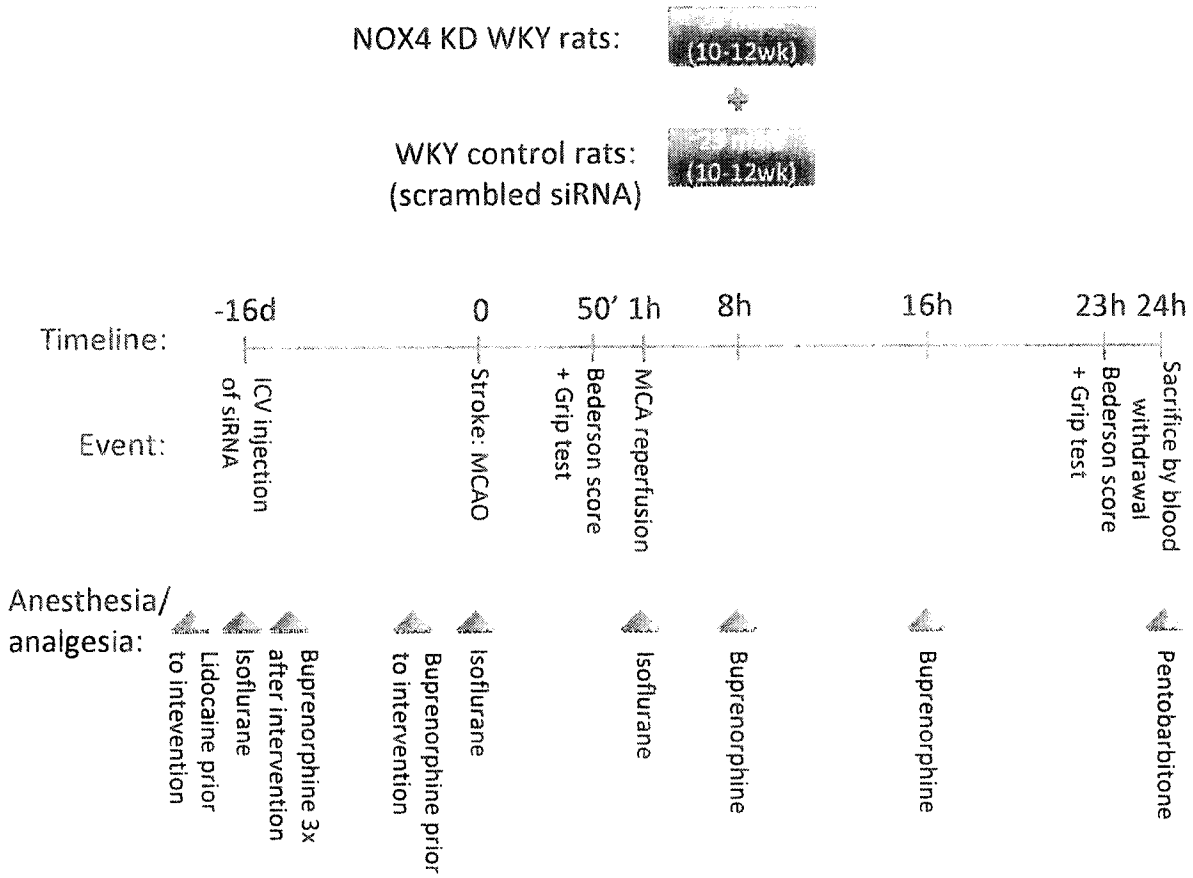
Ex vivo parameters:

- Stroke infarct volume (Histology of the brain: TTC staining)
- ROS staining in brain tissue (IHC, brain homogenate)
- Nitrotyrosin staining
- Apoptosis (TUNEL staining)
- Expression of NOX in brain tissue (qRT-PCR, WB, IHC)

Animal handling procedure in words (briefly):

The experiment will start with the placement of an intracerebroventricular (i.c.v.) cannula (SOP_ICV), 16 days before MCAO. For this, a local analgesic (lidocaine) has to be applied beforehand and then the NOX4KD-lentivirus will be injected into the brain. After immediate removal of the cannula, a painkiller (buprenorphine 0.1mg/kg BW s.c.) will be applied over a period of 1 day after intervention (3x). The animals should be kept in individual cages for at least one day after surgery. For the lentivirus injection we have to work in a MLIII lab, but as induction of stroke will take place 16 days after the injection, we can move to our normal animal lab again to perform MCAO. After previous administration of a painkiller (buprenorphine 0.1 mg/kg s.c.) at day 0, middle cerebral artery occlusion (SOP_MCAO) will be performed under isoflurane anesthesia in male NOX4KD rats and control WKY rats (N=23 each). After closing the wound, animals are allowed to recover in a room with controlled temperature and their neurological outcome will be assessed by Bederson score and Grip test (SOP_functional outcome). One hour after induction of cerebral ischemia the rats are anesthetized again by isoflurane and the occluding filament is removed from the artery to allow reperfusion. Buprenorphine injection will be repeated in the evening and in the morning after MCAO. After 1 day of tMCAO rats will be again assessed for neurological outcome and motor function. Immediately afterwards, they will be anesthetized by pentobarbitone and sacrificed by blood withdrawal. Then the brain will be extracted for histology or brain homogenate. All experimental procedures and the subsequent analyses will be conducted in such a way that the experimenters will be blinded regarding the genotype or drug treatment of the animals. Selection of the animals for experiments will be on an at random basis but taking care that animal groups will be standardized for bodyweights (or (subtle) age differences).

Animal groups, numbers and handling timeline for pilot:



Experiment set 6

Aim:
To evaluate the therapeutic efficiency of specific NOX inhibitors ([redacted] in WKY rats after stroke. Infarct volume will be compared to normal WKY rats or rats injected with siRNA (NOX4 KD).

Parameters to be assessed in vivo:

- Functional neuronal outcome by Bederson score
- Motor function and coordination by Grip test.

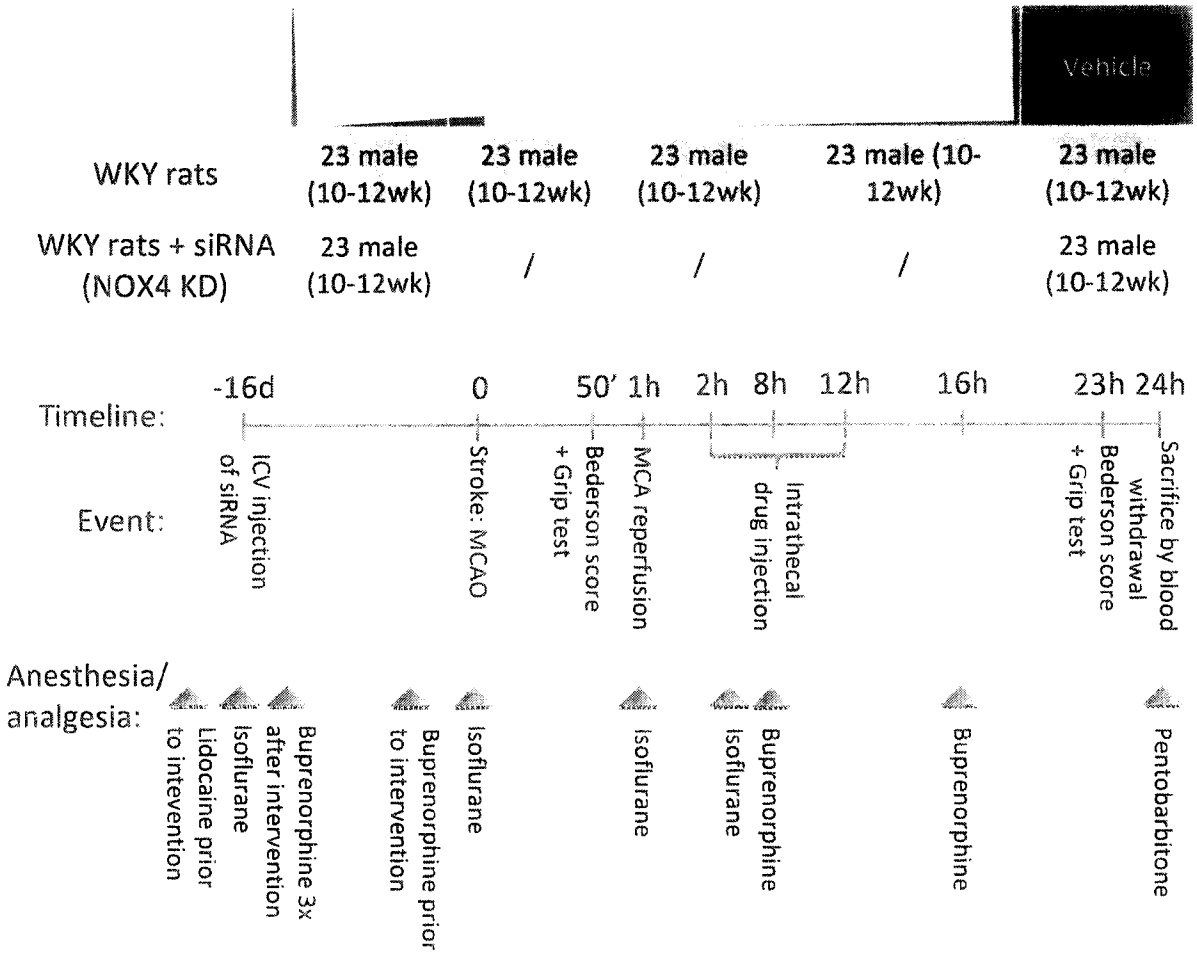
Ex vivo **parameters:**

- Stroke infarct volume (Histology of the brain: TTC staining)
- ROS staining in brain tissue (IHC, brain homogenate)
- Nitrotyrosin staining
- Apoptosis (TUNEL staining)
- Expression of NOX in brain tissue (qRT-PCR, WB, IHC)

Animal handling procedure in words (briefly):

For the group of NOX4KD rats we will perform i.c.v. injection 16 days before onset of MCAO, as described in exp set 4. For the induction of tMCAO we will also proceed exactly as in exp set 4, except that, between 2 and 12h after vessel occlusion, animals will be anesthetized again by isoflurane and one of the 4 drugs or the vehicle will be injected intrathecally (SOP_IT).

Animal groups, numbers and handling timeline for pilot:



Standard Operation Procedure (SOP)

Key data

Code (as appearing in SOP_Overview)	E-IV-7
Title (full text)	Assessment of functional outcome after stroke
Version (major change/minor change)	1
Effective date (not earlier than final signature)	2011-06-22

Signatures

Prof Harald Schmidt		
Head of Group	Signature	Date
Supervisor	Signature	Date
Kim Radermacher		
1st Author	Signature	Date
2nd Author	Signature	Date

Index

1. Update History

This is the first version.

2. Purpose

To determine global neurological and motor function after stroke.

3. Resources

4. Risks and Responsibilities

5. Procedures

5.1. Bederson score

This score is used to determine neurological function according to the following scoring system. First the animal is held by the tail without contact to the ground:

- No obvious deficit:score 0
- Flexion of the body or forelimb flexion to the opposite direction of stroke side:score 1

Secondly, the animal is placed on the ground:

- Decreased resistance to lateral push:score 2
- Unidirectional circling:score 3
- Longitudinal spinning: score 4
- No movement:score 5

Ref.: Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Stroke. 1986 May-Jun;17(3):472-6.

5.2. Grip test

This score is used to evaluate motor function and coordination. The apparatus is a wooden rod (0.5 cm diameters, 50cm length) between two vertical supports at a height of 40 cm over a flat surface. The animal is placed mid-way on this string and is rated according to the following system:

- Falling off: score 0
- Hanging onto the string by one or two forepaws:score 1
- Same as score 1 but at least trying to climb onto the string:score 2
- Hanging onto string by two forepaws and one or two hindpaws:score 3
- Hanging onto the string by all 4 paws and the tail is wrapped around the string:score 4
- Moving along the string an escaping towards support:score 5

Moran PM, Higgins LS, Cordell B, Moser PC. Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5341-5.

6. Abbreviations

7. Protocol Templates

Checklist

Intracerebroventricular injection

1. I.c.v. injection in rat

1. The surgery will be performed under aseptic conditions.
2. Painkillers are administered subcutaneously to the animal prior to intervention (Buprenorphine 0,1mg/kg).
3. Animals are anesthetized with 3-4% isoflurane (maintenance 1.5-2.5%).
4. Rats are shaved at the site of incision and the head is placed in a stereotaxic apparatus. The head is fixed with ear pins and a dental support.
5. After disinfection, a median incision is performed in the skin (±1-1.5 cm).
6. The periosteum is dissected to the border of the skull and the skin is kept aside with wound clips.
7. The scull is dried with cotton swabs.
8. The drill machine is fixed on the stereotaxic apparatus and 3 holes are drilled, one for the cannula and 2 for the screws. The screws are used to reinforce the dental cement that fixes the cannula onto the skull.
9. For i.c.v. injection into the right lateral ventricle a hole is drilled in the skull with the coordinates of 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 4.5 mm ventral to the surface of the skull.
10. A sterile 22-gauge cannula is inserted. Before applying the cement, the skull is dried with cotton swabs. Bleedings of the skin have to be stopped on beforehand.
11. The cannula is then secured to the skull with the dental cement. Be vigilant that no sharp edges are formed.
12. Once the cement is dry, the skin surrounding the cannula can be sutured with a 3-0 Polysorb® suture.
13. A stopper closes the cannula. Via this cannula i.c.v. injections can be performed.
14. Optional: The position of the cannulas can be verified one week after the surgery by applying 20 pmol/5 µl angiotensin II. Animals that drink less than 5 ml within 15 min of injection should be excluded from the experiment.

2. Ic.v. injection in mice

1. Implanting i.c.v. cannulas into the right cerebral ventricle of mice is performed in the same manner as for rats, except that the hole coordinates and the cannula size are different in mice.
2. In mice, the hole will be drilled in the skull at 1.00 mm lateral and 0.5 mm posterior to Bregma and the tip of a 26-gauge stainless steel infusion cannula was placed 2.00 mm below the skull surface into the right ventricle.
3. In mice, the skin is sutured with a 5-0 Polysorb® suture.

Intrathecal (i.t.) injection in mice and rats

1. The surgery will be performed under aseptic conditions.
2. Animals are anesthetized with 3-4% isoflurane (maintenance 1.5-2.5%).
3. Caudal cutaneous incision (1cm) is performed.
4. The syringe is held at an angle of about 20° above the vertebral column.
5. The needle is inserted into the tissue to one side of the L5 or L6 spinous process so that it slips into the groove between the spinous and transverse processes. This site represents a compromise to maximize inter- vertebral accessibility and to minimize the possibility of spinal damage.
6. The needle is then moved carefully forward to the intervertebral space as the angle of the syringe is decreased to about 10°. The tip of the needle is inserted so that approx. 0.5 cm is within the vertebral column.
7. The solution is injected in a volume of 5 µl and the needle rotated on withdrawal.
8. The skin is sutured with a 3-0 Polysorb® suture for rats and a 5-0 Polysorb® suture for mice.

Standard Operation Procedure (SOP)

Key data

Code (as appearing in SOP_Overview)	E-IV-6
Title (full text)	MCAO model in mice and rats (permanent or transient)
Version (major change/minor change)	1
Effective date (not earlier than final signature)	2011-06-22

Signatures

Head of Group	Signature	Date
Supervisor	Signature	Date
1st Author	Signature	Date
2nd Author	Signature	Date

Index

1. Update History

This is the first version.

2. Purpose

To induce ischemic stroke in mice and rats.

3. Resources

3.1 Experimental set-up

- Operating microscope
- Heating device
- Anesthesia unit

3.2 Surgical instruments and materials

- Scissors (delicate curved sharp/blunt iris scissors, Fine Science Tools Inc., Foster City, CA).
- Spring scissors (Vannas spring scissors straight with 3 mm blade, Fine Science Tools Inc., Foster City, CA).
- Two pairs of splinter forceps (30 mm curved splinter forceps, Aesculap AG, Tuttlingen, Germany).
- Two pairs of delicate angled forceps (delicate angled forceps designed for eye surgery, Geuder AG, Heidelberg, Germany).
- Vessel clip and clip applying forceps (micro-serrifine clips and micro-serrifine clip applying forceps, Fine Science Tools Inc., Foster City, CA).
- Occluding monofilament (Doccol Corporation, Redlands, USA; for mice: 60 MyDesign MCAO suture PK10 (60SPPK10), length: 20 mm, coating length: 9-10 mm; tip diameter: 0.23+/-0.02 mm; for rats: MCAO suture filament size 4-0 (403556PK10), length 30 mm, coating length 5-6 mm, tip diameter 0,35+/-0,02 mm)
- Sterile suture material (3-0 (rat) and 5-0 (mice) Polysorb® for wound suture, 4-0 (rat) and 7-0 (mice) silk suture for vessel occlusion)
- Swans
- Gloves
- Eye ointment (Bepanthen Augen- und Nasensalbe).

4. Risks and Responsibilities

5. Procedures

5.1. MCAO in mice

Surgical procedure

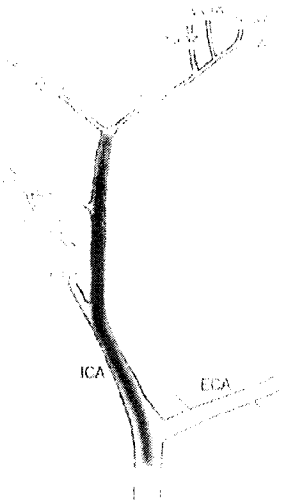
1. Painkillers are administered subcutaneously to the mice prior to intervention (Buprenorphine 0,1mg/kg).
2. Animals are anesthetized with 3-4% isoflurane.
3. After 3-5 min the anesthetized animal is placed under the operating microscope lying in supine position. Adhesive bandages fix the fore- and hind limbs. An appropriate ointment is applied to the eyes prior to surgery to prevent drying out
4. 1.5-2.5% isoflurane is used for maintenance during surgery. After re-checking depth of anesthesia by applying a painful stimulus, a midline skin incision is made in the neck. For this, a scalpel or scissors can be used.
5. Following midline skin incision, the thyroid gland is exposed. The right and left laps of the thyroid gland are mobilized by blunt dissection using two pairs of splinter forceps, separated at the isthmus and moved to the right and left. Underneath the trachea comes into view.
6. The common carotid artery (CCA) is located lateral to the trachea. It can be found in the carotid triangle bound by the sternocleidomastoid muscle, the stylohyoid muscle and the posterior belly of the digastric muscle, and the omohyoid muscle. In principle, the common carotid artery of either side can be operated, though most right-handed experimenters may find it easier to perform occlusion on the right MCA. The common carotid artery can now be removed from adjacent connective tissue by blunt dissection using two pairs of delicate angled forceps. The vagus nerve (in the carotid sheath, lateral to the artery) should be separated carefully from the common carotid artery by using the foreceps.
7. By following the common carotid artery in cranial direction and dissecting this vessel from the surrounded tissue, the point of division into the external and internal carotid arteries (carotid bifurcation) becomes visible. The superior thyroid artery emerges as first branch of the external carotid artery but, unlike the situation in humans, the pterygopalatine artery (Ptyr) emerges from the internal carotid artery. The proximal external and internal carotid arteries are also dissected from surrounding tissue.
8. A permanent ligature is tied around the proximal common carotid artery (CCA) and another ligature around the external carotid artery (ECA). A 7-0 silk suture can be used.
9. A tourniquet (4-0 silk suture) is loosely tied around the distal common carotid artery.
10. A vessel clip is applied to the distal common carotid artery or proximal internal carotid artery (ICA).
11. One third of the vessel diameter is now incised between the proximal common artery ligature and the vessel clip by using spring scissors.
12. The monofilament is inserted through the incised vessel wall into the common carotid artery lumen.
13. The vessel clamp is removed and the monofilament further advanced. The monofilament is carefully pushed up through the internal carotid artery until a gentle resistance is felt. Then, the end of the monofilament should still protrude from the vessel hole and is now secured by tightening the tourniquet suture prepared before to prevent dislocation during the ischemia period. The distance between carotid bifurcation and the origin of the middle artery is usually 0.9 mm in normal-sized C57Bl/6 mice. Hence, when using a 20 mm long occluding filament, a distance of 11 mm from the carotid bifurcation to the end of the monofilament indicates appropriate placement. Given these premises, the tip of the monofilament should now be located intracranially at the origin of the ipsilateral middle cerebral artery and thereby interrupting blood flow. Excessive advancement of the filament may lead to perforation of the vessel wall and thus cause subarachnoid hemorraghe, while insufficient advancement may not induce adequate ischemia.
14. The wound is sutured using a needle holder and a 5-0 Polysorb® suture with attached needle and the mouse is allowed to recover in a heated cupboard. The occluding monofilament may be left in place permanently to induce permanent cerebral ischemia. For permanent occlusion, the procedure can be stopped at this point, for transient ischemia, move on to point 15.

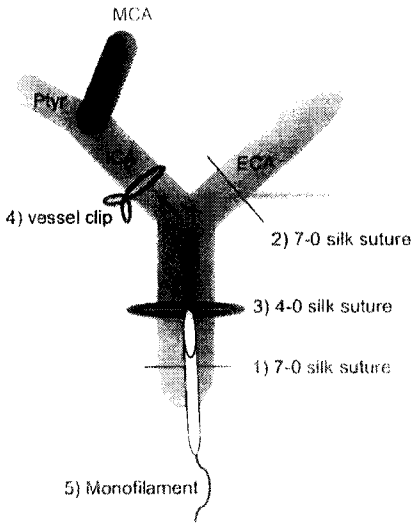
15. The filament may be removed after a desired time interval to model reperfusion. For transient MCAO, the tourniquet suture is loosened again, the occluding filament is removed, and the tourniquet is then secured by knots and thus changed into a ligature to prevent bleeding from the incised vessel.
16. Closure is achieved by standard skin suture using a needle holder and a 5-0 Polysorb® suture material with attached needle. Total time for surgery should usually not exceed 15 minutes.
17. Every twelve hours after the first dose of bupreborphine, the injection should be repeated.

5.2. MCAO in rats

The experimental protocol of MCAO in rats is similar to the one applied for mice,

The only differences to be considered when working in rats are the use of a 4-0 silk suture (instead of 7-0) for permanent ligature of the proximal common carotid artery and the external carotid artery (point 8), a 3-0 Polysorb® suture to close the wound (point 14+16) and a different filament type should be used since the vessel anatomy is different in rats compared to mice (point 13). In rats, the distance between the carotid bifurcation and the origin of the middle cerebral artery is 1.8-2.1 mm. When using a 30 mm long filament, the distance from the carotid bifurcation to the end of the filament should be 9-12 mm.





6. Abbreviations

7. Protocol Templates

Checklist

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

Aan:

voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL 6200 MD Maastricht
Telefoon

Uw referentie: Onze referentie Maastricht, 19-07-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Is NOX4 implicated in detrimental ROS production during ischemic stroke in rats and if so, is this target relevant in stroke therapy?*", is op de DEC vergadering van 15 juli 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De DEC verzoekt de kolommen 2 en 5 op te splitsen in verband met het ongerief.
- Bij punt 4 verzoekt de DEC de zin "We believe that the science, enzovoort", te verwijderen.
- Punt 7c- De DEC vraagt zich af of de 20 ratten om de MCAO operatie te leren niet samenvalt met DEC 2011-106 waarin deze techniek ook al geoefend is.
- De DEC is van mening dat wanneer men de MCAO methode oefent, in hetzelfde dier ook de IT methode geoefend kan worden.
- Bij punt 10a verzoekt de DEC het ongerief per groep aan te geven, ook op het voorblad en het leven na stroke ook te vermelden.
- De DEC verzoekt de dieren zo snel mogelijk sociaal te huisvesten (punt 11) als ze bijgekomen zijn uit anesthesie.
- De DEC wijst erop dat iemand die deze technieken beheerst de deskundigheid dient aan te leren (punt 12).

Gelieve eventuele vragen te beantwoorden in een brief en indien noodzakelijk Uw project aan te passen en duidelijk de aanpassingen grijs te markeren.

Uw project staat bij de DEC geregistreerd onder nummer 2011-107, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

Responses from the researchers to the comments and questions of the DEC committee regarding the DEC protocol 2011-107: "*Is NOX4 implicated in detrimental ROS production during ischemic stroke in rats and if so, is this target relevant in stroke therapy?*") that has been discussed at the DEC meeting the 15 July 2011.

The researchers want to thank the DEC for reviewing this protocol. The questions/remarks from the DEC are cited below with the corresponding replies from the researchers underneath. In the DEC protocol itself, the required changes have been made as requested and have been highlighted in gray.

- De DEC verzoekt de kolommen 2 en 5 op te splitsen in verband met het ongerief.

Response:
For the experimental set 2, the columns have been split up into 2+3 regarding the two different discomfort scores as requested by the DEC. Exp. set 5 (training for intrathecal injection), will be combined with exp. set 1 (MCAO training) to reduce the number of rats as suggested by the DEC (see 4th point of this letter). All mice of this first group will have a discomfort level of 05 due to MCAO operation and thus it not necessary to split them anymore.

- Bij punt 4 verzoekt de DEC de zin "We believe that the science, enzovoort", te verwijderen.

Response:
The sentence has been removed as requested.

- Punt 7c- De DEC vraagt zich af of de 20 ratten om de MCAO operatie te leren niet samenvalt met DEC 2011-106 waarin deze techniek ook al geoefend is.

Response:
As also stated in the response letter to the DEC 2011-106, we did not ask for training mice in the DEC 2011-106 because we are already able to perform the MCAO technique in mice. However, in this DEC (2011-107) only rats will be used and therefore we will need some training rats in order to see if we can perform the MCAO as easily as in mice. Although we will use the same technique to induce stroke, it is of crucial importance to use the appropriate filament size to occlude the middle cerebral artery and as the size is dependent on rat strain, gender, age etc. it is important to test different filament sizes (diameter, length, coating and so on) before starting the real experiments (exp set 4 and 5).

- De DEC is van mening dat wanneer men de MCAO methode oefent, in hetzelfde dier ook de IT methode geoefend kan worden.

Response:
This is a good point, we could combine the training of both techniques. As the mortality rate after MCAO is around 20-30% (especially in the beginning), it

would be better to start first with the i.t. injection and then perform the operation. All rats will have to recover from anesthesia after MCAO operation (in order to check if the MCA occlusion was successful) and thus we do not have to split the experimental set for i.t. injection into different discomfort groups anymore as requested in the DEC's first comment. We combined i.t. injection and MCAO training and made changes in the DEC protocol wherever it was necessary (highlighted in grey).

- Bij punt 10a verzoekt de DEC het ongerief per groep aan te geven, ook op het voorblad en het leven na stroke ook te vermelden.

Response:
Under point 10a we now included both, exp set numbers and group numbers, to make it easier to understand what discomfort will be exactly caused to the different groups. In the summarizing table we listed the different manipulations causing discomfort to the different groups and we also added a row for the discomfort that will be experienced by the mice during the 24h-survival after stroke. All changes have been highlighted in gray.

- De DEC verzoekt de dieren zo snel mogelijk sociaal te huisvesten (punt 11) als ze bijgekomen zijn uit anesthesie.

Response:
If the DEC committee thinks that it is not necessary to keep the animals in individual cages, we will put them in social housing as soon as they recover from anesthesia. Therefore we removed the individual housing from the discomfort table in point 10a and the following sentence from point 11 has been replaced:

"Although the cannula will be removed immediately after injection, animals of exp set 4+5 will be kept in individual cages (discomfort score 2) to avoid mutual nibbling of the wound suture. "

has been replaced by

"Animals will be kept in social housing as soon as they recover from anesthesia."

- De DEC wijst erop dat iemand die de techniek beheerst de deskundigheid dient aan te leren (punt 12).

Response:
As already mentioned in the response letter of the DEC 2011-106, two of the researchers learned the technique on mice 4 month ago from a specialist in the stroke field. Now we just have to find out the correct filament size for rats on our own because every strain has different filament requirements.
We added a corresponding sentence at point 12:

"roup in Würzburg/Germany in April 2011 to learn and train the MCAO technique from a leading scientist in the field of stroke (. "

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
		15-08-2011

Project: *Is NOX4 implicated in detrimental ROS production during ischemic stroke in rats and if so, is this target relevant in stroke therapy?*

DEC-UM
Voorzitter DEC-UM
en
p/a secretariaat DEC-UM

Secretariaat DEC-UM

Bezoekadres

Postadres
Postbus 616
6200 MD Maastricht

Verantwoordelijk onderzoeker (VO):

Namens de Vergunninghouder van de DEC-UM, delen wij u mede dat voornoemd project aan de ethische toetsingscriteria voor proefdiergebruik voldoet.
De DEC maakt geen bezwaar tegen uitvoering van dit project zoals aangevraagd en geeft een positief advies.

Projectnummer:	2011-107
Diersoort:	rat
Aantal dieren:	259
Einddatum:	15-08-2015

Uw project staat bij de DEC en CPV geregistreerd onder bovenstaand nummer. Gelieve dieren, die voor dit project bestemd zijn, ook onder dit nummer aan te vragen.

Voorzitter DEC-UM

Vicevoorzitter DEC-UM