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Titel van het onderzoek:

Which NADPH oxidase isoforms play a role in ischemic stroke in mice. Could they be used as therapeutic target?

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	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. Verantwoordelijk medewerker (VM) GGO[7]				Art.9	
4. overige uitvoerenden				Art. 12	
5. PI and hoofd					

Diergroep	1	2	3	4	5	6	7	8	9
ctrl/exp/sham	Exp set 1	Exp set 1	Exp set 2	Exp set 2	Exp set 2	Exp set 2	Exp set 2	[redacted]	[redacted]
Diersoort	01	01	01	01	01	01	01	01	01
Stam	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6
Construct / mutatie ?	NOX2 KO	WT	NOX4 KO	EC-specific NOX4 KO	NC-specific NOX4 KO	SMC-specific NOX4 KO	WT	WT	WT
Herkomst (leverancier) *	02	02	01	01	01	01	01	01	01
Aantal	40	40	20	20	20	20	20	[redacted]	[redacted]
Geslacht	M	M	M	M	M	M	M	M	M
Dieren immuuncompetent ?	Ja	Ja	Ja	Ja	Ja	Ja	Ja	ja	ja
Leeftijd/gewicht	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks
Doel van de proef *	31	31	31	31	31	31	31	29	29
Belang van de proef *	1	1	1	1	1	1	1	1	1
Toxicologisch onderzoek *	1	1	1	1	1	1	1	1	1
Bijzondere technieken *	1	1	1	1	1	1	1	1	1
Anesthesie *	4	4	4	4	4	4	4	4	4
Pijnbestrijding *	4	4	4	4	4	4	4	4	4
Mate ongerief *	5	5	5	5	5	5	5	[redacted]	[redacted]
Toestand dier einde exp*	1	1	1	1	1	1	1	1	1

* VIII-coderingen zie bijlage

Diergroep	10	11	[redacted]	[redacted]	14	15	16	17	18
ctrl/exp/sham	Exp set 4	Exp set 4	[redacted]	[redacted]	Exp set 6	Exp set 7	Exp set 7	Exp set 8	Exp set 8
Diersoort	01	01	01	01	01	01	01	01	01
Stam	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	129	129
Construct / mutatie ?	NOX4 KO	WT	WT	WT	NOX4 KO	NOX4 KO	WT	NOX5 KI	WT
Herkomst (leverancier) *	01	01	01	01	01	01	01	Harlan	Harlan
Aantal	20	[redacted]	[redacted]	[redacted]	12	40	40	20	20
Geslacht	M	M	M	M	M	M	M	M	M
Dieren immuuncompetent ?	ja	ja	ja	ja	ja	ja	ja	ja	ja
Leeftijd/gewicht	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks
Doel van de proef *	31	31	29	29	31	31	31	31	31
Belang van de proef *	1	1	1	1	1	1	1	1	1
Toxicologisch onderzoek *	1	1	1	1	1	1	1	1	1
Bijzondere technieken *	1	1	1	1	1	1	1	1	1
Anesthesie *	4	4	4	4	4	4	4	4	4
Pijnbestrijding *	4	4	4	4	4	4	4	4	4
Mate ongerief *	5	5	[redacted]	[redacted]	[redacted]	5	5	5	5
Toestand dier einde exp*	1	1	1	1	1	1	1	1	1

* VHI-coderingen zie bijlage

TITEL: ***Which NADPH oxidase isoforms play a role in ischemic stroke in mice. Could they be used as therapeutic target?***

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.

Since there are contradicting publications about the role of NOX2 in ischemic stroke our first aim is to clarify whether NOX2 really plays a significant role in stroke and to find a reasonable explanation for this discrepancy in the literature. One hypothesis is that the NOX2 effect could originate from the influx of peripheral neutrophils occuring when stroke-inducing operations are not performed in aseptic conditions. To exclude confounder effects from systemic neutrophils, **stroke size will be compared after septic versus aseptic operation of NOX2 knockout (KO) and wildtype (WT) mice (exp set 1)**. The research group of recently published that deletion of the NOX4 gene in mice greatly reduced ischemia-reperfusion damage of brain tissue after stroke. In our next experiment **(exp set 2) we want to attribute the observed neuroprotection in NOX4 KO mice to a specific cell type (endothelial cells, smooth muscle cells or neuronal cells)** as it is still unclear if the NOX4-related excessive ROS production is of neuronal or vascular origin. Therefore, different cell specific NOX4 KO models are currently being established at CPV. Moreover, in this same paper of , similar benefits were achieved after administration of a novel NOX inhibitor to normal (wildtype) mice whereas the infarct size could not be further 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 (exp set 3)**. Once the i.t. injections are routine, we want to elucidate (exp set 4) **the therapeutic efficiency of specific NOX inhibitors in ischemic stroke**. To further validate our previous findings, we will restore NOX4 activity in NOX4 KO mice by injecting cDNA-carrying lentivirus into the right lateral ventricle of the brain. Since **the intercerebroventricular (i.c.v.) injection still needs to be established (exp set 5)** and the **dose regimen of the lentivirus to efficiently restore the NOX4 activity needs to be determined (exp set 6)**, we will first establish this model and only then move on to stroke experiments, where **lesion size of restored NOX4 KO mice will be assessed (exp set 7)**. All animal handling that involves lentivirus injection (exp set 6 and 7) will be performed in a MLIII lab. Altough the NADPH oxidase isoform NOX5 is not expressed in mice, it is well present in human vessels. Therefore, it could play a crucial role in human stroke disease. To study the implication of NOX5, transgenic NOX5 knock-in (KI) mice are currently being established to **compare the stroke size in normal mice versus NOX5 KI mice (exp set 8)**.

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 different 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 NOX isoforms 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 4) 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 the 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.

The literature provides conflicting results concerning the role of NOX2 in stroke [9] [10]. For this reason, we will reassess post-stroke brain damage in NOX2 deficient mice versus control mice. As NOX2 is predominantly present in inflammatory cells, several papers mention a possible cofounder effect from peripheral neutrophils that are triggered towards the infarct region if the stroke-inducing operation is not performed under aseptic conditions. We want to verify this hypothesis by comparing lesion size in sterile versus non-sterile operated NOX2 KO and WT mice. Concerning the NOX4 isoform, the research group of 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 [ ]. 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. Now we want to elucidate if NOX4 expression is related to a specific cell type in the brain (endothelial cells, smooth muscle cells or neuronal cells). For this purpose, different cell-specific NOX4 knockout lines are currently established at CPV. Moreover we want to test the efficiency of specific NOX inhibitors such as] and a promising compound from the in stroke since NADPH oxidases may present a future therapeutic target and their inhibition might induce clinically relevant neuroprotection. We also aim to validate the neurotoxic role of NOX4 in stroke more thoroughly by rescuing the deleted NOX4 gene in NOX4 KO mice by intracerebroventricular administration of cDNA. Furthermore, we want to assess the potentially neurotoxic role of NOX5 in stroke by insertion of the human NOX5 gene in mice. These mice will be provided to us by an external supplier (Harlan). If these latter NOX5 experiments show an implication of NOX5 in stroke, NOX5 KI mice could also be injected with NOX inhibitors in future experiments.

6. Wetenschappelijke beoordeling
This specific project has been approved by

Proefdier

7. Proefdier keuze
7a. Soort, stam / herkomst / eindbestemming
For **experiment set 1**, heterozygous NOX2 KO mice and WT (C57Bl6) mice, both can be purchased from Jackson.
For **experiment set 2,** three different cell-specific conditional NOX4 KOs, general NOX4 KO mice and WT littermates will be used. They are currently bred at Maastricht University,

For **experiment set 3**, only normal C57Bl6 mice will be needed.
For **experiment set 4,** homozygous NOX4 KO mice (developed at Monash University and now transferred and bred at Maastricht University,) will be used along with matched WT mice.
For **experiment set 5**, only normal C57Bl6 mice will be needed.
For **experiment set 6,** homozygous NOX4 KO mice (will be used.
For **experiment set 7**, homozygous NOX4 KO mice ( ) will be used along with matched WT mice.
For **experiment set 8,** transgenic NOX5 KI mice (and then transferred to) will be used along with matched WT mice (129), both strains will be purchased from

7b. Sexe

For the preliminary pilots only male mice 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 all 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 $n=2x\ s^2x\ (Z\alpha/2+Z\beta)^2/D^2$(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. Taking into account an animal loss of 20 % due to acute mortality of the infarction (based on experience from the previously published paper [9]), this implies a total animal number of 15.7/0.8 = 19.6 ~ 20 mice.
Thus, as explained above, the total number of animals per group will be 20.
If the first starting experiment of exp set 1, 2, 4, 7, and 8 is successful, we should also repeat the findings in females and in adult animals in future experiments. According to published stroke guidelines, a permanent stroke model and sham-operated mice 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 mice) we see a significant difference in stroke size.

Experiment set 1:
For this experiment mice will be divided into 2 groups: aspetic and septic operation group.
Starting experiment: Each group will contain 20 male NOX2 KO mice (6-8 weeks) + 20 male WT mice (6-8 weeks) = 40 mice/group.
As we have two groups, the total amount of mice needed for this experiment will be 80 mice.
Of course, if the starting experiment shows negative results after 10 mice/group, we will not perform experiments with the remaining 40 mice.

Experiment set 2:
Starting experiment: As we have 3 different cell-specific KO models (endothelial cell-EC, neuronal cell-NC, smooth muscle cell-SMC), one general NOX4 KO model and the matched WT mice, we have 5 groups altogether. As each group should contain 20 mice according to the statistical calculations above, we will need 100 male mice (6-8 weeks) for this experiment.
Of course, if the starting experiment shows negative results after 10 mice/group, we will not perform experiments with the remaining 50 mice.

Experiment set 3:
As the i.t. injection (used for the injection of the different NOX inhibitors) is completely new to us, we would like to have some training mice to get used to this new injection method. As two people have to learn this technique , art. 12) we calculate 10 mice per person. Thus we will need **20 male C57Bl6 mice (6-8 weeks).**

Experiment set 4:

Number of groups: 6

- WT vehicle group
- 4 groups, where WT mice are treated with one of the 4 NOX inhibitors
- NOX4 KO vehicle group

Starting experiment: Each group will contain 20 male mice (6-8 weeks).
As we have 6 groups, the total amount of mice needed for the pilot will be 120 mice.
Of course, if the starting experiment shows negative results after 10 mice/group, we will not perform experiments with the remaining 60 mice.

Experiment set 5:
I.c.v. injection that is needed for the injection of the cDNA-carrying lentivirus has also to be trained by . We first want to have some training mice to get used to this new injection method before starting with the lentivirus: **20 male C57Bl6 mice (6-8 weeks).**

Experiment set 6:
To determine the correct dose of cDNA-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 mice: **12 male NOX4 KO mice (6-8 weeks).**

Experiment set 7:
Number of groups: cDNA group and control group
Starting experiment: Each group will contain 20 male NOX4 KO mice (6-8 weeks) + 20 male WT mice (6-8 weeks) = 40 mice/group.
As we have 2 groups, the total amount of mice needed for this experiment will be 80 mice.
Of course, if the starting experiment shows negative results after 10 mice/group, we will not perform experiments with the remaining 40 mice.

Experiment set 8:
Starting experiment: 20 male NOX5 KI mice (6-8 weeks) + 20 male WT mice (6-8 weeks) = 40 mice.
Of course, if the starting experiment shows negative results after 10 mice/group, we will not perform experiments with the remaining 20 mice.

→ TOTAL: 472 mice

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: To clarify whether NOX2 plays a pathophysiological role in stroke by comparing infarct size after stroke in NOX2-deficient mice and in normal C57Bl6 mice after aseptic operation versus non sterile operation in order to exclude confounder effects from peripheral neutrophils.
Aim experiment set 2: We want to attribute the observed neuroprotection in NOX4 knockouts to a specific cell type (endothelial cells, smooth muscle cells or neuronal cells) as it is still unclear which type of cells is responsible for excessive ROS production by NOX4. Therefore different cell specific NOX4 KO models are currently being established at CPV.
Aim experiment set 3: To practice the intrathecal drug injection.

Aim experiment set 4: To evaluate the therapeutic efficiency of specific NOX inhibitors in stroke. The drug will be injected between 2 and 12h post-stroke and neuronal damage will be compared to NOX4 KO and WT mice.
Aim experiment set 5: Establish the intracerebroventricular cannula insertion.
Aim experiment set 6: Determine the appropriate dose of cDNA-expressing lentivirus that will be injected to restore the NOX4 gene in NOX4 KO mice.
Aim experiment set 7: In this rescue experiment we want to restore NOX4 activity in NOX4 knockout mice by injecting cDNA-carrying lentivirus intracerebroventricularly.
Aim experiment set 8: To evaluate if NOX5 is implicated in stroke, the human NOX5 gene is inserted in the genome of 129 mice and stroke size of these mice will be compared to normal 129 mice.

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 [14]. At least we will avoid anesthetics with a marked intrinsic neuroprotective effect such as ketamine. Since the control mice will also be anesthetized the same way, the impact of isoflurane on our results will be less important.
In experiment sets 1, 2, 4, 7 and 8: 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 1 hour 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), mice will be sacrificed by pentobarbitone injection (60 mg/kg BW i.p.) and blood withdrawal.
In experiment set 5 to 7, a cannula will be installed in the right lateral cerebral ventricle of mice (SOP_ICV) under isoflurane anesthesia (initiating at 3-4 %, maintenance at 1.5-2.5 %). For intrathecal injection as used in exp set 3 and 4, animals will also be anesthetized prior to the injection of the drug. The animals used for training (exp set 3,5+6) will be sacrificed by injecting a pentobarbitone 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, 2, 4, 7 and 8 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 5-7, 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, mice already got painkillers that are effective for the following 12h and thus do not need an additional analgesic (exp set 4). The animals used in exp set 3 are only used for practicing i.t. injection and will not undergo MCAO. These mice will be relieved

from pain the first day after intervention by locally applied lidocaine.

9c. Euthanasie en Humane eindpunten

In exp set 1 we will be particularly vigilant because of the non sterile operation methods and in case of severe sepsis the mice will be sacrificed immediately. We will also 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 mice 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 1,2, 4, 7 and 8 [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 5-7 [redacted] an i.c.v. injection will be performed to inject the cDNA-carrying lentivirus under isoflurane (discomfort score [redacted]). Half of the animals of exp set 5 [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 5 [redacted] have to regain consciousness because we want to check their health status after intervention.

To administer the different drugs in set 3 and 4 [redacted], an intrathecal injection post-stroke (discomfort score 03-04) under isoflurane anesthesia will be necessary. But again, half of the training mice in exp set 3 [redacted] can be sacrificed without regaining consciousness (score 02).

In exp set 2 [redacted] an intraperitoneal injection (score 03) will be necessary to induce the conditional KO in smooth muscle cells.

The mice 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, 2, 4, 7 and 8 [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] stroke	24 h	[redacted]	05
i.c.v. placement of the cannula, lentivirus injection and removal of the cannula	1 h	1x [redacted]	05
[redacted]	1 h	[redacted]	02
i.t. injection of the NOX inhibitor	15 min	[redacted]	03-04
[redacted]	15 min	[redacted]	02
i.p. injection of Tamoxifen	1 min	5x [redacted]	03
Pentobarbitone i.p. and blood withdrawal	10 min	1x [redacted]	02
Pentobarbitone overdose i.p.	1 min	1x [redacted]	02

10b. Welzijnsevaluatie

Mice 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

Animals will be kept in social housing as soon as they recover from anesthesia. Surgery and 1 day of recovery (in a special heated room) of the animals will take place in the animal lab of Pharmacology. Thereafter animals will be sacrificed

12. Deskundigheid

All animal-handling procedures will be conducted by experienced artikel 12 research assistant [redacted] or by [redacted] r (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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11. Wind S, Beuerlein K, Eucker T, et al. Comparative pharmacology of chemically distinct NADPH oxidase inhibitors. Br J Pharmacol, 161(4), 885-98 (2010).
12. ten Freyhaus H, Huntgeburth M, Wingler K, et al. Novel Nox inhibitor VAS2870 attenuates PDGF-dependent smooth muscle cell chemotaxis, but not proliferation. Cardiovasc Res, 71(2), 331-341 (2006).
13. 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).
14. Howells DW, Porritt MJ, Rewell SS, O'Collins V, Sena ES, van der Worp HB, Traystman RJ, Macleod MR. Different strokes for different folks: the rich diversity of animal models of focal cerebral ischemia. J Cereb Blood Flow Metab. 30(8), 1412-31 (2010).

Experiment set 1

Aim:
To clarify whether NOX2 plays a pathophysiological role in stroke by comparing infarct size after stroke in NOX2-deficient mice and in normal C57Bl6 mice after aseptic operation versus non sterile operation in order to exclude confounder effects from peripheral neutrophils.

Parameters to be assessed in vivo:

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

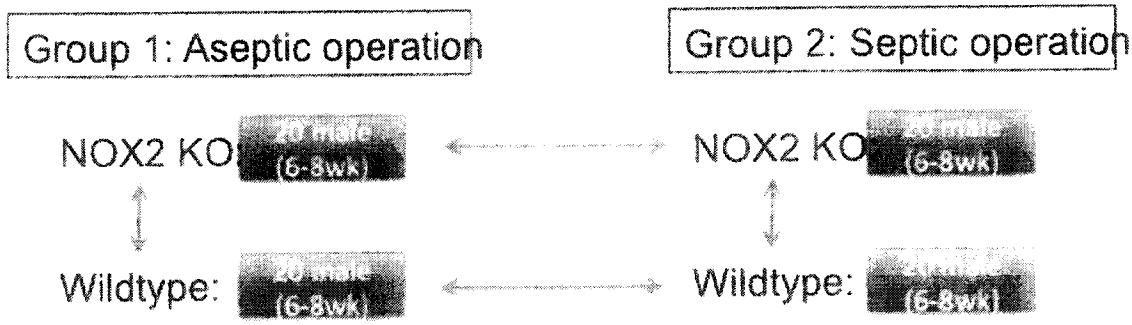
Ex vivo parameters:

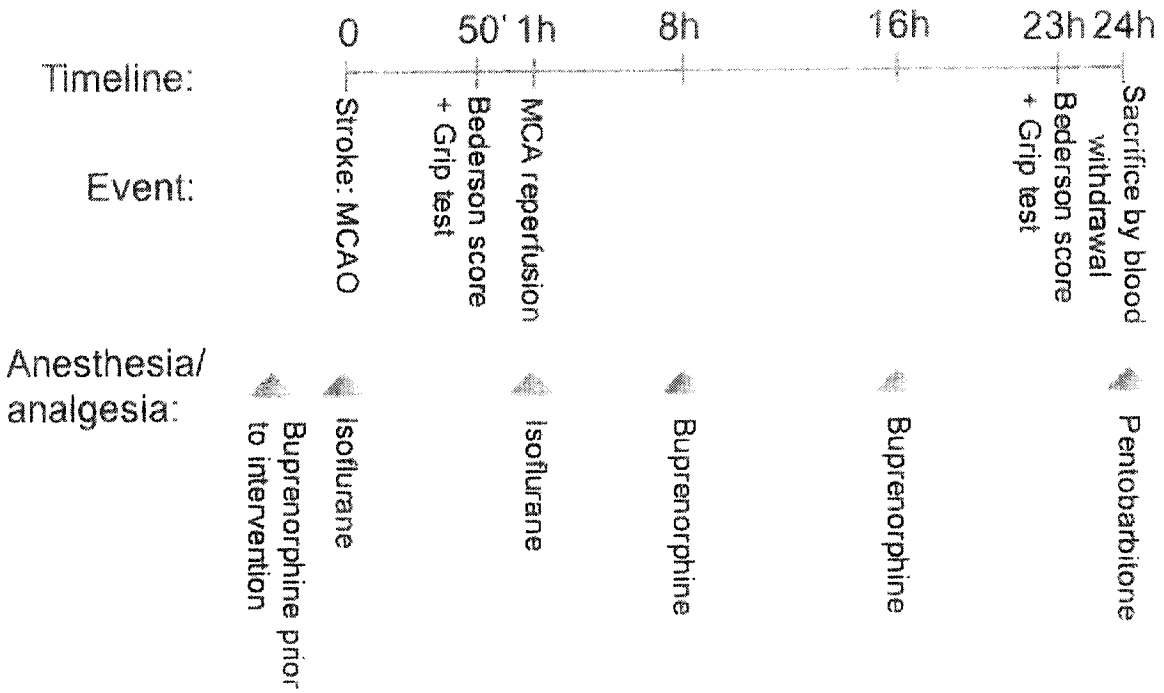
- Stroke infarct volume (Histology of the brain: TTC staining)
- ROS staining in brain tissue (IHC, brain homogenate)
- Nitrotyrosin staining
- Apoptosis (TUNEL staining)
- Expression of NOX in brain tissue (qRT-PCR, WB, IHC)

Animal handling procedure in words (briefly):

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 either under aseptic or under septic operation conditions in male NOX2 KO and WT mice (N=20 each). What we mean by aseptic conditions is that we will not shave the animals before the intervention and we want to operate mice without gloves and with non sterile surgical material. 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 mice 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 mice 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 2

Aim:
We want to attribute the observed neuroprotection in NOX4 knockouts to a specific cell type (endothelial cells, smooth muscle cells or neuronal cells) as it is still unclear which type of cells is responsible for excessive ROS production by NOX4. Therefore different cell specific NOX4 KO models are currently being established at CPV.

Parameters to be assessed in vivo:

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

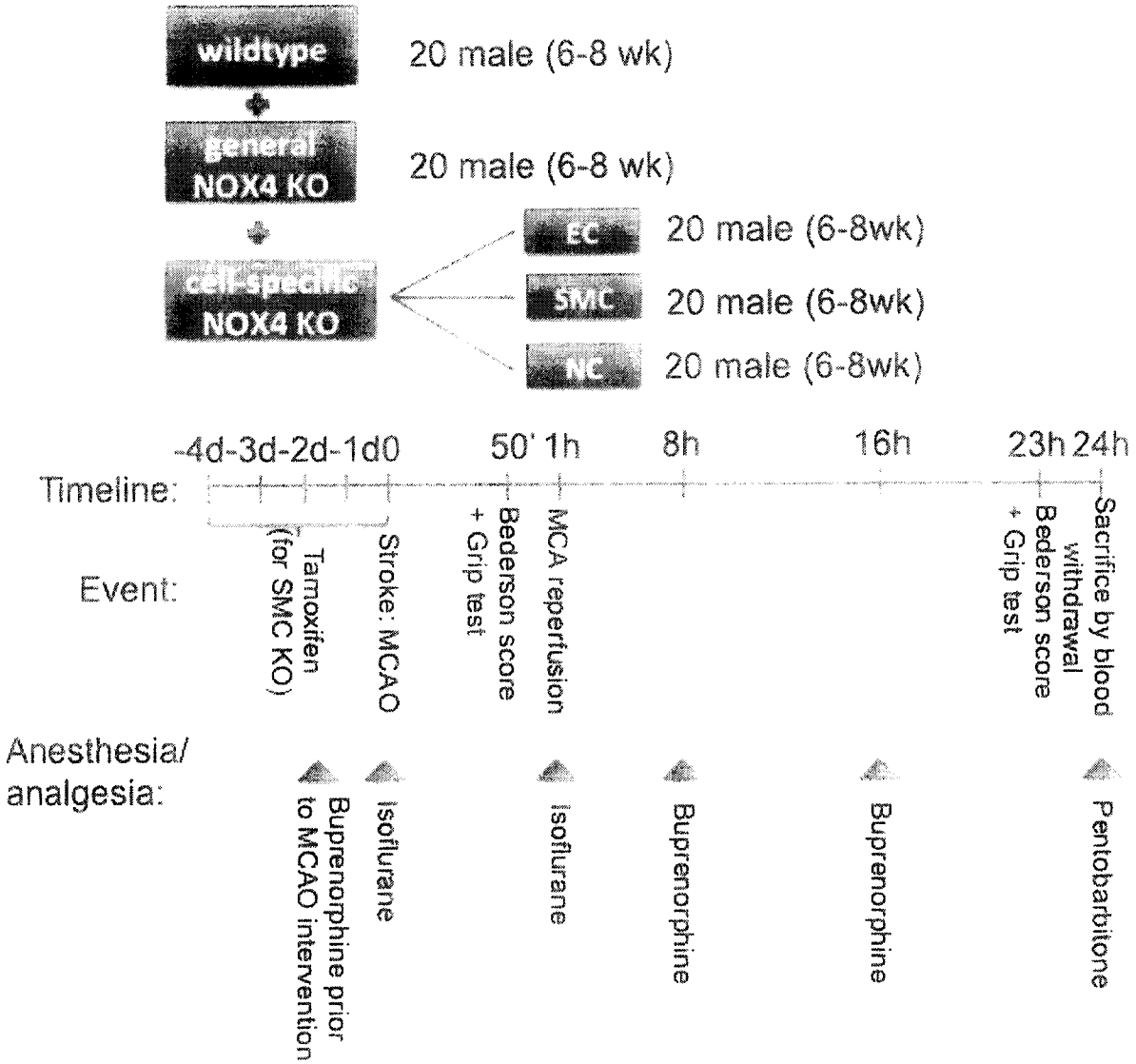
Ex vivo **parameters:**

- 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):

To establish the conditional NOX4 KO models, mice that are floxed for the NOX4 gene will be crossed with mice, in which Cre is expressed in a specific type of cells (endothelial cells-ES, smooth muscle cells-SMC or neuronal cells-NC). To delete the NOX4 gene in smooth muscle cells (SMC), the situation is a little bit different because here the Cre is inducible. Therefore, to knockout NOX4 in SMCs we first have to administer tamoxifen (1mg i.p., 5 consecutive days) in order to induce Cre. For the other cell-specific KO models no further manipulation will be needed. To induce tMCAO (under aseptic conditions of course) and to assess neurological outcome, motor function and ex vivo parameters we will proceed exactly as described in exp set 1.

Animal groups, numbers and handling timeline for pilot:



Experiment set 4

Aim:
To evaluate the therapeutic efficiency of other specific NOX inhibitors in stroke. The drug will be injected between 2 and 12h post-stroke and neuronal damage will be compared to NOX4 KO and WT mice.

Parameters to be assessed in vivo:

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

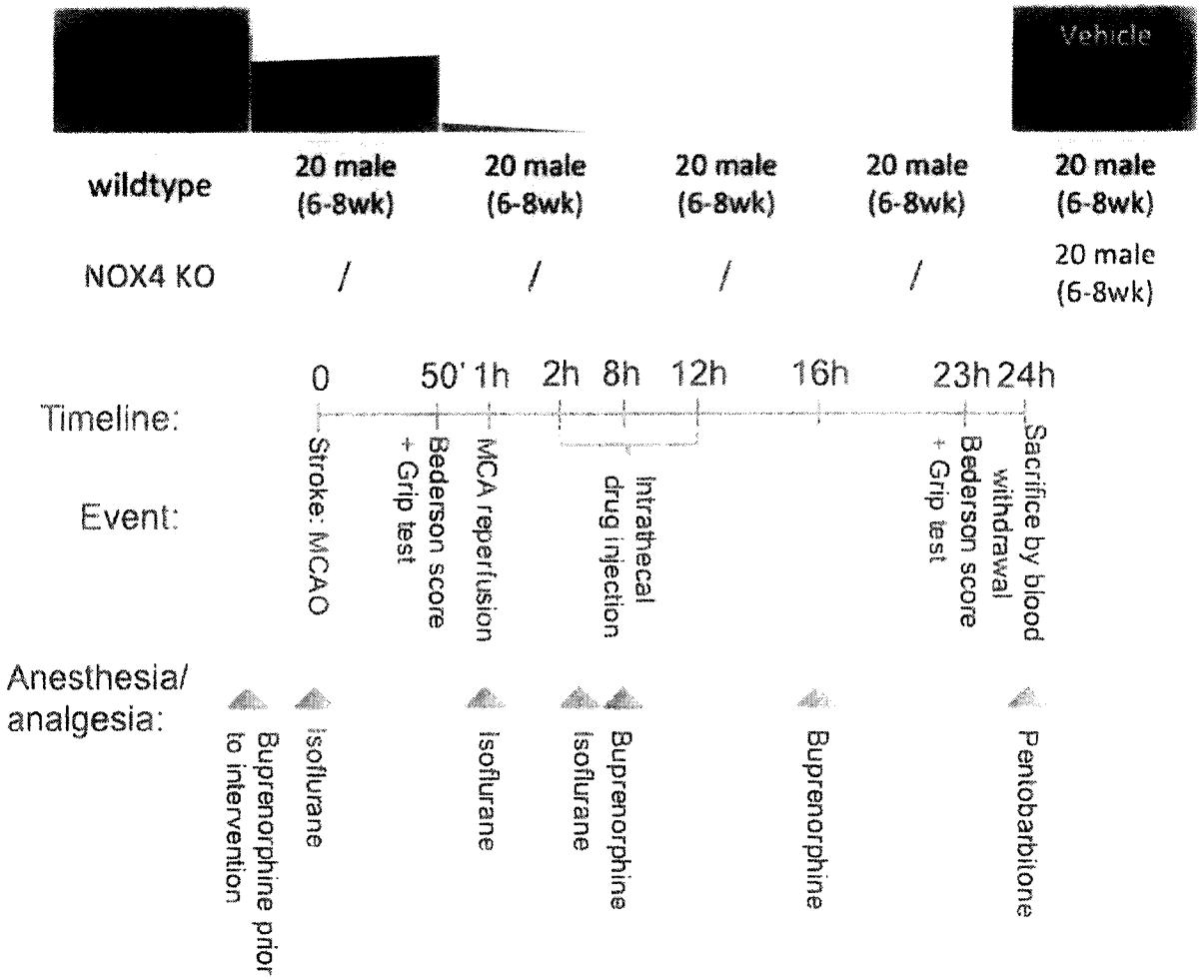
Ex vivo **parameters:**

- 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):

To induce tMCAO and to assess neurological outcome, motor function and ex vivo parameters, we will proceed exactly as described in the previous exp set, except that between 2 and 12 hours post-stroke one of the 4 drugs or the vehicle will be injected intrathecally into the mice.

Animal groups, numbers and handling timeline for pilot:



Experiment set 7

Aim:
In this rescue experiment we want to restore NOX4 activity in NOX4 knockout mice by injecting cDNA-carrying lentivirus intracerebroventricularly.

Parameters to be assessed in vivo:

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

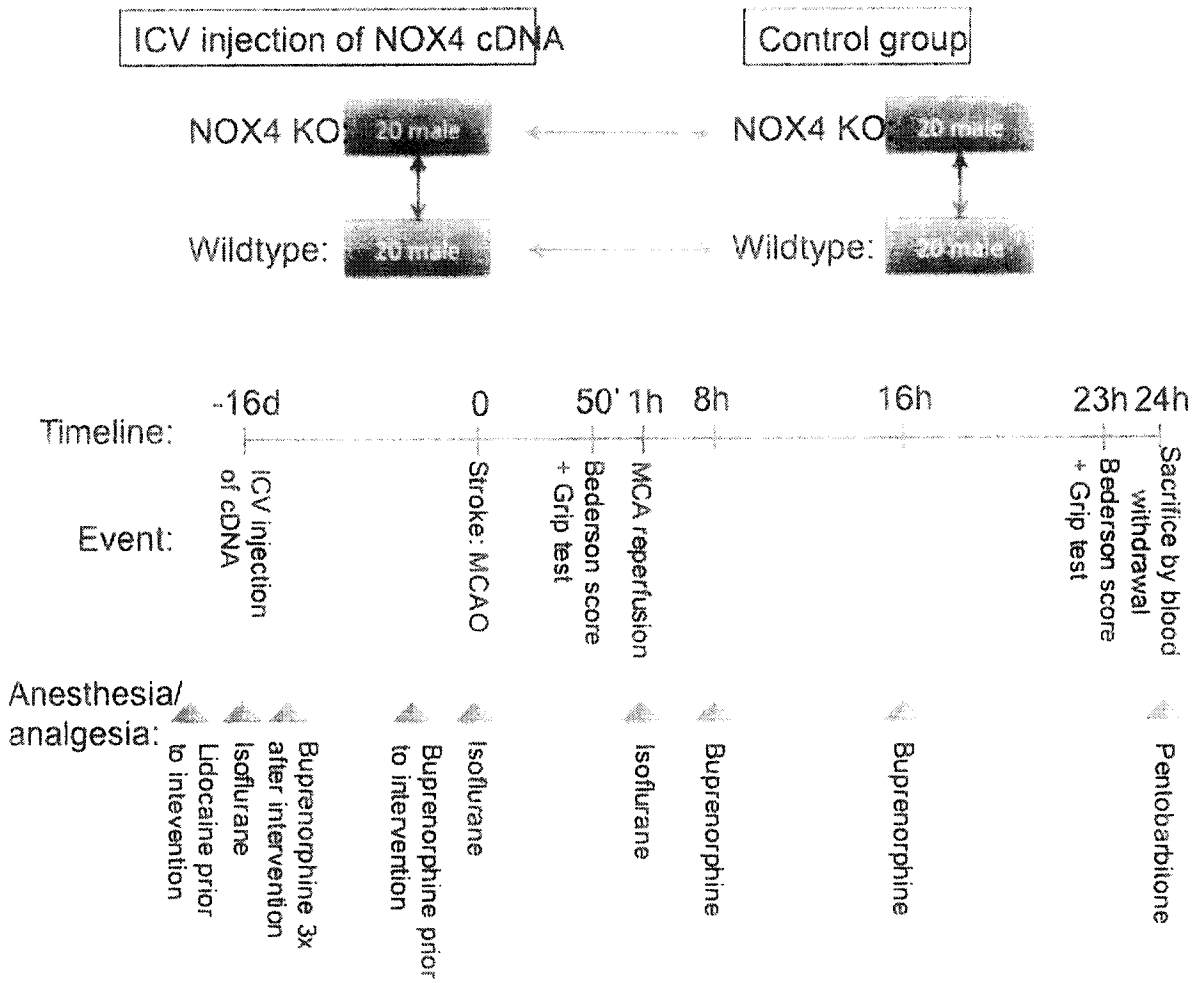
Ex vivo parameters:

- 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 NOX4rescue-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. For the induction of tMCAO we will proceed exactly as described in previous exp sets.

Animal groups, numbers and handling timeline for pilot:



Experiment set 8

Aim:
To evaluate if NOX5 is implicated in stroke, the human NOX5 gene is inserted in the genome of 129 mice and stroke size of these mice will be compared to normal 129 mice.

Parameters to be assessed in vivo:

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

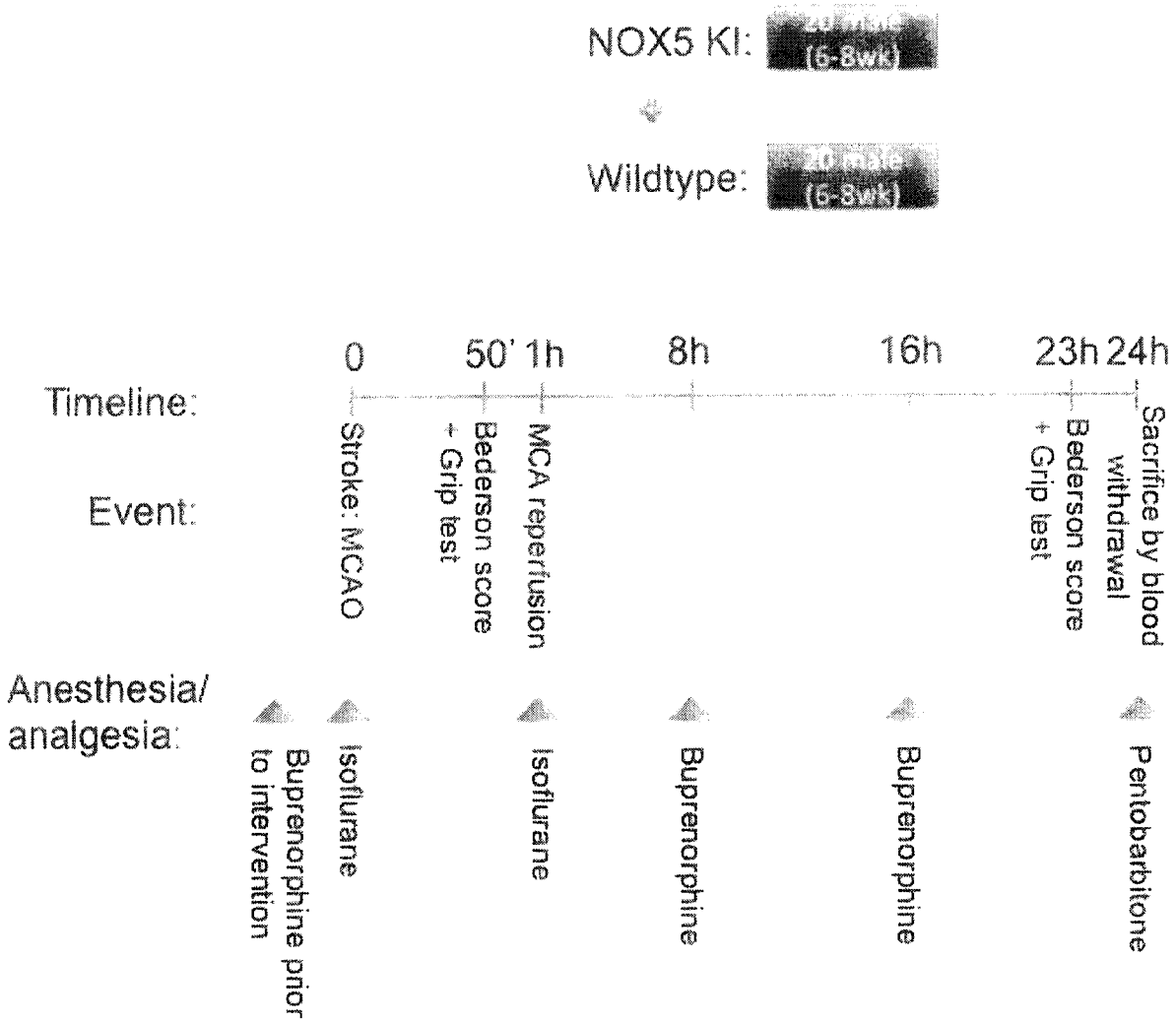
Ex vivo parameters:

- 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):

In this experiment the handling procedure for tMCAO will be exactly the same as previously described. We will operate transgenic NOX5 KI mice and corresponding control mice under aseptic conditions.

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

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 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

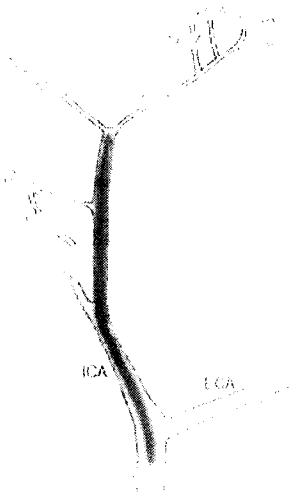
1. Painkillers are administered subcutaneously to the mice prior to intervention (Buprenorphine 0,1mg/kg).
2. Animals are anesthetized with 3-4% isoflurane.
3. After 3-5 min the anesthetized animal is placed under the operating microscope lying in supine position. Adhesive bandages fix the fore- and 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.

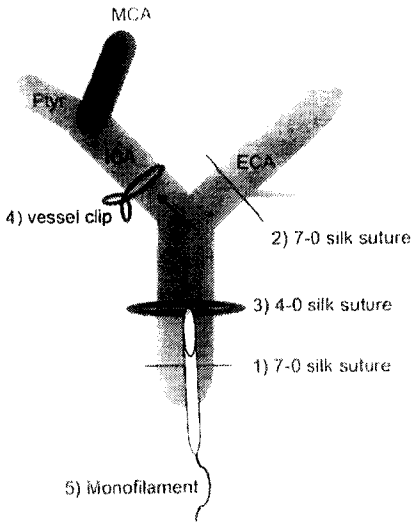
15. The filament may be removed after a desired time interval to model reperfusion. For transient MCAO, the tourniquet suture is loosened again, the occluding filament is removed, and the tourniquet is then secured by knots and thus changed into a ligature to prevent bleeding from the incised vessel.
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: "*Which NADPH oxidase isoforms play a role in ischemic stroke in mice. Could they be used as therapeutic target?*", is op de DEC vergadering van 15 juli 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De DEC verzoekt op het voorblad het ongerief van de groepen 3-5 en 6 aan te passen conform de vermelding bij punt 10a en de groepen 8 en 11 op te splitsen met de juiste ongeriefscores.
- Bij punt 4 verzoekt de DEC de zin "We believe that the science, enzovoort", te verwijderen.
- Bij punt 7c merkt de DEC op dat in kolom 10 het aantal dieren 100 moet zijn.
  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. De DEC merkt op dat het ongerief van kolom 11 waarschijnlijk ongerief code 02 of code 05 moet zijn en verzoekt het leven na stroke ook te vermelden bij het ongerief.
  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 de techniek beheerst de deskundigheid dient aan te leren (punt 12).

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

Uw project staat bij de DEC geregistreerd onder nummer 2011-106, 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 2011-106 ("*Which NADPH oxidase isoforms play a role in ischemic stroke in mice. Could they be used as therapeutic target?*") that has been discussed at the DEC meeting of 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 op het voorblad het ongerief van de groepen 3-5 en 6 aan te passen conform de vermelding bij punt 10a en de groepen 8 en 11 op te splitsen met de juiste ongeriefscores.

Response:
In exp set 2 we will have to use different mouse strains (group 3 to 7). All of these mice will undergo middle cerebral artery occlusion and reperfusion. This operation represents the most important discomfort caused to the animals (score 05), as stated on the front sheet and in point 10a, and for this reason we do not understand why the DEC committee asked us to adapt the discomfort of the groups 3-6 on the front page according to point 10a.
For group 8 and 11, the columns have been split up into 8+9 and 12+13 respectively, regarding the two different discomfort scores as requested by the DEC.

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

Response:
The sentence has been removed as requested.

- Bij punt 7c merkt de DEC op dat in kolom 10 het aantal dieren 100 moet zijn.

Response:
Of course, the number of animals used in column 11 (previously column 10) should be 100 instead of 1000. We corrected our typing error.

- 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, but we believe that there is a little confusion with another DEC protocol that has been sent in at the same time (DEC 2011-107). In the current DEC (DEC 2011-106) we did not ask for training mice for MCAO because we are already able to perform the MCAO technique in mice.
of the researchers,
'Würzburg/Germany), a leading expert of the MCAO

technique , to learn and train the technique in his lab. Therefore, we just need to train intrathecal injections.

- Bij punt 10a verzoekt de DEC het ongerief per groep aan te geven, ook op het voorblad. De DEC merkt op dat het ongerief van kolom 11 waarschijnlijk ongerief code 02 of code 05 moet zijn en verzoekt het leven na stroke ook te vermelden bij het ongerief.

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.
The discomfort of group 13, related to i.c.v. injection, has been changed from 04 to 05 on the front page and in paragraph 10a as requested. As the major discomfort of group 14 is also related to i.c.v. injection, the score of this group has also been changed to 05.

- 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 5+6 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 earlier in this response letter, two of the researchers already learned the technique 4 month ago from a specialist in the stroke field. We added a corresponding sentence at point 12:

in Würzburg/Germany in April 2011 to learn and train the MCAO technique from a leading scientist in the field of stroke (see ref. 9). ".

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

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

Project: *Which NADPH oxidase isoforms play a role in ischemic stroke in mice. Could they be used as therapeutic target?*

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:	2011-106
Diersoort:	muis
Aantal dieren:	472
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

DEC-UM
Voorzitter DEC-UM
t.n.v.
p/a secretariaat DEC-UM

Secretariaat DEC-UM

Bezoekadres

Postadres
Postbus 616
6200 MD Maastricht