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Hoofdproject	CARIM	NUTRIM	**Hersenen en gedrag**	GROW	biomaterialen	Ander UM	Geen UM
Deelproject	1. 2. 3.	1. 2. 3. 4.	1. 2. 3.	1. 2. 3.			

Financieel beheerder		Budgetnummer	30973302N

Titel van het onderzoek:

Phosphodiesterase inhibitors as a treatment option for severe perinatal asphyxia in rats

startdatum Juni 2011 einddatum [9] December 2012 *Duur van de proef [10]:* *8 dagen*

	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	
3. Verantwoordelijk medewerker (VM) GGO[7]					
4. overige uitvoerenden				Art.9	
5.				Art.9	
6.				Art.9	

Diergroep	1	2	3	4	5		
ctrl/exp/sham	Foster mothers	Experimental mothers	Offspring (experiment)	Offspring (exp; rest)	Offspring (foster; rest)		
Diersoort	02	02	02	02	02		
Stam	SD	SD	SD	SD	SD		
Construct / mutatie ?	-	-	-	-	-		
Herkomst (leverancier) *	01	01	01	01	01		
Aantal	14	88	160	800	168		
Geslacht	Female	Female	Female/male	Female/male	Female/male		
Dieren immuuncompetent ?	ja	ja	ja	Ja	Ja		
Leeftijd/gewicht	12 weeks	12 weeks	0 – 7 days	0 days	0 days		
Doel van de proef *	32	32	32	32	32		
Belang van de proef *	01	01	01	01	01		
Toxicologisch onderzoek *	01	01	01	01	01		
Bijzondere technieken *	01	10	10	01	01		
Anesthesie *	01	01	01	01	01		
Pijnbestrijding *	01	02	04	04	01		
Mate ongerief *	02	05	05	01	01		
Toestand dier einde exp*	03	01	01	02	02		

* VHI-coderingen zie bijlage

1 Verantwoording

Aanvraag dierproef DEC-UM (**kaders zijn licht flexibel, maar het geheel is max. 5 pag. versie 2006**)

Titel: Phosphodiesterase inhibitors as a treatment option for severe perinatal asphyxia in rats

1. Doel van de proef.

Asphyxia (hypoxia-ischemia) during fetal and neonatal development is a serious problem, causing each year thousands of victims world-wide. It is the major cause of both short and long-term neurological dysfunction, ranging from structural brain damage and death to neurodevelopmental impairment. Nowadays, no pharmacological treatment is available yet to improve the outcome of infants at risk for post-asphyctic brain damage. Therefore, there is an urgent need for further research to find more ideal drugs and therapies to prevent or treat post-asphyctic encephalopathy. One interesting group of pharmacological compounds are phosphodiesterase isoenzyme inhibitors (PDE-Is), like sildenafil (Viagra) [1]. PDEs belongs to a family of 11 phosphodiesterases that regulate the intracellular levels of cyclic nucleotides (cAMP and/or cGMP) and are for that reason involved in second messenger signalling [2, 3]. This rise in cGMP and/or cAMP can have some beneficial effects in the brain that might be of interest to prevent and/or treat infants with post-asphyctic encephalopathy. Recent data show that PDE-Is can potentially increase post-asphyctic survival in animal models, improve recovery and induce restorative mechanisms in the brain [4-6]. This is a new study in which we would like to test if one of those PDE-Is can improve or prevent brain damage in fetuses and neonates.

2. Maatschappelijke relevantie en/of wetenschappelijk belang

Fetal and perinatal asphyxia are serious problems, causing each year thousands of victims world-wide. The prevalence of birth asphyxia is ±25 in 1000 term births. If you know that in the Netherlands alone 185000 children are born each year, you can calculate that around 4500 of them are affected by neonatal asphyxia every year. Asphyxia can be induced by an unrecognized number of insults, resulting in moderate or severe hypoxic-ischemic encephalopathy, ranging from structural brain damage to neurodevelopmental impairment or death. Nowadays, no pharmacological treatment is available yet to improve the outcome of asphyctic infants. Therefore, testing PDE-Is as a potential treatment option for neonatal asphyxia is of great importance.

3. Alternatieven

In this project, all procedures represent clinical relevant situations. Such situation cannot be simulated by the use of other alternatives. Given that this project studies developmentally and neuroanatomically related events, including long-term effects on the offspring's biochemistry and behaviour, cell culture techniques would be irrelevant. Another reason is that complex mechanisms like placenta-related processes and cell-cell interaction are of great importance.

4. Ethische afweging

The researchers are convinced that the importance of the planned study (see also point 2 'maatschappelijk relevantie' and point 3 "Alternatieven") outweighs the suffering of the animals. Furthermore, all effort is taken to minimize the number of animals needed, as well as to minimize stress/suffering as much as possible.

Wetenschap

5. Wetenschappelijke onderbouwing

An interesting group of pharmacological compounds to treat post-asphyctic encephalopathy in infants are phosphodiesterase inhibitors (PDE-Is), like sildenafil (Viagra) [1]. PDEs regulate the intracellular levels of cyclic nucleotides and are for that reason involved in second messenger signalling [2, 3]. They either hydrolyze both cGMP and cAMP or specifically one of the two. By blocking these PDEs you can increase the intracellular levels of cGMP/cAMP. In turn, cAMP and cGMP can open cyclic nucleotide gated ion channels and stimulate PKA & PKG. The downstream targets of PKA and PKG are ion channels, receptors and transcription factors by which they can modulate neuronal functioning, metabolism and gene expression. In this way a rise in cGMP and/or cAMP can have some beneficial effects in the brain that might be of interest to prevent and/or improve post-asphyctic encephalopathy. One interesting target is PDE5. As will be discussed below, PDE5-I are most frequently cited in literature in relation to brain recovery processes in adult animals. It has been detected in different regions of the brain, like hippocampus and cortex [3]. PDE2A and PDE4D are maybe even more interesting since they are higher expressed in most brain areas than PDE5 in human as well as rats [7].

Why PDE inhibitors might have a beneficial effect after perinatal asphyxia?

First, Sildenafil (PDE5-I) is able to improve survival after asphyxia. For example, Sanchez-Aparicio et al. demonstrated that Sildenafil administered to pregnant guinea pigs increased the survival rate after intrapartum asphyxia in the offspring [8].

Second, PDE5 inhibitors are thought to have beneficial effect on the blood flow. For example, Zhang et al. demonstrated an increased localized cerebral blood flow after the administration of sildenafil in rats [6, 9]. An increased blood flow and concomitantly an increase in glucose metabolism might be helpful in rescuing the brain after/during asphyxia. In addition, PDE5-Is enhance angiogenesis [9], and might have desired effects on the uterine circulation [10].

Third, PDE5-I have been found to induce neurogenesis. For example, Zhang et al. found an increased number of proliferating cells in the subventricular zone, suggesting that Sildenafil augments neurogenesis [11]. In addition, there might also be an important role for cGMP in synaptogenesis during prenatal brain development [12]. There PDE5-Is might also induce syaptogenesis.

Fourth, PDE5-Is increase the total anti-oxidative status [13] as described by Choi et al after chronic treatment with DA-8159. Other protective effects from cGMP on oxidative stress have been reported. McIntyre et al. reviewed that zaprinast attenuated lipid peroxidation-mediated neuron toxicity [14]. Overall, most of those studies demonstrate that an elevated level of cGMP can reduce the excessive ROS generation and elevate the total anti-oxidative status.

Fifth, Sildenafil reduces neurological deficits and improves functional recovery in different adult stroke models. Treatment with Sildenafil for 7 days improved neurological function after stroke in aged rats, as measured by neurological severity scores and foot-fault test [6, 11]. Tadalafil showed similar beneficial effects on functional neurological outcome after embolic stroke [5].

Finally, it is important to mention that PDE5 inhibitors like Sildenafil are already approved by the FDA for the treatment of erectile dysfunction and pulmonary hypertension [2]. Furthermore, Sildenafil was used several times before in pregnant animals proving that this small molecule can most likely be transferred across the placenta. Sildenafil showed no deleterious effects on the offspring in animals studies, human case reports and a phase II clinical trial [4, 8, 15-18].

6. Wetenschappelijke beoordeling

This DEC protocol has been examined and approved by

Proefdier

7. **Proefdier keuze**

7a. Soort, stam / herkomst / eindbestemming

Time-pregnant (E14 on date of delivery) Sprague-Dawley rats derived from Charles River will be used. This strain was also used in our previous studies. Mothers are killed by decapitation at the end of gestation, offspring are anesthetized and perfused at 7 days and foster mothers can be returned to the CPV. Since the animals are only delivered on E14, they have to be immediately released by the CPV for the experiments.

7b. Sexe

Only male offspring will be used for the two aims mentioned in appendix 1, because morphological and behavioural data show a differential vulnerability to a birth insult in males versus females. A greater impact is seen in the male gender, probably due to a protecting role of the circulating oestrogens (El-Khodor et al., 2003; Tanila et al., 1994; Zhang et al., 1998). The female offspring will be used post-mortem by Eduardo Villamor and Evi Vlassaks for alternative experiments (see also point 7c)

7.c. Aantallen

Sample sizes were calculated using the power and sample size program PS version 3.0 (2009) (*www.mc.vanderbilt.edu/prevmed/ps*). From previous studies, we know that the within group standard deviation can be estimated at 17% (σ). The wanted difference between the control group and the treated groups in mortality is 25% (δ). With a type I error probability of $\alpha = 0.05$ and power of 80%, the sample size estimated by the PS program is 8. Therefore, the sample size used in this project is 8 animals per group for immunohistochemistry and 8 pups per group for molecular analysis (2 aims; see appendix 1).

In addition, we know from our previous experiments that the mortality after perinatal asphyxia is around 60% [19, 20]. Taken this into account we need 20 pups per group for the experimental groups instead of 8. For the control groups, there is no mortality and therefore 8 pups is enough. (For overview of the groups see appendix 1)

Only 2 pups per litter per aim can be used to prevent "litter effects" [21]. Therefore, we need 4 mothers per group to become a sample size of 8 pups per group per aim for the controls and 10 mothers for the experimental groups.

Furthermore, pups will be cross-fostered with surrogate mothers (max. 12 pups/dam).
All this is summarized in the table below.

Pilot study

	Sample size (AIM 1)	Sample size (AIM 2)	Mortality	Sample size after mortality correction (AIM 1)	Sample size after mortality correction (AIM2)	Mothers needed
Exp 1	8	8	60%	20	20	10
Exp 2	8	8	60%	20	20	10
Exp 3	8	8	60%	20	20	10
Controls	8	8	0%	8	8	4
Fosters			0%			6
	32	32				40

Large experiment

	Sample size (AIM 1)	Sample size (AIM 2)	Mortality	Sample size after mortality correction (AIM 1)	Sample size after mortality correction (AIM2)	Mothers needed
Exp 1	8	8	60%	20	20	10
Exp 2	8	8	60%	20	20	10
Exp 3	8	8	60%	20	20	10
Exp 4	8	8	60%	20	20	10
Exp 5	8	8	60%	20	20	10
Controls	8	8	0%	8	8	4
Fosters			0%			8
	48	**48**				62

In conclusion: For the whole experiment 102 pregnant dams and 160 offspring will be used.

It is important to note that the rest of those litters will be used post-mortally by our group to make primary cell cultures to do additional **alternative experiments** (DECnr 2010-012; Evi Vlassaks). Therefore, as much pups as possible will be used. Foster mothers can be returned to the CPV.

8. Experiment

First, we would like to test the hypothesis that the administration of a PDE5, PDE2A or PDE4D inhibitor around and after a severe asphyctic insult can prevent or reduce post-asphyctic encephalopathy and neonatal mortality in rats. For that, we would like to use our validated rat model for severe perinatal asphyxia (SPA), in which the uterine horns will be submersed in a water bath of 37°C for 19 minutes at embryonic day 22 [19, 20] (For more information see SOPs in appendix 2)

First, a pilot study will be done to test which PDE-I is the most promising. To test this research question, four groups (n=8/group) will be included: 1) A control group receiving saline injections; 2) a PDE2A-I group receiving BAY60-7550zxc vh (3mg/kg orally for mothers and 1mg/kg s.c. for pups); 3) a PDE4D-I group (0,01mg/kg orally for mothers and 0.003mg/kg s.c. for pups) and 4) a PDE5-I group receiving Vardenafil (3mg/kg orally for mothers and 1mg/kg s.c. for pups [4]). Doses were based on the effective dose in adult animals, as were provided by the companies. For pregnant rats the dose was multiplied by 3 since only around 35% of PDE-Is crosses the placenta [22]. ***Figure 1*** in appendix 1 shows the experimental set-up of this pilot experiment.

The most promising inhibitor will then be tested in more detail in an extensive study. This compound will be administered at different time points before and after the insult to test with dosing regime works best. The experimental set-up is given in ***figure 2*** in appendix 1. Group 1 will be used to test if a single dose can precondition the animal against a subsequent severe asphyctic insult, while group 4 will be used to test if a single dose after the insult can postcondition the animals. Group 2 will test if multiple doses can precondition the animal, while group 5 will test if multiple doses can postcondition the animals. Both groups will be used to see if repeating the treatment for 7 consecutive days is more efficacious than a singe dose. In group 3 a combination of a single dose before and after the insult is used. In the control group repeated injections of saline will be given before and after the insult to test the effect of repeated injections per se.

The outcome measurements for both studies are the same: 1) mortality, 2) neuronal cell death, 3) cell proliferation etc. They are more extensively written down in appendix 1.Below you can find the experiments per group of animals.

Experimental procedures of pregnant dams (control and treatment):
Stress level (VHO): 05

- Single or repeated s.c./oral injections of compound or saline
- Solitary housing during pregnancy
- Decapitation and SPA procedure (See SOP)

Experimental procedures of the foster mothers
Stress level (VHO): 01

- Fostering of pups and solitary housing

Experimental procedures of the offspring:
Stress level (VHI): 05

- Weighed, sexed and marker after birth
- Cross-fostering
- Single or repeated s.c. injections with compound or saline
- Euthanasia with pentobarbital and perfusion at P7

It is not possible to use historical controls.

9. Experimentele condities

9a. Anesthesie

Not applicable in the experiments itself. During euthanasia, offspring will be anesthetised with pentobarbital (pentobarbital; 60mg/kg; i.p.).

9b. Pijnbestrijding

No analgesia will be used during decapitation of the pregnant dam, since these can cross the placenta easily, having even a prolonged half-life in the fetus which has been shown to interrupt with fetal development (for Buprenorphine see [23]. and for NSAIDs see [24]) Therefore, the use of analgesics can interfere with the outcome of our experiments.

9c. Euthanasie en Humane eindpunten

Term pregnant rats are decapitated. Offspring will be either perfused or decapitated at ± 7 days of age. Perfusions will be performed after the pups are anesthetized with pentobarbital (pentobarbital; 60mg/kg; i.p.). Pups presenting uncompleted cardio-respiratory recovery 5 minutes after birth are decapitated. Offspring with a weight loss > 20% body-weight postnatally are decapitated. All decapitations will be executed using a guillotine. More human endpoints: see appendix 3.

Zorg

10a. Ongerief

There are no known side effects for any of these PDE-Is on mother or pups.
For more detailed table see Appendix 3

10b. Welzijnsevaluatie

From previous experiments, we know that the pups that underwent a severe perinatal asphyxia have normal birth weight, but they have a growth retardation (they weigh less between 2-3 days, but have normal weights again after 5 days. During these experiments, we will again check the general condition and weights of both the mother animals and pups. More info see appendix 3.

11. Verzorging en huisvesting

Solitary housing of the pregnant dams during the full extent of the pregnancy CPV. This in order to prevent interference and stress from the other pregnant female, for which we cannot control. Changing cages and feeding of the animals will be taken care for at set-time points by the investigators themselves. The SPA procedures will be performed a CPV. Offspring are cross-fostered to surrogate mothers (max. 12 pups/litter), where they are kept for 7 days. In case of problems, please contact

12. Deskundigheid

have several years of experience with the animal model.

13. Standard Operation Procedures (SOP)

See appendix 2

Relevante literatuur

1. *Chen, J. and M. Chopp. Neurorestorative treatment of stroke: cell and pharmacological approaches. NeuroRx, 2006; 3(4):466-73.*
2. *Uthayathas, S., S.S. Karuppagounder, B.M. Thrash, K. Parameshwaran, V. Suppiramaniam, and M. Dhanasekaran. Versatile effects of sildenafil: recent pharmacological applications. Pharmacol Rep, 2007; 59(2):150-63.*
3. *Reneerkens, O.A., K. Rutten, H.W. Steinbusch, A. Blokland, and J. Prickaerts. Selective phosphodiesterase inhibitors: a promising target for cognition enhancement. Psychopharmacology (Berl), 2009; 202(1-3):419-43.*
4. *Ozdegirmenci, O., T. Kucukozkan, E. Akdag, T. Topal, A. Haberal, H. Kayir, et al. Effects of sildenafil and tadalafil on ischemia/reperfusion injury in fetal rat brain. J Matern Fetal Neonatal Med.*
5. *Zhang, L., Z. Zhang, R.L. Zhang, Y. Cui, M.C. LaPointe, B. Silver, et al. Tadalafil, a long-acting type 5 phosphodiesterase isoenzyme inhibitor, improves neurological functional recovery in a rat model of embolic stroke. Brain Res, 2006; 1118(1):192-8.*
6. *Zhang, R., Y. Wang, L. Zhang, Z. Zhang, W. Tsang, M. Lu, et al. Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. Stroke, 2002; 33(11):2675-80.*
7. *Lakics, V., E.H. Karran, and F.G. Boess. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology; 59(6):367-74.*
8. *Sanchez-Aparicio, P., D. Mota-Rojas, A.A. Nava-Ocampo, M.E. Trujillo-Ortega, A. Alfaro-Rodriguez, E. Arch, et al. Effects of sildenafil on the fetal growth of guinea pigs and their ability to survive induced intrapartum asphyxia. Am J Obstet Gynecol, 2008; 198(1):127 e1-6.*
9. *Li, L., Q. Jiang, L. Zhang, G. Ding, Z. Gang Zhang, Q. Li, et al. Angiogenesis and improved cerebral blood flow in the ischemic boundary area detected by MRI after administration of sildenafil to rats with embolic stroke. Brain Res, 2007; 1132(1):185-92.*
10. *Wareing, M., J.E. Myers, M. O'Hara, and P.N. Baker. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. J Clin Endocrinol Metab, 2005; 90(5):2550-5.*
11. *Zhang, R.L., Z. Zhang, L. Zhang, Y. Wang, C. Zhang, and M. Chopp. Delayed treatment with sildenafil enhances neurogenesis and improves functional recovery in aged rats after focal cerebral ischemia. J Neurosci Res, 2006; 83(7):1213-9.*
12. *Chen, J., Y. Tu, C. Moon, V. Matarazzo, A.M. Palmer, and G.V. Ronnett. The localization of neuronal nitric oxide synthase may influence its role in neuronal precursor proliferation and synaptic maintenance. Dev Biol, 2004; 269(1):165-82.*
13. *Choi, S.M., J.E. Kim, and K.K. Kang. Chronic treatment of DA-8159, a new phosphodiesterase type V inhibitor, attenuates endothelial dysfunction in stroke-prone spontaneously hypertensive rat. Life Sci, 2006; 78(11):1211-6.*
14. *McIntyre, M., D.F. Bohr, and A.F. Dominiczak. Endothelial function in hypertension: the role of superoxide anion. Hypertension, 1999; 34(4 Pt 1):539-45.*
15. *Momma, K., K. Toyoshima, S. Imamura, and T. Nakanishi. In vivo dilation of fetal and neonatal ductus arteriosus by inhibition of phosphodiesterase-5 in rats. Pediatr Res, 2005; 58(1):42-5.*
16. *Villanueva-Garcia, D., D. Mota-Rojas, R. Hernandez-Gonzalez, P. Sanchez-Aparicio, M. Alonso-Spilsbury, M.E. Trujillo-Ortega, et al. A systematic review of experimental and clinical studies of sildenafil citrate for intrauterine growth restriction and pre-term labour. J Obstet Gynaecol, 2007; 27(3):255-9.*
17. *Miller, S.L., J.M. Loose, G. Jenkin, and E.M. Wallace. The effects of sildenafil citrate (Viagra) on uterine blood flow and well being in the intrauterine growth-restricted fetus. Am J Obstet Gynecol, 2009; 200(1):102 e1-7.*
18. *Samangaya, R.A., G. Mires, A. Shennan, L. Skillern, D. Howe, A. McLeod, et al. A randomised, double-blinded, placebo-controlled study of the phosphodiesterase type 5 inhibitor sildenafil for the treatment of preeclampsia. Hypertens Pregnancy, 2009; 28(4):369-82.*
19. *Strackx, E., D.L. Van den Hove, J. Prickaerts, L. Zimmermann, H.W. Steinbusch, C.E. Blanco, et al. Fetal asphyctic preconditioning protects against perinatal asphyxia-induced behavioral consequences in adulthood. Behav Brain Res; 208(2):343-51.*
20. *Strackx, E., B. Zoer, D. Van den Hove, H. Steinbusch, H. Steinbusch, C. Blanco, et al. Brain apoptosis and carotid artery reactivity in fetal asphyctic preconditioning. Front Biosci (Schol Ed); 2:781-90.*
21. *Chapman, R.H. and J.M. Stern. Failure of severe maternal stress or ACTH during pregnancy to affect emotionality of male rat offspring: implications of litter effects for prenatal studies. Dev Psychobiol, 1979; 12(3):255-67.*
22. *Pellicer, B., S. Herraiz, O. Cauli, R. Rodrigo, M. Asensi, J. Cortijo, et al. Haemodynamic effects of long-term administration of sildenafil in normotensive pregnant and non-pregnant rats. Bjog; 118(5):615-23.*
23. *Robinson, S.E. Effects of perinatal buprenorphine and methadone exposures on striatal cholinergic ontogeny. Neurotoxicol Teratol, 2002; 24(2):137-42.*
24. *Alano, M.A., E. Ngougmna, E.M. Ostrea, Jr., and G.G. Konduri. Analysis of nonsteroidal antiinflammatory drugs in meconium and its relation to persistent pulmonary hypertension of the newborn. Pediatrics, 2001; 107(3):519-23.*

Appendix 1

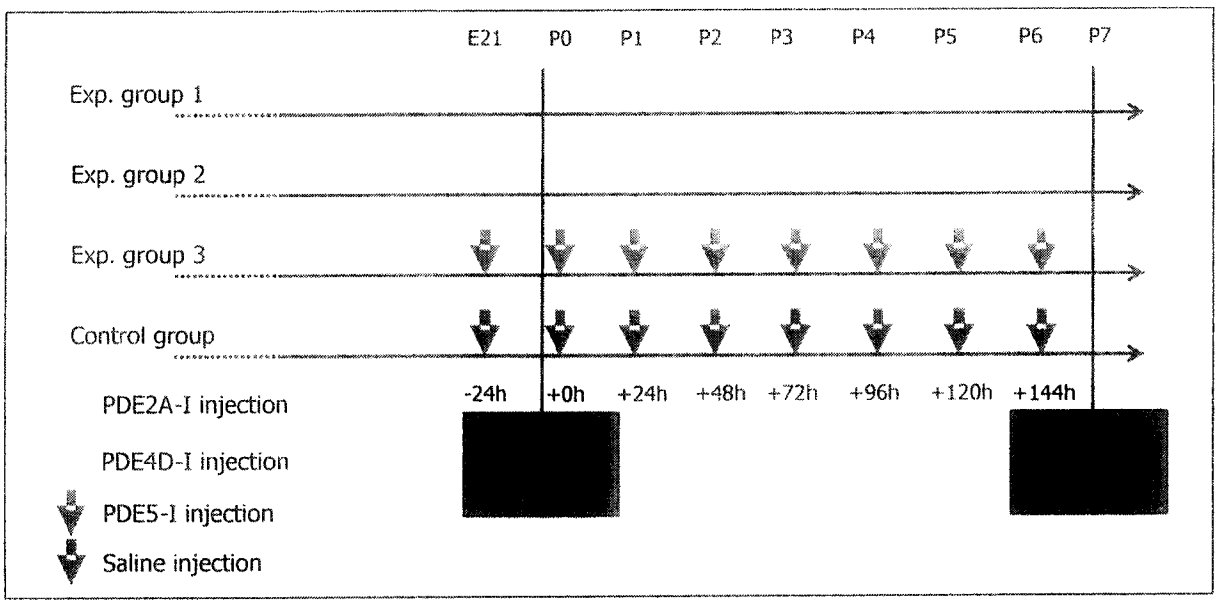

Figure 1: The experimental set-up of the pilot experiments to test which compound works best.

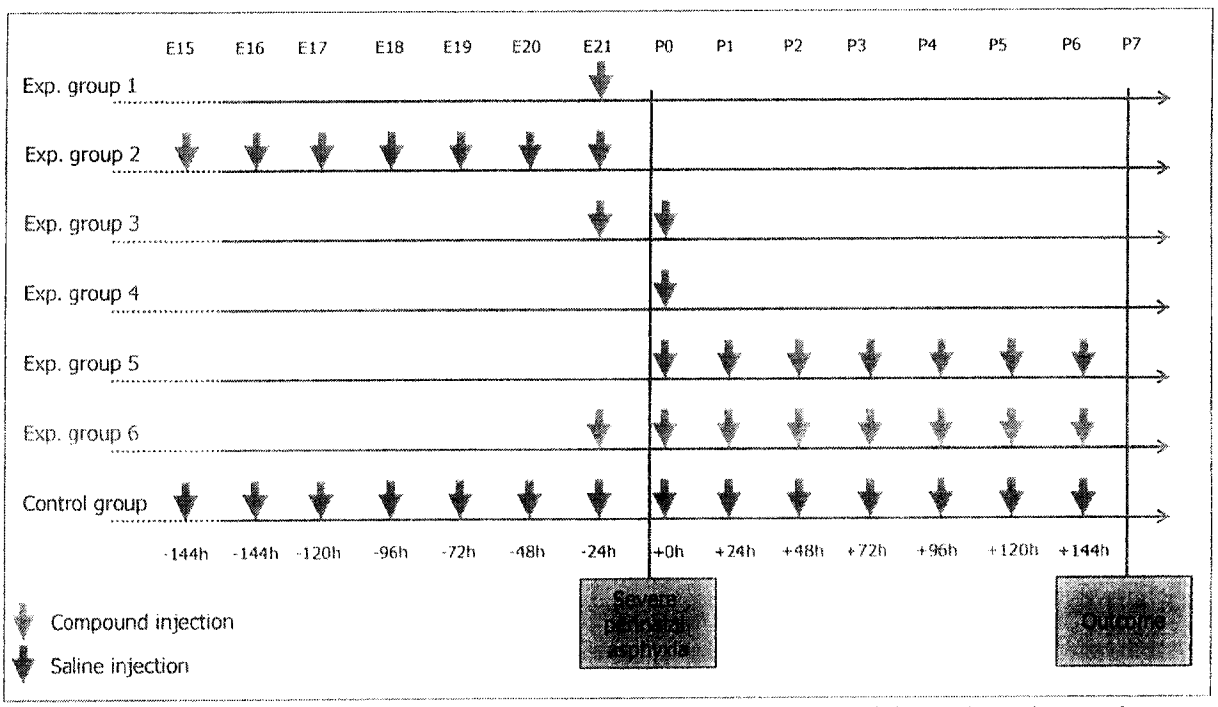

Figure 2: The experimental study with the chosen compound from the pilot study.

Outcome measurements

Eight pups per condition will be used for immunohistochemical analysis (**AIM 1**). Pups will be perfused transcardially with somogyi fixative, brains will be removed and cut on the cryostate. Another eight pups per condition will be used for the above described molecular analysis (**AIM 2**). Brains will be snap frozen until analysed. For each aim to pups per mother will be used.

The first two outcome measures will be the survival rate at birth and during the first week of life as well as the body and organ weights of the offspring to check if the PDE-Is treatment can improve neonatal survival after perinatal asphyxia and if it has any effect on the neonatal body and organ weights

.

In addition, offspring will be sacrificed at postnatal day 7 to analyze all treatment options. Detailed histological and molecular analysis will be performed on their brains as follows:

1. Post-asphyctic encephalopathy will be analysed immunohistochemically by measuring the number of TUNEL+ apoptotic cells in different regions of the brain. We expect less apoptosis in the treated groups compared to the control group.
2. Neurogenesis, synaptogenesis and angiogenesis will be immunohistochemically assessed in different brain regions. Neurogenesis will be evaluated by the Ki67 antibody (a marker for proliferating cells), the MCM2 antibody (a marker for proliferating cells) and the doublecortin antibody (a marker for migrating neuroblasts). Angiogenesis will be analysed by visualizing and counting the number of cerebral blood vessels by the endothelial barrier antigen (EBA) antibody. We expect more. The total number of synapses will be measured using the synaptophysin antibody. We expect more neurogenesis, synaptogenesis and angiogenesis in the treated groups compared to the control group.
3. The total anti-oxidative status will be estimated using the commercially available total antioxidant status assay kit, where we assume to find a higher antioxidative status in the treated animals.
4. To test the underlying mechanism by which PDE-Is might protect the neonatal rat brain against post-asphyctic encephalopathy the level of nitric oxide, nitric oxide synthase and cGMP will be measured in plasma and/or brain also using commercially available kits. The presence of PDE5A en PDE5B will be examined by RT-PCR. Furthermore, Western blot analysis will be performed for phosphospecific Akt, phosphospecific Erk and phospho-GSK3α/β to test if the activation of the PI3-K/Akt/GSK-3 pathway might be the underlying mechanism. We believe that PDE-Is causes neuroprotection via an increase in cGMP in the brain, thereby activating the above mentioned pathway.
5. To test if PDE-Is are able to reopen the ductus arteriosus, pups will be sacrifized at different time points (0h, 24h, 48h, 72h, 96h and 120h) by a rapid whole-body freezing method. The frozen thorax will be cut on a cryostate in the frontal plane and the inner diameters of the ascending aorta, main pulmonary artery and ductus arteriosus will be measured with the stereology system [15].

Appendix2: SOP's

Severe perinatal asphyxia procedure (Embryonic day 22/postnatal day 0)

- Pregnant rats are euthanized by decapitation using a guillotine and rapidly hysterectomized.
- The uterine horns still containing the foetuses are detached and placed in a water bath, precisely calibrated at 37°C, for exactly 18 minutes (counting from cutting off the blood circulation of the uterus until the moment the pups are taken out of the water bath.)
- After 18 minutes, the uterine horn is incised quickly and the pups are removed, cleaned with medical swipes and stimulated manually to breathe to aid recovery. (All steps are executed inside a closed paediatric incubator to aid recovery (37°C, 60-80% humidity and room air). The umbilical cords are ligated and cut to separate the pups from their placentas.
- Then, they are left to recover for 60 minutes in the paediatric incubator.
- Offspring are cross-fostered to surrogate mothers (12 pups/litter), where they are kept for 8 or 15 days.

Euthanasia of pregnant dams at postnatal day 0

- Pregnant rats are euthanized by decapitation and rapidly hysterectomized.
- The uterine horns still containing the foetuses are detached, incised quickly and the pups are removed.
- Pups are euthanized by decapitation. The brains are removed and fixated by an immersion fixation for further immunohistochemical examination.

Perfusion

- Pups are anesthetized with sodium pentobarbital (60mg/kg; pentobarbital; i.p.).
- They are perfused intracardially, first by a tyrode solution, followed by fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.2M phosphate buffer (pH=7.2).
- Afterwards, the brains are removed from the skull and utilized for further immunohistochemical examination.

Appendix 3: Human end points and possible complications

From previous experiments we know that the pups that underwent severe perinatal asphyxia are a bit lighter 3 days after the insult, but their weights are completely normal after 5 days. We will daily check the weights and the overall condition of the pups and mothers during the experiments.

Listed below a list of the possible complication that might occur:

During the experiment:

1. After perinatal asphyxia on postnatal day 0 the following complications might occur in the pups
    a. An insufficient cardio-respiratory response that does not improve in the first 5 minutes after birth
    b. A deviation of more than 20% of the normal growth curve

2. Within the first days of life the following complications can occur:
    a. Weight loss of more that 20% since the start of the experiments or a weight loss of more than 15% in short period of a few days (1-2)

3. Repeated injections orally or s.c. of the PDE-Is mentioned in this study are not known to cause any side effects.

Human end points

If one of the complications listed above occurs, we will first ask for advice from the veterinary of the CPV. If the animal cannot be treated of when the complications are too severe, the animal will be decapitated to prevent needless suffering.

Appendix 3:

	Stimulus	Time point	frequency	Inconvenience score
Foster mothers	Solitary housing	E14–P7 (=14d)	1x	02
Experimental mothers (pilot)	Solitary housing	E14–P7 (=7d)	1x	
	Oral injection PDE-I	E14–E21	7x (1x/day)	
	decapitation	E21	1x	05
Experimantal mothers (rest)	Solitary housing	E14-E21 (=7d)	1x	
	decapitation	E21	1x	
Exp group 1	Oral injection PDE-I	E21	1x	04
Exp group 2	Oral injection PDE-I	E21	1x	04
Exp group 3	Oral injection PDE-I	E15-E21	7x (1x/day)	05
Exp group 4	No injection			04
Exp group 5	No injection			04
Exp group 6	Oral injection PDE-I	E21	1x	04
Control group	Oral injection saline	E15-E21	7x (1x/day)	05
Offspring (exp)	sexing	P0	1x	
	marking	P0	1x	
	Cross-fostering	P0	1x	
	perfusion	P7	1x	
Exp group 1	No injection			04
Exp group 2	No injection			04
Exp group 3	S.C. injection PDE-I	P0	1x	04
Exp group 4	S.C. injection PDE-I	P0	1x	04
Exp group 5	S.C. injection PDE-I	P0-P6	7x (1x/day)	05
Exp group 6	S.C. injection PDE-I	P0-P6	7x (1x/day)	05
Control group	S.C. injection saline	P0-P6	7x (1x/day)	05
Offspring (rest)	/	/	/	01

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

Voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon: 043

Aan.

Uw referentie:

Onze referentie

Maastricht, 29-06-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Phosphodiesterase inhibitors as a treatment option for severe perinatal asphyxia in rats*", is op de DEC vergadering van 24 juni 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De DEC denkt, bij punt 4, dat "outweight suffering" ongerief code 04 moet zijn.
- De DEC verzoekt bij punt 6 de laatste zin te verwijderen en de eerste zin aan te passen in "This **DEC** protocol has been examined and
- Bij punt 7b wenst de DEC de motivering waarom alleen mannelijke dieren gebruikt worden.
- Bij punt 7c vraagt de DEC zich af of de dieren, genoemd in de voorlaatste zin (additional alternative experiments), al verdisconteerd zijn in project 2010-012 of moeten deze op die aanvraag als uitbreiding worden toegevoegd?
- De DEC wil graag een referentie bij de bewering van "litter effects".
  Bij punt 7c verzoekt de DEC de aantallen en de uitval per groep duidelijk te motiveren en te specificeren. De uitval dient direct meegenomen te worden bij de berekening van de groepsgrootte.
- Bij punt 10a verzoekt de DEC in een tabel, de aard, ernst, duur en frequentie beter te definiëren per handeling en groep, en het totale ongerief aan te geven. Het mogelijk effect van de toegediende stoffen op de moeders, dient ook vermeld te worden bij het ongerief.

Conclusie:
Het project wordt aangehouden.

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 2011-069, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend

Voorzitter DEC-UM

Universiteit Maastricht
Centrale proefdiervoorziening
t.a.v.
Voorzitter DEC-UM

Betreft: Aanvraag project: "*Phosphodiesterase inhibitors as a treatment option for severe perinatal asphyxia in rats*" (DEC 2011-069)

Maastricht, 1 juli 2011

Beste

Hierbij de antwoorden op de opmerkingen omtrent DEC 2011-069 (titel: "*Phosphodiesterase inhibitors as a treatment option for severe perinatal asphyxia in rats*") dat werd besproken op de DEC vergadering van 24 juni 2011.

- *Opmerking 1*: De DEC denkt, bij punt 4, dat "outweight suffering" ongerief code 04 moet zijn.
  Antwoord: De onderzoekers begrijpen niet wat met deze opmerking bedoelt wordt. Er staan helemaal geen ongerief codes vermeld bij punt 4.

- *Opmerking 2*: De DEC verzoekt bij punt 6 de laatste zin te verwijderen en de eerste zin aan te passen in "This DEC protocol has been examined and approved by
  Antwoord: Aangepast en in het grijs gemarkeerd.

- *Opmerking 3:* Bij punt 7b wenst de DEC de motivering waarom alleen mannelijke dieren gebruikt worden.
  Antwoord: Enkel mannelijke dieren worden gebruikt omdat zij vatbaarder zijn dan de vrouwelijke dieren. Vrouwtjes worden beschermd door de werking van oestrogenen. Dit werd aangepast en gemarkeerd in het DEC en referenties werden toegevoegd.

- *Opmerking 4:* Bij punt 7c vraagt de DEC zich af of de dieren, genoemd in de voorlaatste zin (additional alternative experiments), al verdisconteerd zijn in project 2010-012 of moeten deze op die aanvraag als uitbreiding worden toegevoegd?
  Antwoord: De aanvraag voor de uitbreiding van dat DEC is ingediend.

- *Opmerking 5:* De DEC wil graag een referentie bij de bewering van "litter effects".
  Antwoord: Een referentie over de litter effects werd toegevoegd:

- *Opmerking 6*: Bij punt 7c verzoekt de DEC de aantallen en de uitval per groep duidelijk te motiveren en te specificeren. De uitval dient direct meegenomen te worden bij de berekening van de groepsgrootte.
  Antwoord: De aantallen en de uitval werden duidelijk gemotiveerd en gespecifieerd per groep. Verder werd alles in een overzichtelijk tabel geplaatst.

- *Opmerking 7*: Bij punt 10a verzoekt de DEC in een tabel, de aard, ernst, duur en frequentie beter te definiëren per handeling en groep, en het totale ongerief aan te geven. Het mogelijk effect van de toegediende stoffen op de moeders, dient ook vermeld te worden bij het ongerief.
  Antwoord: Een uitgebreide tabel werd toegevoegd. Deze stoffen veroorzaken geen neveneffecten bij de moeders en pups. Dit werd ook toegevoegd.

Met vriendelijke groeten,