RESEARCH SUMMARY

Background: Perinatal asphyxia is one of the primary causes of neonatal mortality and morbidity, **accounting for 700.000-1.000.000 neonatal deaths** yearly. Hypothermia has been proven to be a safe and effective therapy after asphyxia; it reduces mortality and improves neurological outcome. Although hypothermia resolved several shortcomings of previous therapeutic approaches, 47% of affected neonates still suffer from neuro-developmental disabilities *(Hochen et al Resuscitation, 2008)*.
Asphyxia is still the main cause of neonatal hypoxic-ischemic encephalopathy and is often associated with multi-organ failure, which **significantly increases the severity of adverse neurological outcomes** *(Nouri et al Arch Pediatr, 2008)*. However, these multiorgan abnormalities often remain undiagnosed due to limited attention focused mainly on neurological pathologies and to the lack of sensitive biomarkers.
While perinatal asphyxia can cause severe life-long disabilities including cerebral palsy or epilepsy, epidemiological studies suggest that even **moderate asphyxia at birth may play a role in the development of cognitive deficits** and psychiatric disorders. Other clinical studies report that renal hypoxia even without alterations in conventional kidney injury parameters (e.g., serum creatinine or BUN) might result in renal functional impairment and **increased susceptibility to hypoxic injury** on the long run. However, **the underlying mechanisms remain elusive** to date which makes it impossible to design rational therapeutic approaches.

Aims: The translatability of the widely used preclinical animal models is very limited for drawing clinically relevant conclusions. Previous attempts to model neonatal asphyxia in mice and rats *(Millar et al, Front Cell Neurosci, 2017)* mostly consisted of surgical occlusion of the carotid artery(ies) followed by exposure to hypoxic gas mixtures to reach a critical threshold for blood flow reduction leading to focal brain injury instead of general hypoxic insult. Several problems arose with the interpretation of experimental data obtained from these models. The nature of brain injury that develops in restricted areas of the brain is markedly different, moreover changes in peripheral organs (kidney, liver, heart, etc.), which are important contributors to the complex pathophysiologies caused by asphyxia could not be appropriately evaluated either. Thus, the main goal of our proposal was **(i) to establish and characterize a new rodent model of moderate perinatal asphyxia** based on the model described by Kaila et al *(Helmy et al, Ann Neurol 2011)*. Secondly, **(ii)** we aimed to investigate **the efficacy of the combination therapy of hypothermia and restoration of normocapnia** on neurological, functional and behavioural outcome measures. The last aim was **(iii) to study the pathological features of acute and long-term multi-organ damage due to perinatal asphyxia** with focus on peripheral organs such as the kidney, liver and heart.

Concise methods: Putative mechanisms through which neonatal asphyxia may contribute to the subsequent CNS, multiorgan and behavioural alterations in adulthood were investigated using Wistar rats. On postnatal day 7 (P7) rat pups were exposed to a gas mixture containing 4% O_2, 20% CO_2 and 76% N_2 for 15 min at 37^{o}C. Control rats were separated from the dams and kept in room air at 37^{o}C for the same period. Rats were subjected to **comprehensive behavioural assessment** from 4h-24h post-asphyxia into adulthood. **Brain perfusion and microglial activation** were investigated with SPECT and MRI *in vivo*. **Inflammation and neuronal injury** were studied by using cytometric bead array, immunofluorescence and confocal/super-resolution microscopy. **Functional, histological injury and organ-specific pathways** associated with asphyxia were investigated in various organs at 4h, 24h, 6wk and 5 months. To assess whether neonatal asphyxia-induced kidney injury increases ischemic susceptibility later in life **mild bilateral renal ischemia** was applied by clamping the renal pedicles for 35 min at 37^{o}C in adult (6-month old) rats.

Results:

1. **Perinatal asphyxia leads to transient, acute activation of HPA – axis.** To investigate acute HPA-axis activation, ACTH and cotricosterone levels were measured in the plasma under baseline conditions and at different time points after the insult. Asphyxic insult resulted in a transient activation of the HPA-axis, confirmed by a rapid and dramatic increase in ACTH and corticosterone levels in asphyxiated (ASX) pups. Corticosterone levels – consistently with the 'stress hyporeactive period' of neonatal rats (*Sapolsky and Meaney Brain Res 1986)* – were very low compared to adult levels and showed an increasing tendency following the insult.

As previous studies suggested that aldosterone might serve as a „stress hormone" during the neonatal period of rodents *(Varga et al, PLOS One ,2018)*, we also measured plasma aldosterone levels and indeed found a transient, significant elevation (**Fig. 1**). Importantly, perinatal asphyxia did not seem to induce lasting perturbations in HPA function as plasma corticosterone levels did not differ between adult ASX and control rats (data not shown).

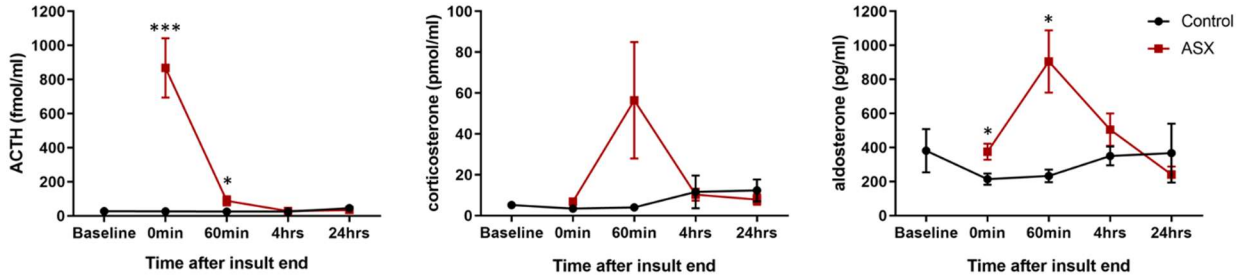


Figure 1. HPA-axis activation after asphyxic insult. The effect of perinatal asphyxia on plasma ACTH, corticosterone and aldosterone levels, measured by radioimmunoassay or ELISA (aldosterone). Data are shown as means±SEMs. *P<0.05; ***P<0.001, unpaired t-test.

2. Perinatal asphyxia leads to region-dependent alterations in the brain

Imaging studies revealed that brain perfusion changes 24h after asphyxia. On T1 weighed MRI, **asphyxia was associated with reduced blood volume in the brain** 24h after the insult **(Fig.2A)**. SPECT imaging (99mTc-HMPAO) showed reduced perfusion in the brainstem 24h after asphyxia, while blood flow did not change in the hippocampus and prefrontal cortex **(Fig.2B).** These alterations appear to appropriately reflect the complex perfusion changes seen in the clinic. In contrast, in experimental models unilateral carotid artery occlusion leads to focal brain injury only resembling that seen in models of experimental neonatal stroke, with corresponding perfusion deficits.

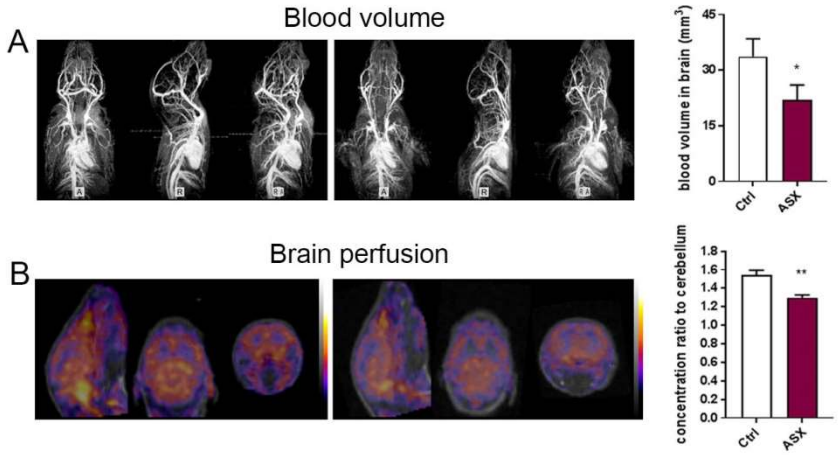


Figure 2. Changes in blood volume and cerebral perfusion in the brain after asphyxic insult. A. The effect of perinatal asphyxia on cerebral blood volume was assessed by dextran-coated iron oxide nanoparticles visualized with T1-weighted magnetic resonance imaging 24h after the insult. **B**. Reduced perfusion in the brainstem is seen 24h after asphyxia by SPECT imaging based on the uptake of 99mTc-HMPAO. *P<0.05; **P<0.001, unpaired t-test.

3. Perinatal asphyxia leads to region-dependent vascular and inflammatory alterations in the brain

Next, we assessed histological changes occurring 24h post-asphyxia and investigated whether any of these alterations are present in adult animals. We found that **perinatal asphyxia resulted in a marked disturbance in the development of neuronal populations** in the brain. The number of c-Fos-positive cells increased in the prefrontal cortex particularly in the infralimbic and prelimbic cortex, **(Fig.3A)**, similarly to that seen in the hippocampus (data not shown). Notably, the prefrontal cortex and the hippocampus are both sensitive to hypoxic injury, which is linked to the development of different forms of psychiatric and mood disorders in humans *(Brockmann et al., PLOS ONE 2013; Salmaso et al., Nat Neurosci 2014.)*. Injury in the prefrontal cortex **(Fig.3B)** and the hippocampus was indicated by the increased number of cleaved Caspase-3-positive cells, which was also observed in other areas of the brain, including the striatum and cerebral cortex. However, no significant reduction of neuronal numbers was seen in adult animals based on the quantification performed on cresyl violet–stained brain sections, indicating that **lasting focal neuronal loss does not develop in this experimental model (Fig.3C)**.

Remarkably, **perinatal asphyxia resulted in neuroinflammatory changes in the brain.** Vascular activation as indicated by increased ICAM-1 levels in cerebral blood vessels was observed primarily in the prefrontal cortex **(Fig.3D)**, which was not seen in adult animals. In line with this, microglial activation was seen in most brain areas 24h after asphyxia. In the prefrontal cortex, increased microglial Iba1 immunopositivity was partially preserved in adult rats **(Fig.3E)**, while the GFAP signal remained unchanged in pups or adult rats **(Fig.3F)**.

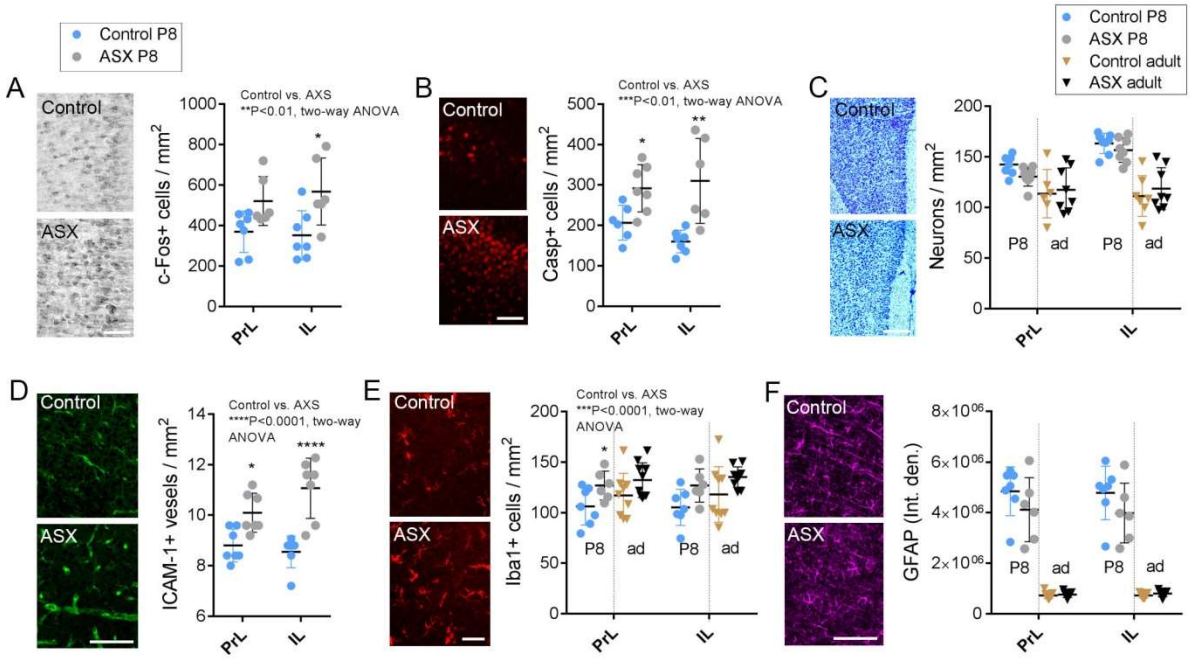


Figure 3. **Histological and inflammatory changes in the prefrontal cortex after perinatal asphyxia**. **A**. c-Fos immunopositive nuclei in the prelimbic (PrL) and inflalimbic (IL) cortex in control rats (Control P8) and 24h after perinatal asphyxia (ASX P8). **B**. Cleaved Caspase-3-positive cells in the PrL and IL 24h after asphyxia. **C**. Neuonal loss as assessed on cresyl violet -stained brain sections in the prefrontal cortex 24h (Control P8 and ASX P8) and 5 months (Control adult and AXS adult) after perinatal asphyxia. **D**. ICAM-1 immunostaining reveals vascular inflammation 24h after asphyxia. **E**. Microglial activation as assessed by Iba1 immunostaining in the prefrontal cortex. **F**. GFAP-positive astrocytes in the prefrontal cortex 24h and 5 months after asphyxia. *P<0.05; **P<0.001, ***P<0.001, two-way ANOVA followed by Sidak's post hoc comparison. Scale bars: A - 50µm; B - 50µm; C - 100µm; D - 100µm; E - 50µm; F - 100µm.

In subsequent studies, we focused on the prefrontal cortex to investigate whether disturbances in neuronal development and inflammatory changes after asphyxia resulted in lasting alterations at the synaptic level. To this end, we used STORM superresolution microscopy allowing the assessment of synaptic proteins at 20 nm lateral resolution *(Dudok et al., Nat Neurosci 2015)*.

In adult rats, the total number of synaptophysin-positive terminals was not altered, but asphyxia-induced increases in vesicular glutamate transporter 1 (VGlut-1) levels in synaptic terminals were found in the hippocampus and in the prefrontal cortex (most markedly in the prelimbic cortex) (**Fig.4B**). In line with this, vesicular GABA transporter (VGAT) levels decreased in the prefrontal cortex, with corresponding reduction in VGLUT-1/VGAT ratio (**Fig.4D**) along with decreased CB1 signal (not shown).
This suggested a long-lasting increase in excitatory/inhibitory balance as a result of perinatal asphyxia. In addition, disturbances in myelination were found as indicated by increased thickness of the myelin sheet around cortical axons (**Fig.4A**). This phenomenon could not be seen by conventional confocal imaging, indicating the advantages of high-resolution STORM imaging in identifying pathophysiological alterations at the molecular level **(Fig.4C)**. Collectively, we concluded that moderate neonatal **asphyxia evoked acute neuroinflammation in the absence of major neuronal injury and resulted in the dysfunction of myelination, glutamatergic terminals and alterations in inhibitory neuronal networks**.

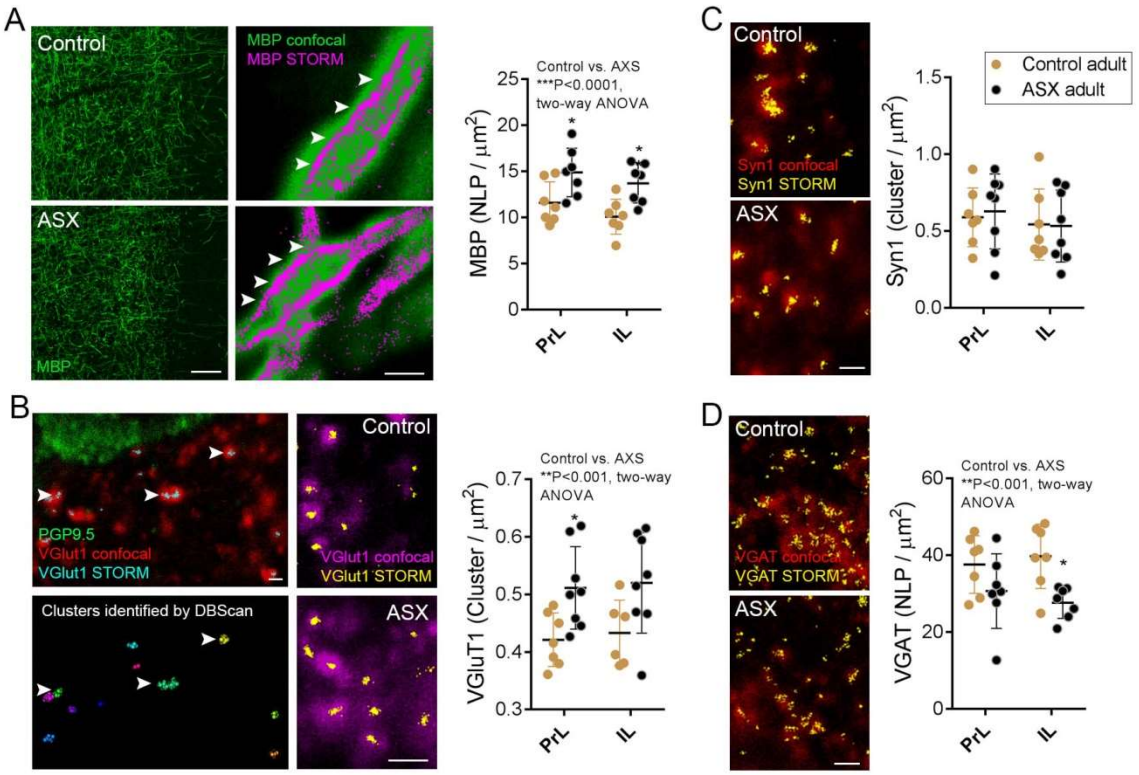


Figure 4. Superresolution microscopy revealed lasting asphyxia-induced changes at nanoscale level in the prefrontal cortex of adult rats. **A**. Myelin basic protein (MBP) confocal (left) and combined confocal / STORM images (right) in control and adult (5-month) rats after perinatal asphyxia. **B**. Combined confocal/STORM images of VGlut1-positive terminals. Neuronal cell bodies were visualized by PGP9.5 immunostaining. **C**. Combined confocal/STORM images of synaptophysin (Syn1)-positive terminals. **D**. Combined confocal/STORM images of vesicular GABA transporter (VGAT) immunofluorescence. Number of Localization Points (NLP) or clusters identified by DBScan are shown / µm^2. *P<0.05; **P<0.001, ***P<0.001, two-way ANOVA followed by Sidak's post hoc comparison Scale bars: A – 100 µm (left panels) and 1 µm (rigth STORM panels); STORM images on B-D – 1 µm.

4. Perinatal asphyxia results in long-term inflammatory changes and dysregulation of neuronal networks in the prefrontal cortex

To further investigate changes caused by perinatal asphyxia, we performed full transcriptome sequencing (Illumina) on prefrontal cortex samples 24h and 5 months after asphyxia. We found that while acute inflammatory changes were apparent at 24h, increased mRNA espressions mostly returned to the level of controls in adult rats. However, levels of the proinflammatory cytokine interleukin-1β (IL-1β) were still higher in adult rats after asphyxia compared to controls (data not shown). In line with this, changes in myelination, interneuron markers and glutamatergic transmission were observed in adult rats, indicating that **perinatal asphyxia results in lasting inflammatory changes in the prefrontal cortex, which parallels dysregulation of excitatory and inhibitory neuronal networks.**

5. Perinatal asphyxia does not have acute or long-term functional neurological or behavioural consequences

To investigate functional consequences of the asphyxic insult, rats were subjected to comprehensive behavioural and neurological tests from 24h after insult into adulthood. Perinatal asphyxia did not cause neuromotor deficits either acutely (24h after insult), or later (in juvenile and adult rats) as measured by testing neonatal reflexes, general locomotory pattern in juvenile and adult animals **(Fig 5A)**, as well as motor coordination of adult rats in the accelerating rotarod **(Fig 5B)**. **The absence severe neurological consequences confirmed that our model is highly suitable for the study of perinatal asphyxia-related behavioural alterations, as motor deficits would potentially mask fine-tuned emotional and cognitive disturbances**.

6. Perinatal asphyxia leads to emotional deficit and enhanced impulsivity in adulthood

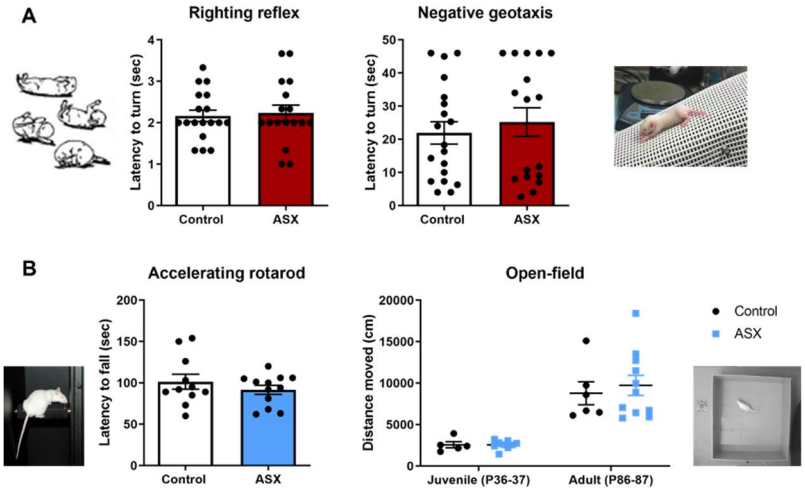


Figure 5. Perinatal asphyxia did not induce acute (A) or lasting (B) changes in neuromotor function. A. Neonatal reflexes 24hrs after asphyxic insult: righting reflex (left) and negative geotaxis (right). **B.** No changes in motor coordination in adults (left) or locomotor activity in juveniles or adults (right).

Subsequently, we tested cohorts of juvenile and adult rats for behavioural alterations in emotional, social and cognitive domains. We found that perinatal asphyxia resulted in elevated anxiety levels as revealed by reduced time spent in the open arms of the elevated plus-maze (**Fig. 6A)** and significant alterations in marble burying behaviour (data not shown). Moreover, adult post-asphyxia rats showed increased number of inadequate responses in the delay-discounting paradigm, indicating enhanced motor impulsivity **(Fig. 6B).** Thus, **perinatal asphyxia resulted in emotional deficits and enhanced impulsivity**, a prefrontal cortex-dependent externalizing symptom that is characteristic of several psychiatric disorders, including attention deficit hyperactivity disorder (ADHD).

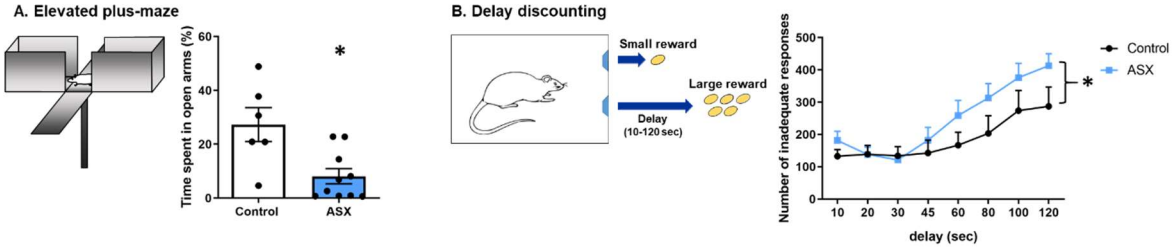


Figure 6. Enhanced anxiety (A) and impulsivity (B) after perinatal asphyxia. A. Time spent in the open arms of the elevated plus-maze. *P<0.05; Mann-Whitney U test. **B.** Rats were trained to make nose pokes for small or large food reward in operant chambers. ASX rats did not differ from controls in operant learning during training (data not shown), however, by increasing the delay of receiving the large reward during delayed discounting phase, they showed more inadequate responses. Data are shown as means+SEMs. Repeated measures ANOVA P(delay)<0.001; P(treatment)=0.2; P(interaction)=0.08. *P(slope)=0.05; unpaired t-test

Social function of asphyxiated animals seemed to remain intact as no changes were observed in the sociability, social avoidance, social interaction and resident-intruder tests that measure various aspects of social behaviour (data not shown).

7. Perinatal asphyxia influences cognitive function in later life

Remarkably, perinatal asphyxia resulted in lasting and specific changes in cognitive function. Although we found no changes in working memory in the Y-maze and operant learning (data not shown), perinatal asphyxia resulted in hippocampus-dependent spatial learning and memory deficits: juvenile rats showed impairments in the modified hole-board test **(Fig. 7A)**, while adults had difficulties in navigating in the Morris-water maze as revealed by longer latencies required to find the hidden platform over training days **(Fig. 7B).**

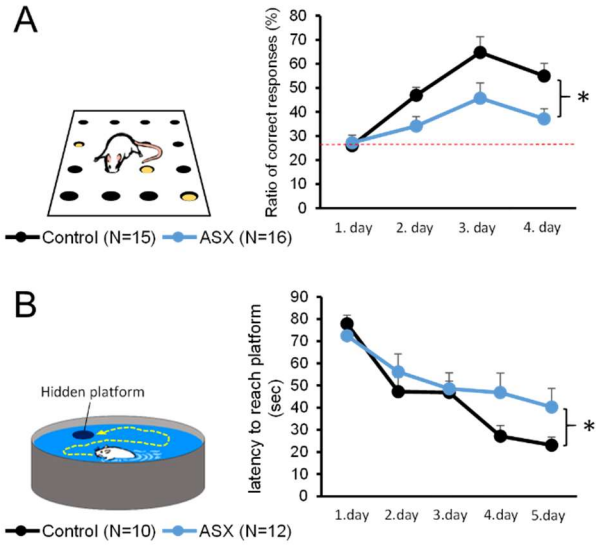


Figure 7. Impairments in spatial learning and memory after perinatal asphyxia. A. Impaired juvenile spatial learning and memory. Juvenile (P31-34) ASX rats made less visits to baited holes compared to empty ones. Dashed line illustrates the ratio of random choice. Repeated measures ANOVA P(days)<0.001; *P(treatment)<0.01; P(interaction)=0.06. **B.** Impaired adult learning and memory. ASX rats found the hidden platform significantly slower over training days. Repeated measures ANOVA P(days)<0.001; P(treatment)=0.2; P(interaction)=0.02; unpaired t-test *P(slope)=0.01. Data are shown as means+SEMs.

Moreover, perinatal asphyxia resulted in marked attention deficits in the 5-choice serial reaction time task **(Fig 8A)**: asphyxiated rats were slower in acquiring the task and were not able to proceed to more difficult stages that would have required better attention performance indicating severe prefrontal cortical dysfunction. As attention deficits together with enhanced impulsivity reveal core symptoms of ADHD, we tested adult ASX rats in the rodent version of the Go/no-Go task, in which ADHD patients often show poor performance. We found that perinatal asphyxia resulted in diminished prefrontal cortex-dependent inhibitory function in the Go/no-Go task **(Fig 8B).**

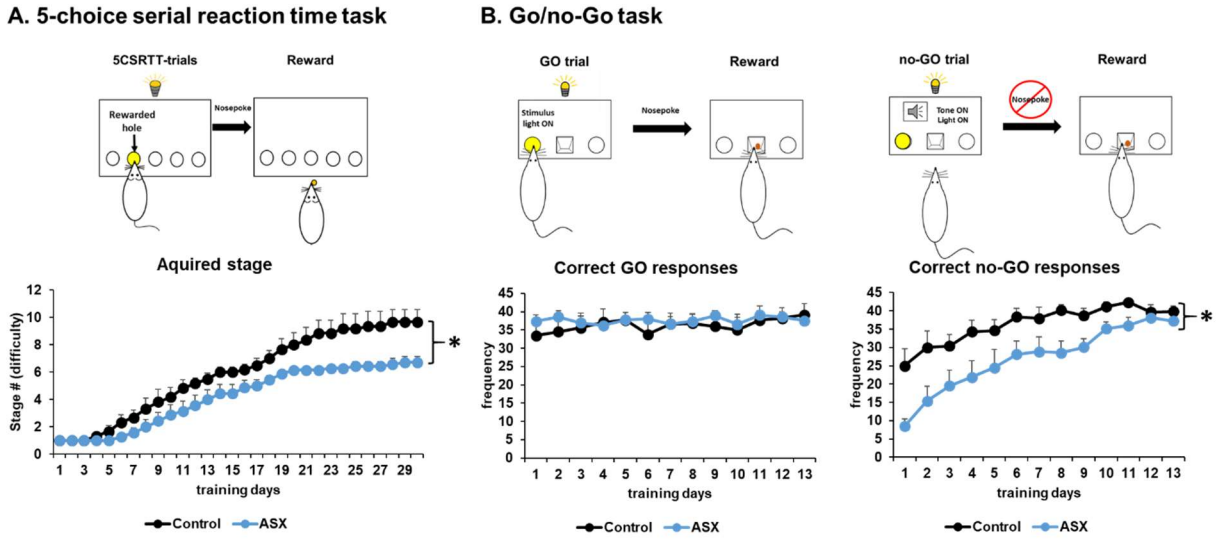


Figure 8. Perinatal asphyxia results in sustained attention deficits (A) and diminished inhibitory function. A. Attention deficits after perinatal asphyxia in the 5-choice serial reaction time task. ASX rats proceeded slower into consecutive difficulty stages (requiring better attention performance) of the test. Repeated measures ANOVA P(days)<0.001; *P(treatment)<0.01; P(interaction)=0.03. Data are shown as means+SEMs. **B.** Diminished prefrontal inhibitory function in the Go/no-Go test. ASX rats made significantly less correct 'no-Go' responses that requires the inhibition of a previously learned motor response (nose poke) upon presentation of a novel stimuli (tone). Repeated measures ANOVA P(days)<0.01; *P(treatment)<0.01; P(interaction)<0.001. Data are shown as means+SEMs.

8. Interventions to minimize asphyxia related long-term functional, neurological or behavioural consequences

A. Graded restoration of normocapnia

Compromised or ceased gas exchange during asphyxic insult results in progressive hypoxemia and hypercapnia, leading to profound metabolic acidosis, a major diagnostic criterion of birth asphyxia. Metabolic acidosis evokes hyperventillation, which in turn, together with therapeutic hypothermia-evoked drop in metabolic rate, induces hypocapnia. Hypocapnia has been shown to be associated with adverse short- and long-term outcome of asphyxiated neonates *(Robertson et al, Annals Neurol, 2012)*.

In joint studies with our collaborator Prof. Kai Kaila, a leading expert of neonatal asphyxia, we investigated the effects of graded restoration of normocapnia in experiments in which P7 asphyxiated rat pups inhaled hypercapnic gas mixtures during the recovery phase immediately after insult. Gas mixtures consisted of either 5% CO_2, 21%O_2 and 74% N_2 or 10% CO_2, 21% O_2 and 69% N_2; pups inhaled either 5% CO_2 for 60min, or 10% CO_2 for 30min, followed by another 30min exposure to 5% CO_2.

We found no acute effects of hypercapnic gas mixtures on neurology scores or behaviour of rat pups, however, both treatments were able to counteract asphyxia-induced long-term attention deficits (data not shown). These results suggest that **graded restoration of normocapnia may prevent hypocapnia-induced detrimental effects on long-term cognitive outcomes**.

B. Interleukin 1-alpha antagonist therapy

Collectively, we have shown that perinatal asphyxia results in lasting inflammatory changes in the hippocampus and prefrontal cortex, paralleled by dysregulation of excitatory/inhibitory balance and marked hippocampus- and prefrontal cortex-dependent emotional and cognitive deficits. In order to investigate the causative relationships between acute neuroinflammation and subsequent behavioural deficits, interleukin-1 receptor antagonist (IL-1RA; Kineret, 100 mg/bwkg, *s.c.*) was administered acutely after asphyxic insult in a therapeutically relevant time window, and attention was measured in adult animals in the 5CSRTT test. We found that IL-1RA administered after the insult significantly ameliorated attention deficit in adult ASX rats **(Fig 10),** suggesting that **IL-1RA might provide an effective treatment opportunity for perinatal asphyxia-induced cognitive deficits**.

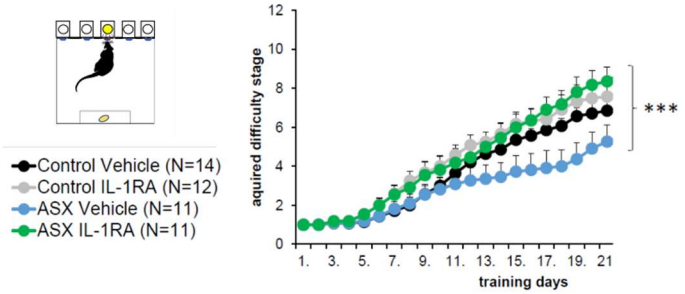


Figure 9. Long-term cognitive deficits can be prevented by acute administration of interleukin-1 receptor antagonist. Interleukin-1 receptor antagonist was administered 1 and 20h after perinatal asphyxic insult and attention was measured in adult animals in the 5CSRTT test. IL-1RA treatment significantly ameliorated attention deficit in adult ASX rats. Repeated measures ANOVA P(days)<0.001; *P(treatment)<0.01; P(interaction)<0.01. Data are shown as means+SEMs.

9. Multiorgan damage following asphyxia

Perinatal asphyxia is an extensive insult, resulting in multi-organ damage to various degrees. Conversely, previous preclinical models focused almost exclusively on brain injury, ignoring most of the systemic and peripheral effects. Our novel translational model allowed us to study the effect of various therapeutic interventions on asphyxia induced multi-organ damage.

A. Asphyxia results in subclinical elevation of classic parameters of renal and hepatic injury

Acute kidney injury (AKI) occurs in at least 50% of asphyxiated newborns, however the prevalence might be even higher, thus AKI often remains undiagnosed due to unsensitive and unspecific biomarkers. Mid - to long-term outcomes were also investigated, of which little is known to-date.

	Control P7 4h	ASX P7 4h	Control P8 24h	ASX P824h	Control Juvenile	ASX Juvenile	Control Adult	ASX Adult
BUN	7.29 ± 1.17	9.48 ± 2.27	10.73 ± 3.12	9.09 ± 2.65	6.25 ± 0.83	6.66 ± 0.79	6.62 ± 0.43	9.38 ± 2.97*
GOT	173.4 ± 15.71	157.4 ± 15.37	175.5 ± 23.48	165.9 ± 23.63	202.1 ± 47.52	222.6 ± 56.02	116.3 ± 14.75	192.7 ± 129.5
GPT	33.57 ± 3.53	28.98 ± 4.84§§	43.95 ± 15.99	26.06 ± 6.07+++	78.54 ± 10.33	81.14 ± 12.12	62.56 ± 15.49	69.23 ± 34.65

Table 1. **Laboratory parameters of newborn, juvenile and adult rats**. Repeated measures ANOVA §§p<0.01 *vs.* Control 4h; *p=0.07 *vs.* Control Adult; BUN-blood urea nitrogen, N=11/group in newborns, 6/group in juvenile and adults Data are shown as means+SEMs.

A transient, moderate elevation of BUN and isolated increase in GPT (indicating liver dysfunction) were detected in newborn rats after asphyxia **(Table 1)** and mild alterations in renal function disappeared in juveniles. Interestingly enough renal impairment was implied by increased BUN levels in adult rats. This is in line with clinical experience illustrating that neonatal AKI may have long-lasting renal damage leading to chronic kidney disease in adult life *(Carmody et al, Pediatrics, 2013)*.

Structural lesions of hypoxia-induced acute tubular necrosis was not detectable in any of the kidneys **(Figure 10A),** while liver sections of asphyxic animals at 24h after the insult showed more prominent cytoplasmic feathery granulated degeneration than their control counterparts **(Figure 10B).**

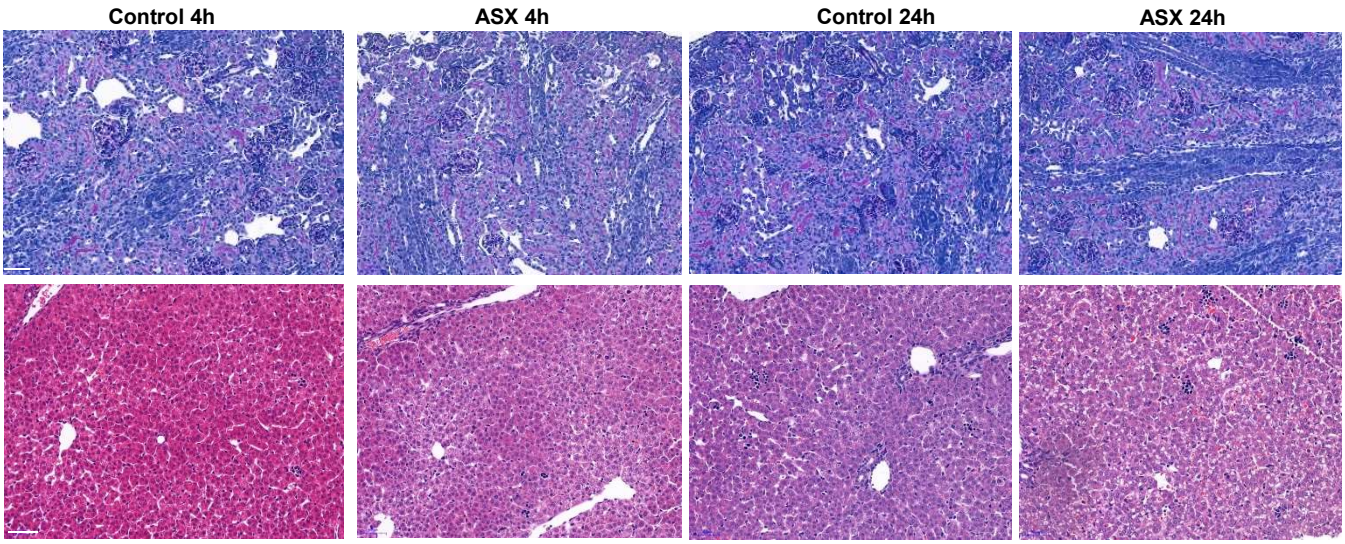


Figure 10. Acute histopathological changes in kidney and liver after asphyxic insult. A: Upper panel: representative images of structural damage after asphyxia on PAS–stained kidney sections of control 4h, ASX 4h, or control 24h, and ASX 24h rats. scale bar, ×100. B: Lower panel: Representative images of structural damage after asphyxia on hematoxylin eosin–stained liver sections of (A) control-4h, (B) ASX 4h, or (C) control-24h, (D) ASX 24h rats. scale bar, ×50.

Various literary data as well as clinical experience suggest that significant liver or kidney dysfunction can occur in the setting of normal transaminase levels *(Karlson et al, Neonatology, 2009)* or renal retention parameters *(Askenazi DJ, Pediatr Nephrol, 2009)*. Therefore we measured additional parameters such as Kidney Injury Molecule-1 (KIM-1) and Neutrophil gelatinase–associated lipocalin (NGAL) as more sensitive, specific markers of organ injury. We also performed a complex analysis of various asphyxia-induced molecular pathways to assess organ-specific changes following the asphyxic insult.

B. KIDNEY

mRNA expression of proximal tubular injury marker *Kim1* increased as early as 4 hours after the asphyxia insult, suggesting massive proximal tubular damage **(Figure 11)**. NGAL and MCP-1 are also specific indicators of kidney injury that correlate with the severity of renal impairment. A robust elevation in *Ngal* and *Mcp1* mRNA expressions was seen after 24 hours. Hypoxia–inducible factor–*α* (HIF-1*α*) mediates cell protection and epithelium recovery after renal hypoxic injury; its mRNA expression began to increase at 4h, and was even higher at 24h.

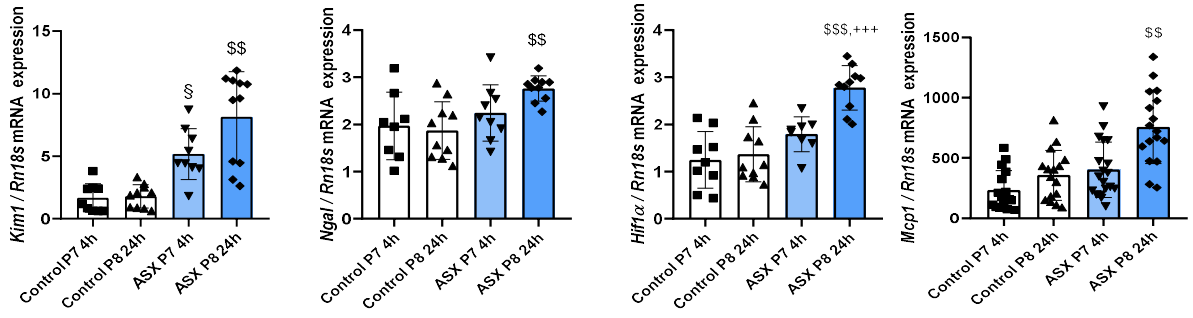


Figure 11. **Asphyxia leads to acute tubular injury in the kidney**
(N=8-11/group) Data are shown as mean+SEM. Repeated measures ANOVA followed by Bonferroni post hoc test; §p<0.05 vs. Control P7 4h; $^{\$\$}$p<0.01 *vs.* Control P8 24h; $^{\$\$\$}$p<0.001 *vs.* Control P8 24h; $^{+++}$p<0.001 *vs.* ASX P7 4h.

Cytokine profile of the kidney verified an increased inflammatory response. In the brain samples we confirmed IL-1*α* as an early proinflammatory mediator of cerebral asphyxia and a key driver of cerebrovascular inflammation; however, its role in renal hypoxia has not been investigated yet.
Here we showed that mRNA expression of IL-1*α* was massively elevated in the kidneys of asphyxic rats. We also detected a robust increase in other proinflammatory cytokines (IL-1*ß*, IL-6 and TNF-*α*,) with less regulation associated with worse outcomes. In parallel, the serum level of IL-10 - the powerful regulatory cytokine, that can block proinflammatory processes and inhibit leukocyte activation - was also decreased in ASX 24h vs. controls measured by cytometric-bead array. There was no difference between the groups in the serum levels of other cytokines (data not shown).

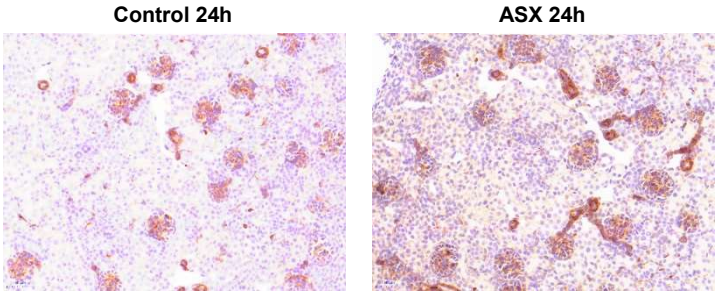
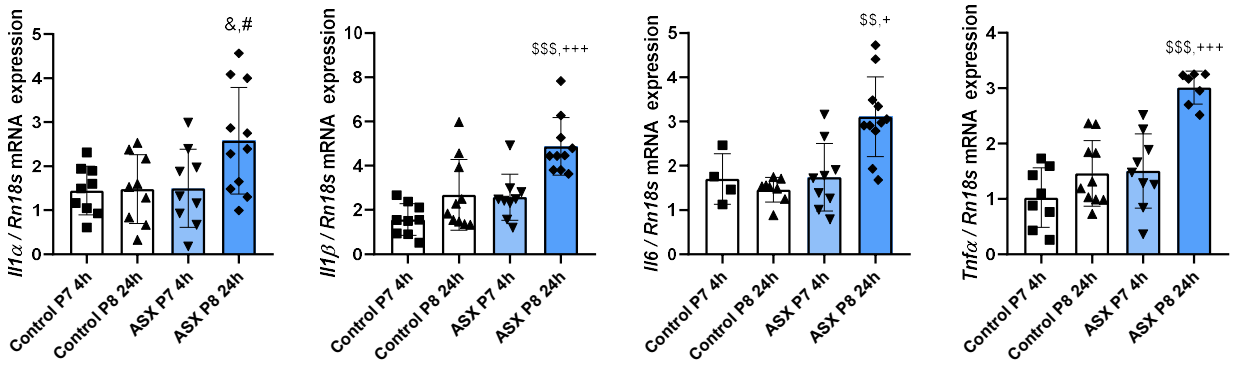


Figure 12. **Asphyxia results in increased inflammatory response in the kidney. Representative pictures of CD45 immunohistochemisry** scale bar x 20
(N=8-11/group) Data are shown as means+SEMs. Repeated measures ANOVA followed by Bonferroni post hoc test; §p<0.05 vs. Control P7 4h; $^{\$\$}$p<0.01 *vs.* Control P8 24h; $^{\$\$\$}$p<0.001 *vs.* Control P8 24h; $^{+}$p<0.05 *vs.* ASX P7 4h; $^{+++}$p<0.001 *vs.* ASX P7 4h; $^{\&}$p=0.06 *vs.* Control P8 24h; $^{\#}$p=0.05 *vs.* ASX P7 4h



Several other factors (e.g.TLR receptors) and pathways have been evaluated such as angiogenetic (HIF2, HIF3 VEGF, EPO, GATA) proapoptotic (Bax, Bcl-2 etc.) and chaperone proteins (Sigma-1 receptor, HSPs); data are not shown due to lack of space.

C. HEART AND MYOCARDIAL DAMAGE

Cardiac troponins released from injured cardiomyocytes are high sensitivity markers of myocardial cell injury in neonates. In animal studies it has been reported that the level of Troponin I starts to rise 1h after cardiac injury with a peak value at 3h, which is normalized within 48h after the insult *(Liu et al, Pediatr Res, 2008)*. Recently proBNP has also been shown to be a stronger predictor of ischemic heart injury than cardiac troponin T (cTnT), or creatine kinase MB, but there is no data about its value in asphyxia *(Cao et al, Int J Mol Sci, 2019)*.
Here we showed that both increased in response to myocardial injury associated with asphyxic insult at 4h and returned to normal levels after 24h **(Figure 13)**.

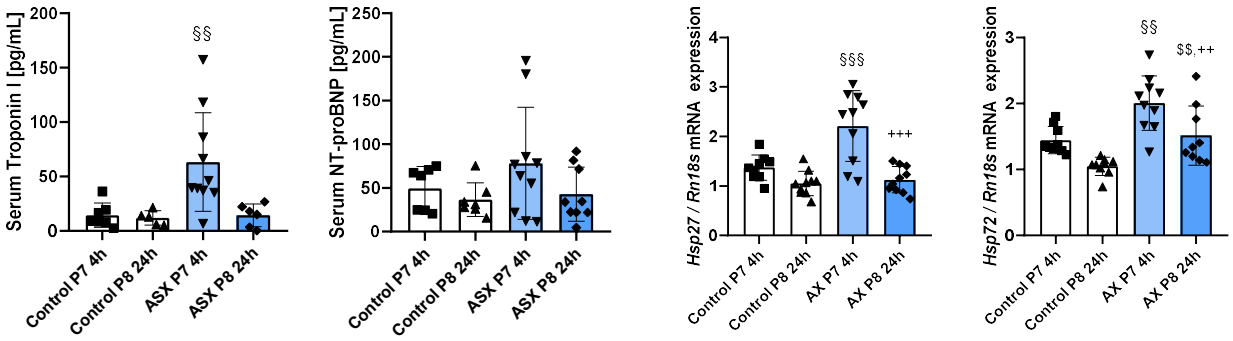


Figure 13. **Asphyxia results in increased level of cardiac injury markers and activated heat shock response** $^{\$\$}$p<0.01 vs. Control P7 4h; $^{\$\$\$}$p<0.01 vs. Control P7 4h; $^{\$\$\$}$p<0.001 vs. Control P7 4h; $^{\$\$}$p<0.01 *vs.* Control P8 24h; $^{+++}$p<0.001 *vs.* ASX P7 4h

In line with measurements in adult rats with early cardiac failures *(Li et al. PLOS One, 2013)*, we proved that cardiac HSP72 and HSP27 level increase shortly after birth asphyxia that can be associated with cardiomycyte hypertrophy. (data not shown).

D. LIVER

Liver injury is likely due to hypoperfusion rather than hypoxia. Inflammatory cytokines, HSPs, as well as the HIF-1α pathway were induced rather due to separation from the dam (C 4h vs. C 24h; ASX 4h vs. ASX 24h), while there was no difference between control and asphyxic animals (data not shown).

10. Perinatal asphyxia results in increased ischemic vulnerability of the kidney in adulthood

Over the past few years, a few data from experimental animals as well as human studies showed that neonatal AKI is not a limited insult and may result in permanent kidney damage *(Nada et al. Semin Fetal Neonatal Med, 2017.)*. However studies to explore the long-term impact of neonatal AKI are lacking. Therefore, first we assessed renal function in control and ASX adult rats, then we performed an additional insult of mild bilateral ischemia to evaluate the ischemic vulnerability of the kidney. We found that neither conventional retention parameters, nor specific tubular injury markers differ between control and postasphyxic rats without a second insult. However, if additional renal injury is present – modeling clinical situations such as supraaortic surgery, severe hypovalemia, burns etc. - all markers are increased in ASX animals, confirming increased tubular hypoxic susceptibility **(Figure 14)**. This is the first experimental evidence for the long-term consequences of subclinical renal injury in asphyxic newborns. More studies are needed to explore the long-term sequela of AKI and to inform appropriate follow-up guidelines to promote the early detection and management of CKD.

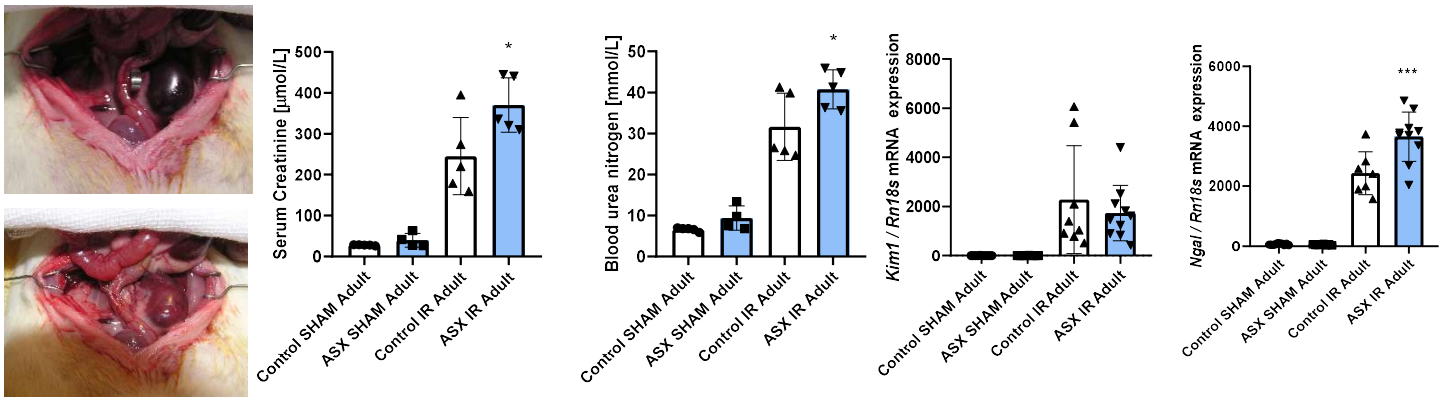


Figure 4. **Perinatal asphyxia increases renal susceptibility to ischemia/reperfusion injury in adulthood** (***p<0.001 vs. Control IR Adult (N=5-8/group) Data are shown as means+SEMs. Repeated measures ANOVA followed by Bonferroni post hoc test,

SUMMARY

We succesfully completed the planned studies. In collaboration with Prof. Kaila's group we established a novel model of mild perinatal asphyxia in rodents. Thus, this experimental setup is suitable for investigating long-term neuronal, behavioural and multi-organ consequences and we sucessfully excluded the limitations of previous studies. We concluded that even mild perinatal aspyhxia leads to impaired cerebral vasoregulation, neuroinflammation resulting in dysfunction of myelination, and alterations in inhibitory neuronal networks. All these factors lead to cognitive dysfunction on the long-term. We also showed that perinatal asphyxia evokes acute hypoxic injury in other organs, such as the kidney and heart in the absence of clinical symptoms. However, even these subclinical injuries increase ischemic susceptibility in adulthood.

We presented our results at several international and domestic congresses and published parts of the results in a peer-reviewed nephrology journal. Two additional publications are right before submission. Some of the data have been included in a PhD thesis successfully defended this year, moreover two PhD works are still ongoing. With the help of this grant we established a truly translational preclinical model of birth asphyxia that could help to test effective therapeutic approaches towards clinical testing not only in the brain, but also in other organs. Our results with indicate that IL-1R antagonist could serve as a new target for future drug development.