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Interaction of Hydrogen Sulfide and the Oxytocin System in Trauma and Shock

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List of Abbreviations

3MST	3-mercaptopyruvate-sulfurtransferase
ACTH	adrenocorticotropic hormone
Akt	protein kinase B serine/threonine-specific protein kinase
AMPK	adenosinmonophosphat-activated protein kinase
AQP4	aquaporin 4
ASDH	acute subdural hematoma
AVP	arginine vasopressin
AVPR	arginine vasopressin receptor
BBB	blood brain barrier
BMW	Bretoncelles-Meishan-Willebrand
C1QBP	complement component 1 Q subcomponent-binding protein
C1q	complement component 1q
iC3	induced complement component 3
C5aR	complement component 5a receptor
CBS	cystathionine-β-synthase
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CPP	cerebral perfusion pressure
CSE	cystathionine-γ-lyase
DMN	dorsal motor nucleus
ELS	early life stress
ERK1/2	extracellular regulated kinase 1/2
FBM	Familial Hypercholesterolemia-Bretoncelles-Meishan
GC	glucocorticoid
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
GYY	GYY4137
H_2S	hydrogen sulfide
HE	hematoxylin and eosin
HPA	hypothalamic pituitary adrenal
HS	hemorrhagic shock
I/R	ischemia-reperfusion
ICP	intra-cranial pressure
ICU	intensive care unit

IHC	immunohistochemistry
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IP	immunoprecipitation
mRNA	messenger ribonucleic acid
NeuN	neuronal nuclei
NO	nitric oxide
NOS	nitric oxide synthase
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OT	oxytocin
OTR	oxytocin receptor
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinases
PICU	porcine intensive care unit
PMN	polymorphonuclear neutrophil
PTSD	post traumatic stress disorder
PVN	paraventricular nuclei
RISK	reperfusion injury salvage kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMCs	smooth muscle cells
SON	supraoptic nuclei
$Na_2S_2O_3$	sodium thiosulfate
TBI	traumatic brain injury
TBS	tris buffered saline
TNFα	tumor necrosis factor-α
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2

List of Symbols

min	minute
h	hour
l	liter
ml	milliliter
μl	microliter
kg	kilogram
g	gram
mg	milligram
μg	microgram
M	molar
mM	millimolar
μM	micromolar
nM	nanomolar
$\%$	percent
°C	degree celsius
$mmHg$	millimeter of mercury

1 Introduction

This thesis describes the interaction of the hydrogen sulfide (H_2S) and oxytocin (OT)/oxytocin receptor (OTR) systems, in the heart and the brain in response to trauma, in clinically relevant, resuscitated, long-term, porcine models. H_2S is a freely diffusible gas and is endogenously produced by three different enzymes [244, 270], while OT is a peptide hormone that acts *via* the OTR, a membrane-bound receptor [8, 237]. The two systems play an important role in the heart [95, 123, 268] and the brain [131, 226], by regulating heart rate [108, 179], blood pressure [22, 103, 285] and blood volume [50, 226]. Physical trauma can arise from a severe injury to the body that may comprise life-threatening organ or circulatory failure [60], pathophysiological events that often lead to circulatory failure are barrier dysfunction, inflammation, and oxidative stress [2, 273]. In case of a polytrauma, hemorrhage and traumatic brain injury (TBI) are the main determiners of morbidity and mortality [180]. In contrast to physical trauma, psychological trauma can be the consequence of a strong emotional assault, perceived as life-threatening [60]. Recent reports emphasize that physical and psychological trauma share somatic effects [14, 54, 231]. Psychological trauma in childhood is associated in adulthood with cardiovascular diseases (91.7%) and hypertension (61.5%) [18, 167]. H_2S and OT assume importance in both physical and psychological trauma, inasmuch by mediating organ protection by antioxidant, anti-inflammatory, neuro- and vasculo-protective properties [8, 93, 119, 145, 161, 173, 181, 203, 240, 270, 293]. Chronic pre-existing diseases, raise mortality in critically ill patients between 24-60 years, by a factor of four [12, 288]. The most prevalent co-morbidities affect the cardiovascular system [42, 58]. 73% of trauma patients above 45 years and up to 90% of patients above 85 years present with underlying atherosclerosis [58, 73]. In fact, co-morbid trauma patients more often require intensive care unit (ICU) admission and

stay significantly longer in the ICU [266]. One pitfall of the many unsuccessful pre-clinical intensive care studies, is that they fail to mimic the pathophysiology of the relevant patient populations [132, 264]. Adult pigs with pre-existing atherosclerosis, are clinically relevant large-animal models that have similar pathophysiological responses to trauma compared to humans [55, 56, 100, 132, 264].
Hence, the aim of this thesis was to characterize the injury and the interaction of the H_2S and the OT systems in the heart and the brain in three different resuscitated, long-term, adult pig models of physical trauma, two of which had pre-existing atherosclerosis. These insights into the interaction of the endogenous H_2S producing enzymes and the OT system in the heart and the brain may improve the understanding of parallel molecular mechanisms of physical and psychological trauma and their interdependencies.

1.1 Physical Trauma

Post-traumatic multi-organ failure is decisively determined by hemorrhage, which causes 30-40% of the mortality after polytrauma [180]. hemorrhagic shock (HS) represents the most acute blood volume change possible, and is due to massive blood loss [43, 92, 263]; sepsis is caused by blood poisoning by infection and leads to barrier dysfunction with subsequent fluid shifts [15, 77, 235]. Both can ultimately lead to circulatory shock, which affects approximately one third of the patients who need ICU treatment [263]. According to Vincent *et al.*, "circulatory shock represents an imbalance between oxygen supply and oxygen requirements", which can lead to organ injury [263]. Key features of circulatory shock are systemic arterial hypotension, tissue hypo-perfusion and hyper-lactatemia [43, 263]. There are four different types of circulatory shock, depending on the underlying cause of hypotension [263]. HS belongs to hypovolemic shock, and septic shock belongs to distributive shock [263]. In addition, 58% of deaths after polytrauma are due to TBI [62, 180]. A TBI, can induce an acute subdural hematoma (ASDH) and can be, but is not necessarily associated with circulatory shock [39, 52, 105, 107, 132, 175, 264]. A look at the

literature on the role of the H_2S and OT systems with regard to trauma, reveals that there are limited reports regarding OT in physical trauma, whereas reports on the roles of H_2S in physical trauma are abundant. Their respective roles are further introduced in section 1.6. The three porcine models that constitute this thesis comprised: HS, septic shock and ASDH.

Hemorrhagic Shock

HS is caused by profound blood loss [43]. A loss of more than 30% of total blood volume leads to hypotension and subsequent hypo-perfusion of vital tissue and organs (ischemia), which impairs oxygen supply [43, 92, 116, 120, 197, 247, 263]. The limited oxygen supply is associated with tissue hypoxia that leads to inflammation and oxidative stress and ultimately to tissue damage in the peripheral organs and the brain [43, 68, 263].

Septic Shock

Septic shock falls under the category of distributive shock, which is the most prevalent type of circulatory shock in the clinic and is characterized by profound cellular, metabolic and circulatory abnormalities [235, 263]. Septic shock entails a higher risk of mortality compared to patients with sepsis, which is defined by Singer *et al.* as "life-threatening organ dysfunction caused by a dysregulated host response to infection" [235]. Systemic infection leads to inflammation, a host defense, in order to protect tissue and organs from damage upon pathogen-induced injury. Inflammation causes vasodilation, leading to a profound drop of blood pressure (hypotension), which provokes a cardiac response in an effort to maintain blood pressure. However, if the heart starts failing to compensate for the severe vasodilation due to a dysregulated inflammation in sepsis, myocardial dysfunction can occur, which in turn, is associated with an increase of mortality up to 50 % [15, 77, 97]. Myocardial dysfunction occurs in up to 70% of septic patients and can be significantly aggravated by underlying atherosclerosis, thereby

worsening the patient outcome [12, 20, 77, 221]. In fact, 44% of the patients with severe sepsis and septic shock present with atherosclerotic vascular disease [206]. Thus, atherosclerosis is an important co-morbidity, which needs to be taken into account in clinically relevant pre-clinical experiments of septic shock. It is not clear up to date what precise pathophysiological mechanisms elicit myocardial dysfunction in septic shock [113].

Acute Subdural Hematoma

Trauma-induced acute subdural bleeding originates from brain contusion, tearing of the bridging veins, or less frequently from arterial bleeding [39, 175, 198]. After TBI, up to 45% of the patients develop an ASDH [39, 107] with concomitant mortality up to 60% [142]. Patients under 70 years more often experience severe TBI compared to mild TBI, while elderly patients (above 70 years) in general show a higher incidence of TBI and mostly experience mild TBI [52, 139]. The elderly population has a higher incidence of developing an ASDH, related to fall-induced head injury [69, 139]. Key pathophysiological events during ASDH are barrier dysfunction [224], inflammation and oxidative stress [68], which ultimately lead to edema formation and swelling. Edema formation can be defined as pathological increase of water content in the interstitial compartment [241]. As a consequence of edema, tissue swelling can take place, which is defined as intracellular fluid accumulation and entails volume expansion [241]. The space-occupying volume expansion of the accumulated blood in the subdural space elicits an increase in intra-cranial pressure (ICP) [241]. At a certain cut off point, when the ICP is higher than the cerebral perfusion pressure (CPP), vessels can collapse, leading to a lack of local tissue oxygen supply (hypoxia) [68, 175, 241] and vascular leakage in brain tissue [175, 207, 241, 261].

1.2 Psychological Trauma

Psychological trauma is defined as a mental injury or insult to the mind of a person, caused by an extremely distressing event, which is above the threshold of a person's capability to cope with the overwhelming negative emotions. The perception of life-threatening psychological trauma can lead to negative long-term consequences on the somatic as well as psychological level. Whether a situation is recognized and interpreted as life-threatening is decided in our brain, especially in the prefrontal cortex [31]. So called 'adverse childhood experiences' and early life stress (ELS), which are caused by neglect, abuse, or other distressing events in childhood or adolescence can lead to long lasting physical effects as cardiovascular diseases as reviewed recently by McCook *et al.* (see figure 1) [45, 167, 259]. Additionally, it is reported that ELS increases the predisposition to develop post traumatic stress disorder (PTSD) [45], in which patients suffer from intrusive recollections of distressing traumata, months to years after the traumatic event had occurred [37, 223]. Classical causes of PTSD are e.g. experiencing natural catastrophes, warfare or traffic accidents. Recently, medical conditions such as intensive care admission, which can be life-threatening for critically ill patients, are also acknowledged as causes of PTSD [223]. Righy *et al.* showed that the ICU admission could elicit PTSD symptoms in one out of five adult cases [40, 223]. Auxéméry *et al.* report that a physical injury, which occurs at the same time with a traumatic psychological exposure, leads to a higher risk for developing PTSD [14]. Furthermore, there is a biological link between physical and psychological trauma, although the causal relationship has not been fully elucidated yet [223], they share common pathophysiological mechanisms such as oxidative stress and inflammation as reviewed by Prasad *et al.* [219].

TBI has been recognized as diagnostic risk factor for developing PTSD (with a prevalence up to 27% [166]) and long-term cognitive deficits in addition to physical disabilities after TBI [11, 30, 37, 139, 166, 217, 219]. Interestingly, TBI and PTSD share both: injury or dysfunction of the prefrontal areas of the brain [31, 78, 83, 166], and common pathological conditions such as oxidative stress and inflammation [219]. It is, however, poorly understood how physical trauma and in particular brain injury lead to psychological long-term consequences

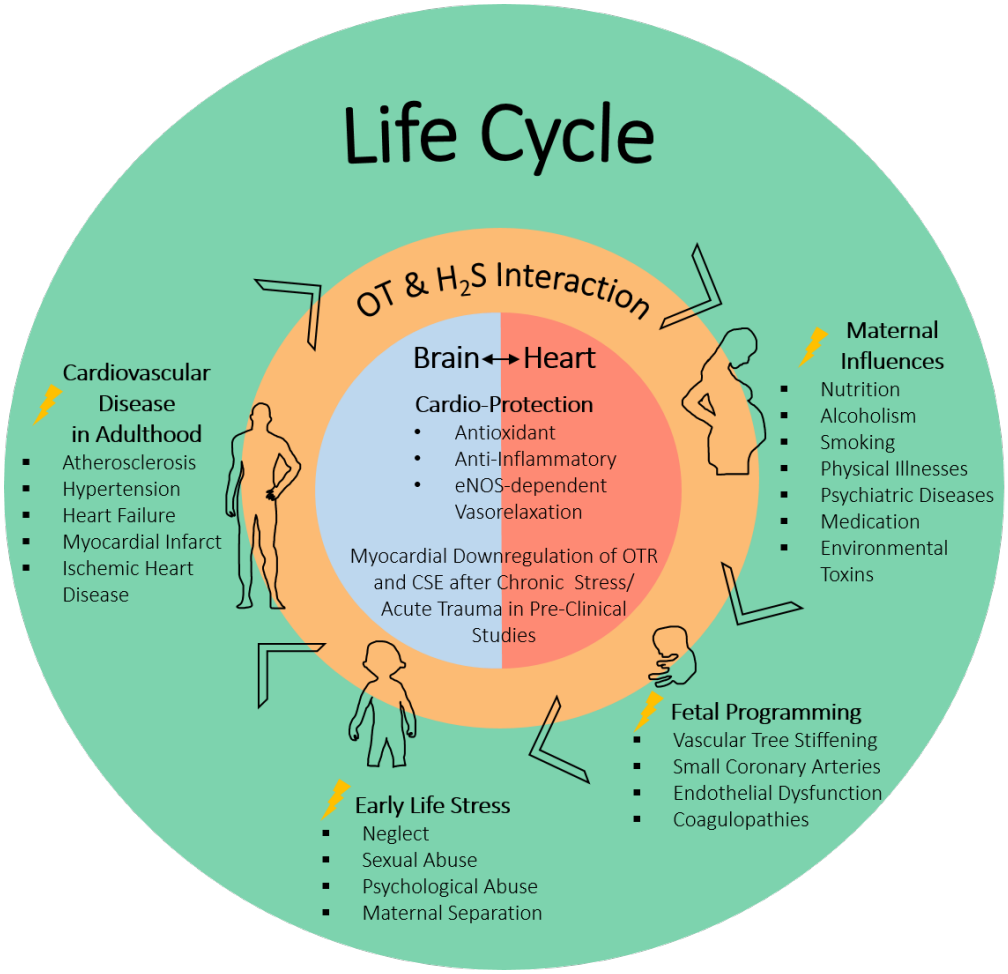

Figure 1: Interaction of OT and H_2S in the brain and the heart: contribution to physical manifestation/protection of ELS-induced cardiovascular disease in adulthood. ELS affects cardiovascular programming and the development of cardiovascular disease in adulthood. Pregnant women who experienced ELS may pass on ELS-triggered behavior/physical conditions: nutrition, smoking, alcoholism, medication/drugs, and illness. Long-term effects on the cardiovascular system of the fetus can be morphological and functional adaptations, stiffening of the vascular tree, small coronary arteries, endothelial dysfunction, reduced number of cardiomyocytes, atherogenic blood lipid profiles, and coagulopathies. Bringing together literature reports on OT and H_2S suggests that both are significant common mediators of cardio-protective effects during development as well as in adulthood through their antioxidant and anti-inflammatory effects and endothelial nitric oxide synthase (eNOS)-dependent vasorelaxation. Pre-clinical studies suggest that chronic stress and/or acute trauma leads to a downregulation of the OTR and cystathionine-γ-lyase (CSE) (the main endogenous H2 S-producing enzyme in the vasculature). The figure and the figure legend are taken from McCook *et al.* [167] under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license (https:// creativecommons.org/licenses/by/ 4.0/).

[37]. There is recent evidence that the H_2S and the OT systems are involved in psychological trauma: in a maternal separation model (a rodent model of human ELS), our work group showed that long-term ELS in mice led to downregulated protein expressions of both systems in the heart [281]. The putative role of the H_2S and OT systems with regards to ELS and the predisposition to cardiovascular disease has been recently reviewed (see figure 1) [167].

Research publications on OT in psychological trauma are ample, whereas literature reports regarding H_2S in psychological trauma are scarce [60, 145, 167, 203, 281]. Since the protein expression changes of both systems in trauma are of particular interest for this thesis, their respective roles are further introduced in section 1.6. Thus, this thesis explores the role of the H_2S and OT systems in the context of brain and heart connection in trauma.

1.3 Co-morbidity

Co-morbidity is defined as one or multiple chronic pre-existing medical conditions (physical or psychological) in addition to an acute primary injury or disease. The presence of co-morbidities has a decisive influence on mortality and morbidity after physical trauma [12, 42, 58, 73, 155, 168, 266]. Regarding physical co-morbidities, atherosclerosis is one of the most commonly occurring co-morbidities in critical care patients, its prevalence increases with age, and it is characterized by cardiovascular disorders and increased mortality (see figure 1) [73, 96, 168, 180]. Atherosclerosis is defined as chronic regional inflammation of the coronary arteries, carotids and peripheral vessels, accompanied by decreased high density lipoproteins, increased low density lipoproteins, oxidative stress and endothelial dysfunction [168, 181]. Lipid-laden macrophages infiltrate into the vascular wall and take up low density lipoproteins and turn into so called foam cells [5, 101]. Foam cell accumulation and subsequent plaque formation contributes to the stiffening of the vessels and decreases blood flow [168, 181]. Blood vessels that are affected by atherosclerosis are prone to develop vasospasm, meaning that the arteries are susceptible to vasoconstriction, since endothelium-

dependent relaxation is progressively lost [165]. Inappropriate vasoconstriction can lead to ischemic events, which can result in dysfunction of tissue and organs [165]. Nitric oxide (NO) is a gas, which is recognized as an endothelium derived relaxing factor and is produced within the vasculature by three different nitric oxide synthases (NOSs) and most prevalently by constitutive eNOS [181, 287]. Under normal conditions, NO elicits its vasodilatory effects *via* the cyclic guanosine monophosphate (cGMP) pathway, thereby protecting against antioxidant injury, adhesion of platelets and immune cells to the endothelial wall [113]. Atherosclerosis is associated with a reduction of endogenous NO availability and increased superoxide production due to dysfunctional eNOS, thereby contributing to endothelial dysfunction [130, 168, 279]. Interestingly, downregulation of the H_2S or OT systems, respectively, leads to higher heart rate and hypertension (see more details in section 1.6) [35, 60, 103, 179, 285], and atherosclerosis is associated with downregulated levels of cystathionine-γ-lyase (CSE) (an endogenous H_2S-producing enzyme, see figure 1) [158, 270, 285].

There is growing awareness that ELS and psychological diseases in adulthood are risk factors that can lead to the development of chronic cardiovascular co-morbidities such as atherosclerosis (see figure 1)[9, 17, 54, 71, 73, 110, 152, 171, 176, 257, 267]. According to a current meta-analysis from Basu *et al.*, child maltreatment was significantly associated with the risk for developing cardiovascular diseases in adulthood in twenty-two out of twenty-four studies examined [18]. Furthermore, psychological trauma (in childhood and in adulthood) contribute equally to an increased risk of adverse complications after trauma as somatic pre-existing co-morbidities [180]. In particular ELS elevates the likelihood to develop PTSD, and trauma patients with TBI significantly more often develop PTSD compared to trauma patients without TBI [180]. Patients with a history of psychological trauma and/or chronic cardiovascular co-morbidities, who are admitted to the ICU due to physical injury, tend to stay longer in the ICU and have a higher rate of morbidity and mortality [3, 42, 73, 155, 258, 266]. That is one of the reasons why in this thesis, animal models with co-morbidity have been included to closely mimic the pathophysiology of the relevant patient population and clinical scenario.

1.4 Hydrogen Sulfide

H_2S is a gas, which was recognized as a neurotransmitter or neuromodulator by Abe and Kimura in 1996 [1], and, hence, has been entitled a gasotransmitter since 2002 by Wang *et al.* [268]. Gasotransmitters are endogenous signaling molecules that pass through cellular membranes by diffusion, independently of a receptor ligand system [226, 244, 268, 270]. Endogenous H_2S production is strictly regulated by enzymatic and non-enzymatic pathways, although the enzymatic H_2S production is predominant (see figure 2) [180, 270]. In mammalian cells, H_2S is produced enzymatically by CSE, cystathionine-β-synthase (CBS) or 3-mercaptopyruvate-sulfurtransferase (3MST) [270]. The clearance of H_2S mostly happens in the mitochondria where the stepwise enzymatic sulfide oxidation pathway takes place (see figure 2) [60, 109, 162, 180, 205]. H_2S can be oxidized non-enzymatically, to polysulfide (at high oxygen concentrations) or to thiosulfate (at low oxygen concentrations) [161]. Hypoxia changes the mitochondrial matrix towards a reduced membrane potential, which stimulates non-enzymatic H_2S production from thiosulfate [161, 205].

H_2S exerts vasodilatory, antioxidant, oxygen sensing, cyto-protective, anti- and pro-inflammatory actions [74, 114, 161, 205, 270, 292]. The anti- or pro-inflammatory role of H_2S is complex and depends on whether it is endogenously produced or exogenously administered, whether local or systemic infection takes place, and may vary depending on species. In septic shock for example, patients tend to have elevated endogenous plasma H_2S levels. In HS, endogenously released H_2S is associated with anti-inflammatory effects [151]. Moreover, endogenous H_2S can mediate the activation of angiogenesis [256, 270] and acts as a basal vasorelaxant [57, 150]. H_2S-induced vasoactive effects are mediated by downstream signaling cascades that activate eNOS *via* the reperfusion injury salvage kinase (RISK) pathway to produce NO [48, 147, 178, 216, 269, 270, 285]. More detailed information on the RISK pathway is given in section 4.2 and figure 19 [60].

In the HS and the ASDH parts of this thesis project, the focus will be on the brain, thus the roles of H_2S in the central nervous system (CNS) are briefly introduced

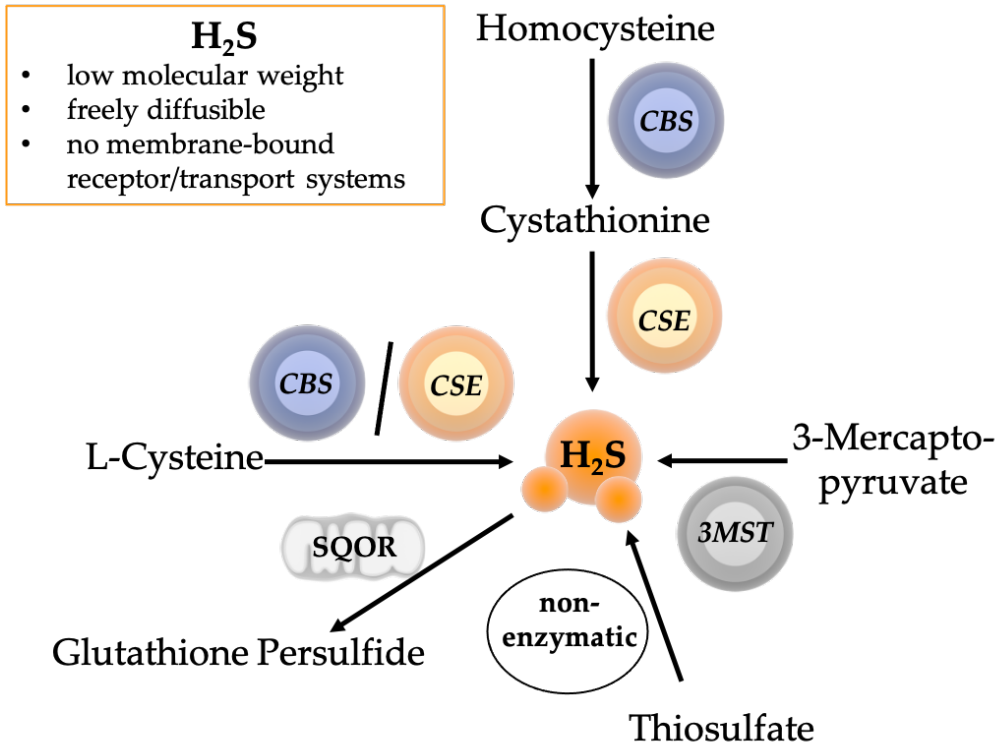

Figure 2: H_2S Metabolism. H_2S is produced by CSE, CBS or 3MST. L-cysteine, homocysteine and 3-mercaptopyruvate are the essential substrates for enzymatic H_2S production. Non-enzymatic H_2S generation *via* the utilization of thiosulfate is possible during hypoxic events. The enzymatic sulfide oxidation pathway takes place in the mitochondria. H_2S is enzymatically oxidized *via* the sulfide:quinone oxidoreductase (SQOR) to glutathione-persulfide. The figure is taken from Merz *et al.* [180] under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license (https:// creativecommons.org/licenses/by/ 4.0/).

here. In the CNS, H_2S plays an anti-inflammatory role, in particular through reducing pro-inflammatory (tumor necrosis factor-α (TNFα), interleukin-1β) and promoting anti-inflammatory mediators (interleukin-4, interleukin-10) [292]. The brain was the first organ where endogenous sulfide levels were detected (in rats and humans) by Goodwin *et al.* in 1989 [86]. H_2S is considered as a neuromodulator, neurotransmitter and neuro-protective agent [1, 161, 162, 270, 293] and is directly involved in the regulation of homeostatic processes in the hypothalamus including fluid shifts, water and electrolyte balance, blood pressure, blood volume, heart rate, respiration, temperature and pH control [161, 162, 226]. Furthermore, H_2S has been reported to reduce edema formation in response to brain injury [82, 126, 146, 292, 293] and improve blood brain barrier (BBB) integrity [82, 126, 146, 272].

As mentioned above in section 1.3, downregulation of CSE is associated with hypertension and atherosclerosis and, thus, potentially lowered vascular H_2S availability (see figure 4) [158, 180, 270, 285]. In fact, Familial Hypercholesterolemia-Bretoncelles-Meishan (FBM) swine with pre-existing atherosclerosis (used in two parts of this thesis and introduced more in detail in section 2.2.1) show reduced CSE expression within the coronary arteries [181]. Therefore, administration of an exogenous H_2S donor could be beneficial after HS for patients suffering from atherosclerosis, considering its above-reported systemic and neuronal antioxidant, anti-inflammatory, cytoprotective and vasorelaxant effects [74, 161, 270, 292, 293].

Regarding the impact of H_2S administration in experimental HS, there are ambivalent reports; as much beneficial [46, 79, 80, 189, 228], harmful [186, 187, 277], or neutral effects were reported [65, 90]. There are different kinds of H_2S releasing donors. H_2S-releasing salts, e.g. NaSH (sodium hydrosulfide) and Na_2S (sodium sulfide), are known for adverse side effects due to potentially high H_2S peak concentrations. Slow-releasing H_2S donors can be disadvantageous under circulatory shock conditions, due to vasodilatory effects, which can aggravate the shock state [177, 180]. In contrast, sodium thiosulfate ($Na_2S_2O_3$) is devoid of of major adverse side effects, can liberate H_2S [236] and is a clinically approved drug for the treatment of calciphylaxis [227, 230], cisplatinum intoxication [33]

and cyanide poisoning [19]. It has antioxidant properties [236] and is even readily available [172, 227]. Marutani *et al.* proposes thiosulfate (see figure 2) as "circulating carrier molecule" which mediates the beneficial effects of H_2S [162]. Intraperitoneal administration of $Na_2S_2O_3$ in a murine model of cerebral ischemia-reperfusion (I/R) injury in the brain did not elevate H_2S itself but elevated levels of thiosulfate systemically and in the brain tissue, increasing survival and ameliorating neurological injury [161, 162]. In a rat model of fluid deprivation, intracerebroventricular injection of an H_2S donor directly stimulated hypothalamic OT release from magnocellular neurons [50]. Furthermore, exogenous H_2S administration can induce upregulation of the endogenous H_2S producing enzymes [38, 232] as an adaptive stress response [169].

Recently, our work group showed an organ-protective effect of $Na_2S_2O_3$ in the lungs of FBM pigs that underwent HS [55]. $Na_2S_2O_3$ attenuated the acute lung injury after HS, which was associated with reduced CSE and significantly higher glucocorticoid receptor (GR) expression in the lung tissue [55]. In line with these findings, our work group also showed the lung-protective properties of $Na_2S_2O_3$ in mice with a genetic deletion of CSE that underwent combined blunt chest trauma and HS. This positive effect was accompanied, by higher GR expression in lung tissue, similar to the pig model [89]. Moreover, $Na_2S_2O_3$ administration led to improved hemodynamic stability, reduced inflammation in the kidney, and attenuated liver cell damage [89]. However, so far it is unknown what effects systemic $Na_2S_2O_3$ administration elicits within the brain after HS [21, 161, 162, 194].

1.5 Oxytocin and its Receptor

OT is a peptide hormone and elicits its effects systemically and in the CNS through the G-protein-coupled OTR [8, 237]. OT is produced by peripheral organs as the heart [95, 123], but primarily in the brain by the magnocellular neurons of the paraventricular nuclei (PVN) and supraoptic nuclei (SON), which

are located in the hypothalamus. In the hypothalamus, OT is released *via* the posterior pituitary into the circulation [226], or through dendritic release, directly in the brain parenchyma and extracellular space (see figure 3) [23].

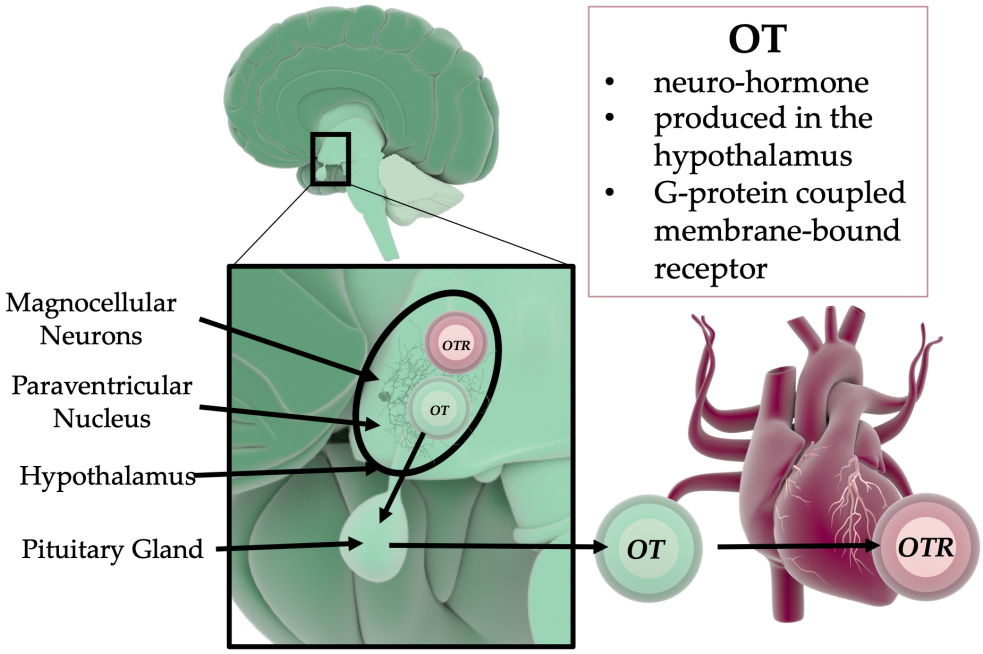

Figure 3: OT Release. The peptide hormone OT is produced in the brain by the magnocellular neurons of the hypothalamic PVN. OT is released *via* the posterior pituitary into the systemic circulation. OT elicits its effects *via* the G-protein-coupled OTR. The figure is modified from Merz *et al.* [180] under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license (https:// creativecommons.org/licenses/by/ 4.0/).

OT is mainly known for its role in interpersonal relationships, psychological trauma and PTSD [28, 203, 281], but it is also relevant in physical trauma [179] as further described in section 1.6. Besides the classically known actions of OT, e.g. stimulation of uterus contraction after labor [84] and protection from severe blood loss after delivery [88], OT has a wide range of other physiological functions: blood pressure lowering and vasodilatory capacities, negative cardiac inotropic and chronotropic, natriuretic, anti-inflammatory, antioxidant and endothelial cell growth effects [41, 93, 122, 188]. High doses of OT reduce heart rate in rats [108]. Furthermore, OT acts in concert with NO to regulate vasodilation *via* the RISK pathway, similar to H_2S as mentioned above (see section 1.4). More detailed information on the RISK pathway is given in section 4.2 and figure 19 [60]. OT has been suggested in 1991 by Loup *et al.* to have neuromodulator

and neurotransmitter function, since it has binding sites in autonomic and limbic areas of the human CNS [153]. OT has neuro-protective effects like repression of oxidative stress by upregulation of antioxidants [8] and attenuation of inflammation [121, 129, 291].

1.6 Interaction of Hydrogen Sulfide and Oxytocin

As briefly mentioned before, literature on H_2S in physical and OT in psychological roles are abundant, whereas information regarding H_2S in psychological and OT in physical trauma, respectively, is scarce [60]. There is a substantial dearth of information on the interaction of the H_2S and the OT systems, and the lack of research on that topic led to a review (Denoix *et al.*) of the current literature bringing together direct and indirect evidence for their interaction in the heart and the brain with respect to regulation of fluid balance, blood volume, blood pressure and heart rate [60]. Moreover, Wigger *et al.* reported that naive OTR knock out mice show reduced cardiac CSE expression [281]. In turn, results from Denoix *et al.* showed that naive CSE knock out mice lack OTR expression in the heart [60]. This crucial reciprocal loss of cardiac CSE/OTR expression in the respective knock out mice underscores the interdependency of the H_2S and OT systems in the cardiovascular system at a basal, uninjured state [60, 281]. Interestingly, naive mice with a genetic deletion of CSE, display significant higher blood pressure and heart rate compared to naive wild-type mice [103, 179, 285], suggesting that a lack of CSE may cause a predisposition to develop cardiovascular co-morbidities [60]. In addition, genetic mutation of 3MST causes hypertension and cardiac hypertrophy and downregulation of CSE expression in the heart of aged mice [211, 252]. Downregulation of the OT/OTR system is also associated with hypertension (see figure 4) [35]. Furthermore, pregnant CSE knock out mice develop symptoms of pre-eclampsia (a pregnancy complication characterized by hypertension), show impaired contraction of the mammary myoepithelial cells (the authors speculated that this was due to decreased expression of the OTR in the mammary glands), and have a lower frequency of uterine contraction upon stimulation with OT [7]. In the human myometrium, CSE and

OTR protein levels show a reciprocal correlation, at the time point immediately before and after the onset of labor [290]. Experiments on human myometrium biopsies revealed that both exogenous administered and endogenous produced H_2S could suppress OTR levels [290]. Finally, exogenous administration of an H_2S releasing salt (NaHS) has been reported to lower the rate of OT-stimulated myometrial contractions in isolated human myometrium strips [115].

Interaction of H_2S and OT in Trauma

Merz *et al.* demonstrated an interaction of the H_2S and the OT system in mice with cigarette smoke exposure-induced chronic lung disease that underwent acute blunt chest trauma [179]. This "acute-on-chronic disease" injury, led to reduced cardiac OTR expression, which was further enhanced in CSE-knock-out mice. The cardiac OTR expression was restored to wild-type levels by exogenous H_2S supplementation using GYY4137 (GYY), a slow H_2S-releasing compound [179]. As mentioned in section 1.2, Wigger *et al.* showed that after long-term ELS in mice, cardiac CSE and OTR were downregulated [281]. Furthermore, Li *et al.* report attenuated colitis by administration of H_2S in a rodent maternal separation model [145]. The similar loss of cardiac OTR in both psychological and physical trauma and the restoration of OTR expression in the heart after physical trauma upon GYY administration, suggests a mutual relationship of the H_2S and the OT systems (see figure 4) [60, 179, 180, 281].

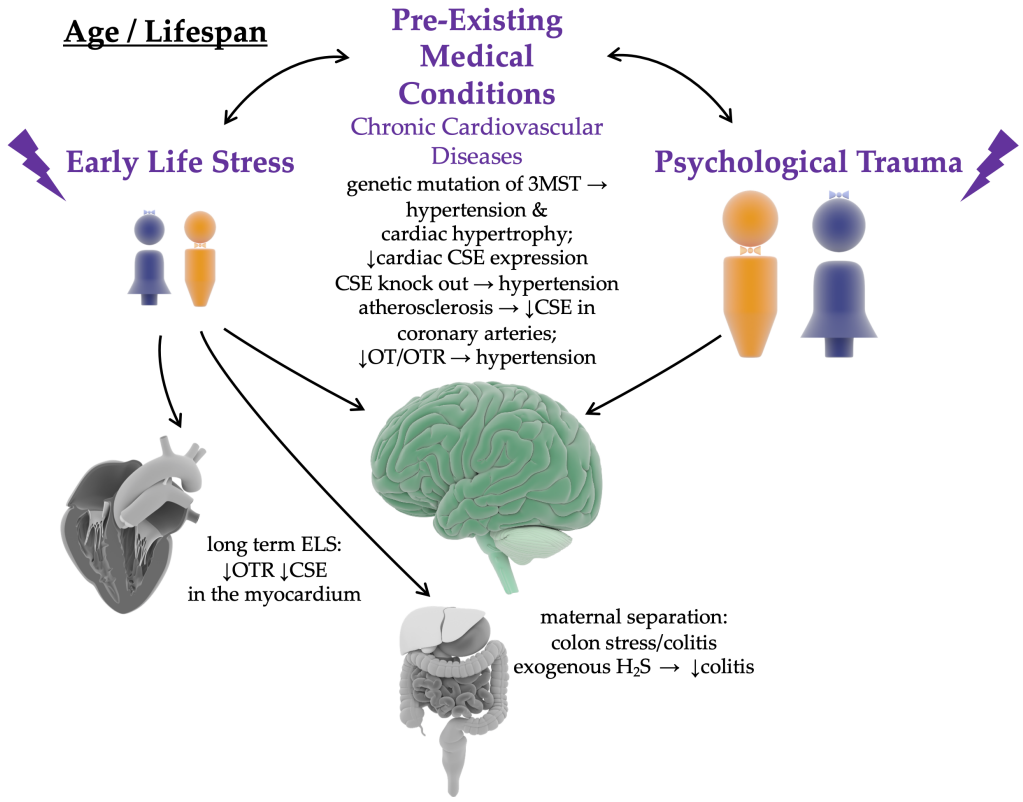

Figure 4: The Interaction of the H_2S and OT Systems in Trauma. ELS and psychological trauma in adulthood can cause the development of pre-existing medical conditions. Chronic cardiovascular co-morbidities are one of the most common pre-existing medical conditions. ELS and chronic cardiovascular co-morbidities are associated with a dysregulation of the H_2S and OT systems in the peripheral organs. Their interplay in other peripheral organs and the brain are unknown so far. (↓ = reduced). The figure is modified from Merz *et al.* [180] under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license (https:// creativecommons.org/licenses/by/ 4.0/).

Interaction of H_2S and OT in the Hypothalamus

A fundamental function of the body in response to injury is to maintain the volume and osmolarity of body fluids. The hypothalamus regulates the systemic fluid homeostasis and is part of the hypothalamic pituitary adrenal (HPA) axis, which regulates stress responses *via* the glucocorticoid (GC)/GR system [225]. Changes in plasma osmolarity and volume, e.g. due to HS, are a strong stimulus for the activation of the highly vascularized PVN of the hypothalamus (see figure 5) [60, 124, 220].

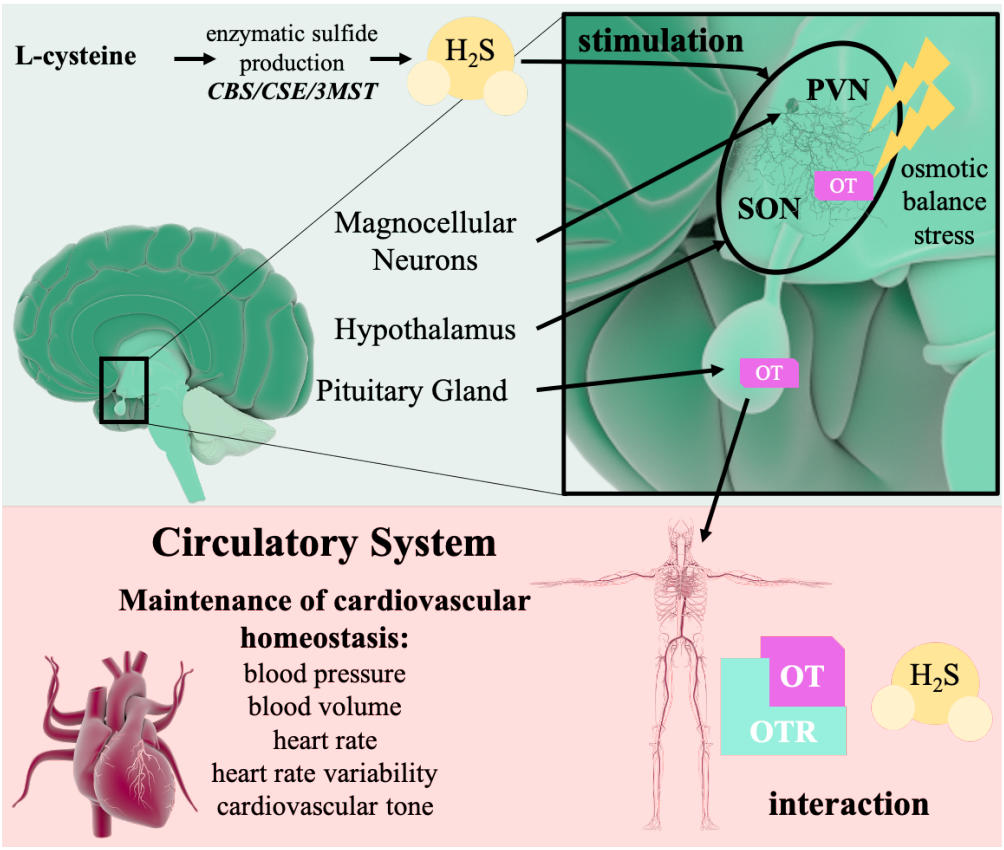

Figure 5: Interaction of H_2S and OT in Hypothalamic Regulation of Fluid Homeostasis. H_2S, endogenously produced by CSE, CBS and 3MST, can directly stimulate hypothalamic OT release from magnocellular neurons under osmotic balance stress. OT is released into the pituitary gland, from where it is liberated into the circulatory system. H_2S and OT can interact in the heart and contribute to preservation of cardiovascular homeostasis. The figure is modified from Denoix *et al.* [60] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

The activated PVN can release OT into the circulation [226, 238], where OT is involved in the elimination of sodium [124]. Sodium homeostasis affects

the osmotic pressure between compartments and therefore water and volume distribution [124, 226]. A very similar peptide hormone to OT, which only differs in two amino acids at position three and eight, is arginine vasopressin (AVP) and can be also released from the PVN [242, 297]. AVP secretion elicits antidiuretic effects and thus increases the blood volume [124, 225]. Moreover, AVP can bind the OTR, while *vice versa* OT can bind the arginine vasopressin receptor (AVPR). This cross-talk is possible due to the high sequence and structural homology of the both ligands and receptors [237, 297]. Due to this reciprocally binding, there have been many speculations about the respectively elicited effects, which might rather depend on receptor activation and localization than the ligand binding itself [93, 136, 212, 237]. This issue will not be further elucidated here, since this thesis focuses on the interaction of OT and H_2S based on the findings from Merz *et al.* [179]. These authors did not show any significant association of H_2S and the AVPR in physical trauma [179].

Osmotic stress (induced by intravenous/intraperitoneal injection of hypertonic saline, hyponatremic diet, or subcutaneous osmotic minipumps) can induce activation and continuously firing of oxytocinergic neurons in the PVN [143, 144, 214]. As reported by Ciosek *et al.*, hemorrhage leads to decreases of OT levels in the hypothalamus and in the posterior pituitary, associated with simultaneous increases in plasma OT [49]. Coletti *et al.* reported that H_2S can directly stimulate hypothalamic OT release from magnocellular neurons under osmotic balance stress in a rat model of fluid deprivation (see figure 5) [50]. Osmotic balance stress describes the dysbalance of fluid osmolarity between intracellular and extracellular compartment and is due to an altered osmotic pressure gradient [34]. The consequences of osmotic balance stress are changes in cell volume (e.g. due net water change) and cellular homeostasis [34]. Additionally, microinjection of an H_2S donor directly in the PVN elevates blood pressure and heart rate and is beneficial for the cardiac sympathetic afferent reflex. Inhibition of OT in the PVN impairs signaling of autonomic control in cardiovascular disease (see figure 6) [167].

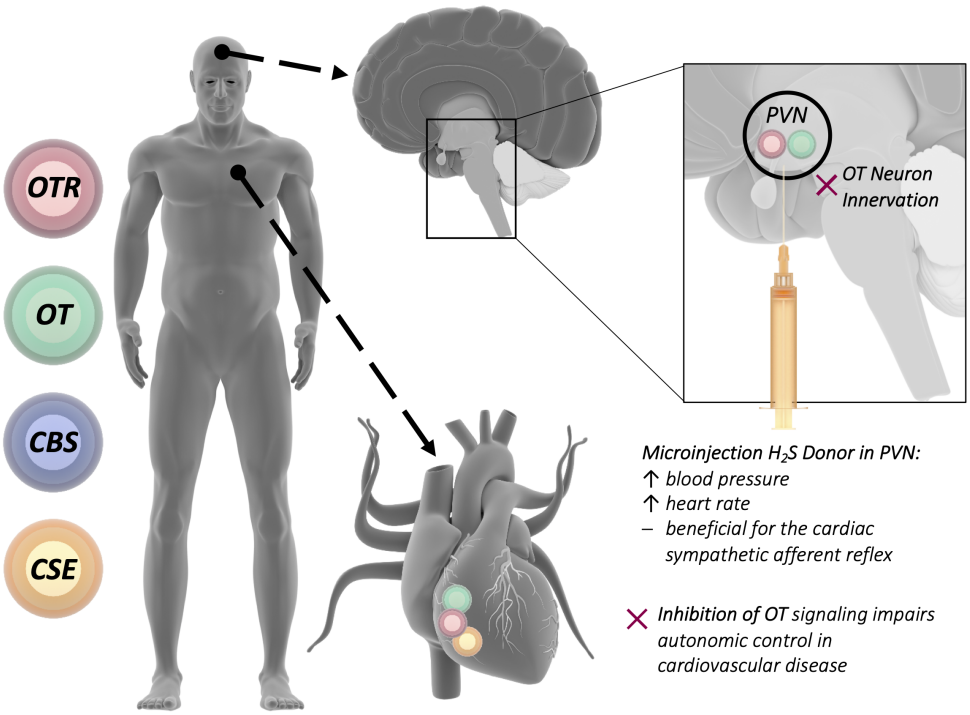

Figure 6: Interaction of H_2S and OT in Hypothalamic Regulation of Cardiovascular Homeostasis. OT and the OTR are expressed in the hypothalamic PVN. Microinjection of an H_2S donor elevates blood pressure and heart rate and is beneficial for the cardiac sympathetic afferent reflex. Inhibition of OT-innrevation impairs signaling of autonomic control in cardiovascular disease. The figure is modified from McCook *et al.* [167] under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license (https:// creativecommons.org/licenses/by/ 4.0/).

Interaction of H_2S and OT in the Vasculature

CSE is present in the vascular endothelium (shown both in rodent tissues as well as in human umbilical vein endothelial cells) and produces H_2S to elicit vasorelaxation in smooth muscle cells (SMCs), in particular in small-resistance arteries. In contrast, NO contributes to vasorelaxation primarily in large arteries [269, 270, 285]. Similar to H_2S, the OTR is found in the endothelium and SMCs of the vasculature and especially in small-resistance vessels, which contribute to the regulation of blood pressure [281]. Their mutual expression and functional similarity in the vasculature imply a crucial interplay in vascular function and blood pressure regulation. In the vasculature, OT has been associated with limiting the formation of atherosclerotic plaques, especially by eliciting anti-inflammatory effects and upregulation of the OTR [5, 245]. A limited bioavailability of H_2S and, in particular, a loss of CSE in the vasculature [29, 240] is linked to dysfunction of endothelial cells [10, 271], development of hypertension [285] and atherosclerosis [158].

1.7 Aims of this Thesis

The CSE/H_2S, OT/OTR and the GC/GR systems are all implicated in the regulation of local (in the heart) and central (in the brain) fluid homeostasis and vascular tone [51, 178, 225].

Since there are very few readily available antibodies for immunohistochemical detection of injury markers for the pig and in particularly for the porcine brain [264], the first aim (1) was to validate novel immunohistochemical markers of injury (with relevance to edema, inflammation and barrier dysfunction) and antibodies for the specific detection of the endogenous H_2S producing enzymes, OT, its receptor and the GR in the porcine heart and the brain.

The second aim (2) was to analyze in what pattern markers of injury and CSE, CBS, OT and the OTR are expressed in the porcine brain after ASDH without

systemic compromise, and if the CSE/H_2S and OT/OTR systems are associated with pathophysiological changes.

The third aim (3) was to analyze, if hypovolemic shock without acute brain injury, leads to hypoxic-events in the porcine brain on neuro-histopathological level. Since the hypothalamic regulation of blood volume and blood pressure is the linking mechanism of the interaction of CSE/H_2S, OT/OTR and the GC/GR systems, the aim (3A) was to analyze if resuscitated HS, as the most acute blood volume change possible, affects the expression patterns of the endogenous H_2S producing enzymes, OT, the OTR and the GR in the PVN, in pigs with underlying atherosclerosis.

Due to the fact that atherosclerotic FBM pigs have a vascular CSE-deficiency and that the H_2S donor $Na_2S_2O_3$ (H_2S-releasing sodium thiosulfate, see figure 2) previously showed organ-protection after resuscitated HS, it was concurrently analyzed (3B) whether systemic $Na_2S_2O_3$ administration has an effect on the neuro-histopathology and/or the protein expression levels of CSE, CBS, OT, OTR, GR, glial fibrillary acidic protein (GFAP) or nitrotyrosine in the PVN, in comparison to vehicle treated animals after resuscitated HS.

Since the $Na_2S_2O_3$-mediated organ-protection in the lung after resuscitated HS was associated with higher GR protein expression, the fourth aim (4) was to investigate what role the OTR and the GR play in the heart after resuscitated septic (distributive) shock in pigs with underlying atherosclerosis [182, 199, 276].

2 Materials and Methods

2.1 Materials

2.1.1 Instruments

Table 1: List of Instruments

Gewebeinfiltrationsvollautomat MTM I & II	SLEE medical GmbH, Mainz, Germany
Vorwärmeinheit MPS/W	SLEE medical GmbH, Mainz, Germany
Dispensiereinheit MPS/P	SLEE medical GmbH, Mainz, Germany
Cooling Plate MPS/C	SLEE medical GmbH, Mainz, Germany
Microtome RM2255	Leica Biosystems GmbH, Nussloch, Germany
Waterbath HI1220	Leica Biosystems GmbH, Nussloch, Germany
Microwave: Privileg 8521 (Typ MO-0927 BVG-SP)	Gustav Schickedanz KG, Fürth, Germany
Refrigerator/Freezer	Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany
Innova 4230 - Refrigerated incubator shaker	New Brunswick Scientific Co. Inc., Edison, USA
Zeiss Axio Imager A1 Microscope (2.5X, 10X, 20X, 40X objectives)	Carl Zeiss AG, Oberkochen, Germany
Eppendorf Pippettes Research® (0.5-10μl, 2-20μl, 50-200μl, 10-100μl, 100-1000μl)	Eppendorf AG, Hamburg, Germany
Ikamag® RCT (Magnetic Stirrer)	Janke & Kunkel GmbH & Co. KR, Staufen i. Br., Germany
Quintix® Analysewage	Satorius Lab Instruments GmbH & Co. KG, Göttingen, Germany

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Sprout® Mini Centrifuge	Heathrow Scientific, Illinois, USA
Vortex-Genie 2	Scientific Industries, Inc., New York, USA
Electrophorese	Mini-Protean Tetra cell, Bio-Rad Laboratories GmbH, Munic, Deutschland
Electrophorese Chamber	Sub-Cell GT Basic, Bio-Rad, Hercules, Kalifornien, USA
ChemiDoc MP imaging system	Bio-Rad Laboratories GmbH, Munic, Germany
Eppendorf® Centrifuge 5804R	Eppendorf AG, Hamburg, Germany

2.1.2 Consumables

Table 2: List of Consumables

Embedding Cassettes (plastic)	Kabe Labortechnik GmbH, Nümrecht-Elsenroth, Germany
Embedding Cassettes (metal)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Microtome Blade: Leica DB80LS	Leica Biosystems GmbH, Nussloch, Germany
Adhesion Slide, Superfrost® Plus	VWR International, Leuven, Belgium
General purpose Cover Glasses (24x60mm)	Thermo Fisher Scientific Inc., Waltham, USA
Cellstar® Tubes (15ml, 50ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Falcon® 10ml Polystyrere Centrifuge Tube	Corning Incorporated, Corning, USA
Duran® Measuring Cylinder	DWK Life Science GmbH, Wertheim, Germany
Eppendorf Tubes® with Snap Lid 5ml	Eppendorf AG, Hamburg, Germany
Reaction Tubes SafeSeal 1.5 ml, 2.0ml	Sarsted AG & Co. KG, Numbrecht, Germany
epT.I.P.S. LoRetention Reloads (0.1-10μ, 0.5-20μ, 2-200μ, 50-1000μ)	Eppendorf AG, Hamburg, Germany
Rotilabo® Dye Box	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Rotilabo® Slide Holder	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

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Rotilabo® Slide Box	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Rotilabo® Cotton Swab	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sekuroka® Waste Bags	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Super PAP Pen Liquid Blocker	Science Services GmbH, Munic, Germany
VWR® Slide Box	VWR International, Radnor, USA
pH-Indikatorstäbchen pH 0-14 Universalindikator	SupelcoSuperclo™ Analytical, Munic, Deutschland

2.1.3 Tissue Processing and Immunohistochemistry

Table 3: List of Materials for Tissue Processing and Immunohistochemistry

Formalin: Formaldehyde Solution 3.5-3.7% buffered	Otto Fischar GmbH & Co. KG, Saarbrücken, Germany
H_2O bidest.	Pharmacy of the University Medical Center Ulm, Germany
Ethanol 99.8%	Sigma-Aldrich, Steinheim, Germany
Xylene	Fisher Scientific GmbH, Schwerte, Germany
Citric Acid 99%	Sigma-Aldrich, Steinheim, Germany
Sodium Hydroxide Solution (Natronlauge, c(NaOH)=2mol/l (2N))	Superclo™ Analytical, Munic, Germany
Tris buffered saline (TBS) pH 8	Sigma-Aldrich Co., St. Louis, USA
Tween® 20 (Polysorbate)	Sigma-Aldrich, Steinheim, Germany
Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse	DAKO Deutschland GmbH, Hamburg, Deutschland
Paraffin: Paraplast Plus ®	Leica Biosystems GmbH, Nussloch, Germany
Normal Goat Serum (unconjugated)	Jackson ImmunoResearch Laboratories, Inc., West Grove, USA
CSE fusion protein: Ag2872	Proteintech, Manchester, United Kingdom
Mayer's Hematoxylin Solution	Sigma-Aldrich, Steinheim, Germany
Neo-Mount®	Sigma-Aldrich, Steinheim, Germany

2.1.4 Immunoprecipitation

Table 4: List of Materials for Immunoprecipitation

CaCo2 cells	Institute for Pathology, University Medical Center Ulm, Germany
Liquid Nitrogen	MTI Industriegase, Ulm, Germany
Phosphate buffered saline (PBS) (50mM Sodium Phosphate, pH 7.4, 200mM NaCl)	Sigma-Aldrich, St.Louis, USA
T25 Cell Culture Flask	Thermo Fisher Scientific Inc., Waltham, USA
RIPA lysis and extraction buffer, Pierce™	Thermo Fisher Scientific Inc., Waltham, USA
HALT™ Protease Inhibitor Cocktail	Thermo Fisher Scientific Inc., Waltham, USA
Pierce™ Protein-A/G-Agarose Kit	Thermo Fisher Scientific Inc., Waltham, USA
Amicon® Ultra 15ml Centrifugal Filters	Merck Millipore, Darmstadt, Germany
Oxytocin Receptor Antibody, rabbit polyclonal	#23045-1-AP, Proteintech, Manchester, United Kingdom
Oxytocin Receptor Antibody C-4, rabbit monoclonal	#sc-515809, Santa Cruz Biotechnology Inc., Heidelberg, Germany

2.1.5 SDS-Page and Western Blot

Table 5: List of Materials for SDS-Page and Western Blot

ReBlot Plus Mild Antibody Stripping Solution	Sigma-Aldrich, Deisenhofen, Germany
Precision Plus Protein™ Kaleidoscope™ standards	Bio-Rad Laboratories GmbH, Munic, Germany
Tris (Tris-(hydroxymethyl)-aminomethan 2M)	Sigma-Aldrich, Deisenhofen, Germany
Tris-HCl (Tris-(hydroxymethyl)-aminomethan Hydrochloride 1M)	Sigma-Aldrich, Deisenhofen, Germany
SDS (Sodiumdodecylsulfate)	Sigma-Aldrich, Deisenhofen, Germany

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Acrylamide (Bisacrylamid Solution 30%)	Sigma-Aldrich, Deisenhofen, Germany
Ammoniumpersulfat (10%)	Sigma-Aldrich, Deisenhofen, Germany
TEMED (Tetramethylethylendiamin)	Sigma-Aldrich, Deisenhofen, Germany
Glycin	Sigma-Aldrich, Deisenhofen, Germany
Methanol	Sigma-Aldrich, Deisenhofen, Germany
NaCl (Sodium Colride)	Sigma-Aldrich, Deisenhofen, Germany
4x Laemmli Sample Buffer	Bio-Rad Laboratories GmbH, Munic, Germany
β-mercaptoethanol	Sigma-Aldrich, Deisenhofen, Germany
Pure Nitrocellulose membrane (0.2 μ, 0.45 μ)	Sigma-Aldrich, Deisenhofen, Germany
Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad Laboratories GmbH, Munic, Germany
Whatman 3M filter sheets	GE Healthcare, Munic, Germany
Milk powder	Roth, Karlsruhe, Deutschland
Clarity™ Western ECL Substrate	Bio-Rad Laboratories GmbH, Munic, Germany
Secondary Antibody HRP (horseradish peroxidase) swine anti-rabbit	Dako, Agilent, Santa Clara, USA

2.1.6 Buffers

Table 6: List of Buffer Compositions

Buffer	Composition
Separation gel buffer (pH 8.8)	75ml Tris, 4ml 10% SDS, 21ml H_2O bidest
Collection gel buffer (pH 6.8)	50ml 1M Tris-Cl, 4ml 10% SDS, 46ml H_2O bidest
Separation gel (12.5%, pH 8.8)	5ml Acrylamide Solution, 3ml 4X Trenngelpuffer, 4ml H_2O bidest, 60μl 10% Ammoniumpersulfat, 6μl TEMED
Separation gel (10%, pH 8.8)	4ml Acrylamide Solution, 3ml 4X Trenngelpuffer, 5ml H_2O bidest, 60μl 10% Ammoniumpersulfat, 6μl TEMED

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Buffer	Composition
Collection gel (5%, pH6.8)	670μl Acrylamide Solution, 1ml 4X Sammelgelpuffer, 2.3ml H_2O bidest, 30μl 10% Ammoniumpersulfat, 5μl TEMED
Transfer buffer (pH8.3)	6.06g Tris (25mM), 28.8g Glycin (192mM), 400ml Methanol, 2l H_2O bidest
10X Elektrophoreses buffer	30g Tris, 144g Glycin, 10g SDS, 1l H_2O bidest
1X Elektrophoreses buffer	100ml 10X Elektrohoresepuffer, 1l H_2O bidest
10X TBS pH 7.4	61g Tris (500mM), NaCl (1.5M), use HCl to get pH8, 1l H_2O bidest
TBS pH 8	100ml 10X TBS, 1l H_2O bidest

2.1.7 Antibodies

Table 7: List of Antibodies. This table is modified from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

Primary Antibody (Source, Catalog Number, RRID)	Host Species	Query Cover(%)	Homology(%)	Immunogen Sequence	Concentration used for Immunohistochemistry
anti-CSE (Proteintech, 12217-1-AP, RRID:AB_2087497)	Rabbit Polyclonal	100	87.05	Gamma cystathionase fusion protein Ag2872	1:200
anti-CBS (Proteintech, 14787-1-AP, RRID:AB_2070970)	Rabbit Polyclonal	100	90.46	CBS fusion protein Ag6437	1:100
anti-OT (Millipore, AB911, RRID:AB_2157629)	Rabbit Polyclonal	100	100	CYIQNCPLG (Synthetic oxytocin (Sigma) conjugated to thyroglobulin)	1:500
anti-OTR (Proteintech, 23045-1-AP, RRID:AB_2827425)	Rabbit Polyclonal	92	83.05	Oxytocin Receptor fusion protein Ag19074	1:100
anti-AQP4 (Proteintech, 16473-1-AP, RRID:AB_2827426)	Rabbit Polyclonal	100	94.83	Aquaporin 4 fusion protein Ag9561	1:2000
anti-NeuN (Cell signaling, 24307, RRID:AB_2651140)	Rabbit Monoclonal	98	97.08	human full length protein: Swiss-Prot Acc.: A6NFN3	1:400
anti-C5aR (Proteintech, 21316-1-AP, RRID:AB_10733105)	Rabbit Polyclonal	100	65.00	C5aR fusion protein Ag15968	1:100
anti-iC3 (Hycult biotech, HM2168, RRID:AB_10130959)	Mouse Monoclonal	100	75.06	human full length protein: UniProtKB - P01024 (CO3_HUMAN)	1:50

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Primary Antibody (Source, Catalog Number, RRID)	Host Species	Query Cover(%)	Homology(%)	Immunogen Sequence	Concentration used for Immunohistochemistry
anti-C1QBPBP (Proteintech, 24474-1-AP, RRID:AB_2827427)	Rabbit Polyclonal	100	85.11	C1QBP fusion protein Ag19773	1:100
anti-Il-6R (Proteintech, 23457-1-AP, RRID:AB_2827428)	Rabbit Polyclonal	100	77.21	IL-6R fusion protein Ag18263	1:100
anti-TNFR1 (Proteintech, 21574-1-AP, RRID:AB_10734433)	Rabbit Polyclonal	100	69.30	TNFR1 fusion protein Ag16112	1:100
anti-TNFR2 (Proteintech, 19272-1-AP, RRID:AB_10640674)	Rabbit Polyclonal	100	67.73	TNFR2 fusion protein Ag5866	1:100
anti-GFAP (Abcam, ab7260, AB_305808)	Rabbit Polyclonal	100	92.13	full length GFAP sequence UniProt ID: 14136	1:2500
anti-GR(D8H2) (Cell Signaling Technology, #3660, AB_11179215)	Rabbit Monoclonal	100	89.40	Glucocorticoid receptor UniProt ID: P04150	1:100

2.1.8 Software

Table 8: List of Software

Axio Vision 4.8 Software	Carl Zeiss AG, Oberkochen, Germany
Zen Image Analysis Software	Carl Zeiss AG, Oberkochen, Germany
GraphPad Prism 4.00/8.00	GraphPad Software Inc., La Jolla, USA

2.2 Methods

2.2.1 The Pig as Animal Model

The pig has been proven to resemble human CSE and OTR expression patterns in peripheral organs of clinically relevant ICU models undergoing circulatory shock [169, 178, 199]. Moreover, the pig as an animal model in pre-clinical intensive care research brings along various advantageous features. First of all, the porcine inflammatory and redox profile is very close to humans [70, 234]. Fairbairn *et al.* reported that the immune system of the pig is closer to the human immune system than the murine immune system in 80% of the parameters examined [70]. In particular, porcine models show a similar response to sepsis as humans with regards to elevated pro-inflammatory cytokines as TNFα, interleukin-1β and interleukin-6 (IL-6) [70]. In addition, the anatomy of the organs is similar to humans, which allows for the same instrumentation and surgical procedures as applied in humans, when pigs with human-like size/body weight are used, obviating the need for special miniaturized instruments like for the mouse. These characteristics facilitate a human-like ICU treatment of the experimental pigs. For example, the porcine kidney resembles the multilobular and multipapillar kindey anatomy of humans, whereas the rodent kidney is unilobular and unipapillar [85]. With regards to the porcine brain, there are gross anatomical similarities to the human brain: size, skull and gyrencephalic structure (see figure 7 [170]) [111, 132, 264]. The gyrencephalic structure describes the characteristic gyri and sulci, which are similar in the porcine and human brain [111, 132, 264]. In contrast, the lissencephalic rodent brain lacks gyri and sulci (see figure 7 [170]) [111, 132, 264]. The size of the pig brain allows for intracranial instrumentation and monitoring as applied in human neurosurgical intensive care [56, 264]. The ratio of grey to white matter is very close to humans, since the adult human and porcine white matter makes 60% of brain matter compared to approximately 12% in adult rats (see figure 7 [170]) [132]. Moreover, pigs and humans share similarities in the cerebral vascularization [229], cerebral autoregulation of blood pressure [27, 210], neurophysiological processes and brain development [111].

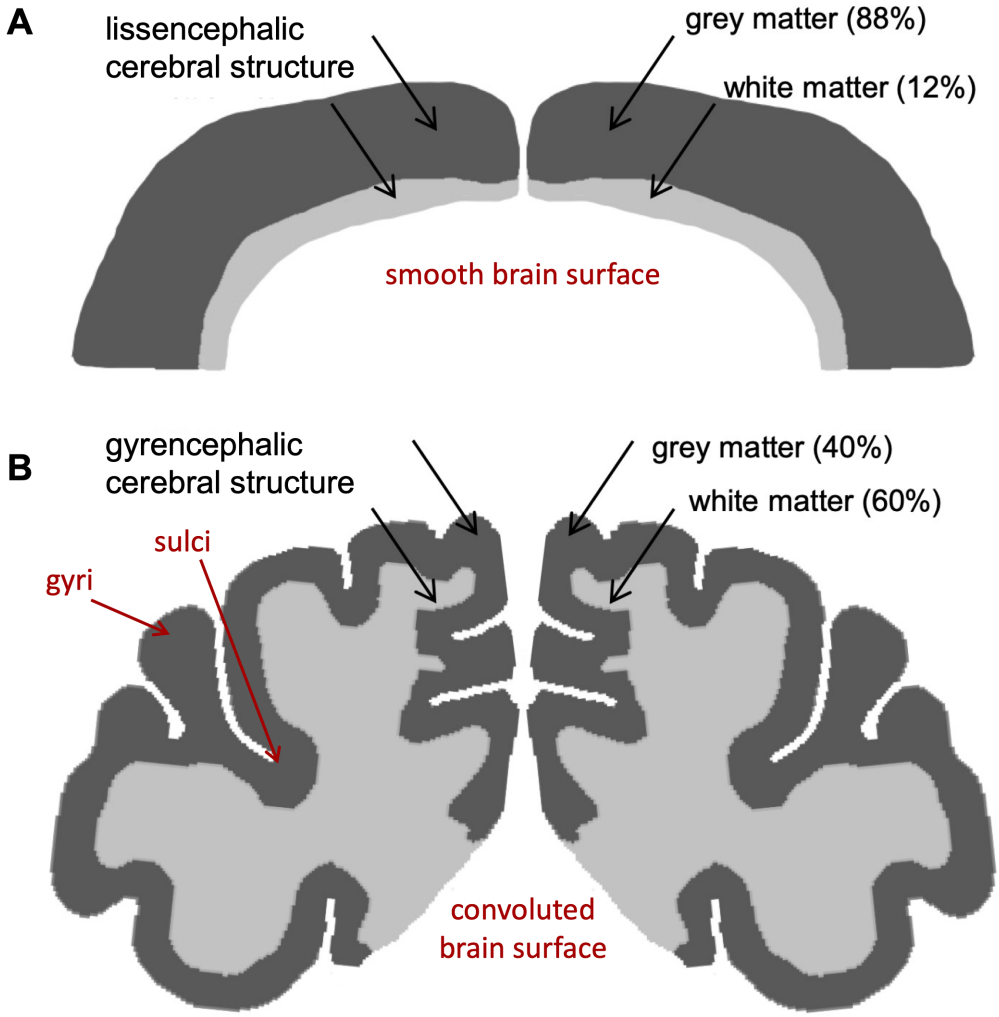

Figure 7: Lissencephalic *vs.* Gyrencephalic Cerebral Structure. The lissencephalic brain (A) has a smooth brain surface, whereas the gyrencaphalic brain (B) has characteristic gyri (ridges) and sulci (depressions) (indicated by red arrows). The lissencephalic rat brain (A) consists of 88% grey matter and 12% white matter and the gyrencephalic human and pig brain (B) consists of 40% grey matter and 60% white matter (indicated by black arrows). The figure is modified from McCook *et al.* [170] under the Creative Commons Attribution- NonCommercial-ShareAlike 4.0 License (CC BY-NC-SA 4.0) (https://creativecommons.org/licenses/by-nc-sa/4.0/).

Bretoncelles-Meishan-Willebrand Pigs

Compared to humans, pigs are by nature hypercoagulative [135, 204]. Therefore, in order to resemble the human blood coagulation pattern, Bretoncelles-Meishan-Willebrand (BMW) pigs are used [195] to properly mirror the temporal pattern of subdural clot formation in response to ASDH [56]. Furthermore, the type of injury, investigated in pre-clinical research should be well-reproducible and minimize the number of animals needed in oder to adhere to the 3R principles (replacement, reduction, refinement). In the ASDH model, anesthesia and surgical instrumentation allow for specific ASDH induction above the parietal cortex of the left hemisphere, and the right hemisphere is used as control tissue. Hence, additional control animals are not necessary, and reproducibility is given by the method of choice [56]. Clinically relevant long-term observation (54h) and multimodal brain monitoring are possible due to the chosen study design [56].

Familial Hypercholesterolemia-Bretoncelles-Meishan Pigs

In order to reflect a patient population with underlying chronic cardiovascular co-morbidity, pigs of the FBM strain are investigated in the septic shock and the HS models respectively [55, 199]. This strain has a spontaneous mutation of the low density lipoprotein receptor (R84C) and is crossed with Bretoncelles and Meishan mini-pigs [249]. FBM pigs develop ubiquitous "human-like coronary atherosclerosis" [248, 249] with characteristic asymmetric plaque formation within the coronary arteries, upon feeding an atherogenic diet composed of 10% lard, 2% cholesterol, 1.5% cholate [164, 181, 199, 249]. These pigs present with a biomarker pattern similar to the profile of patients with hypercholesteremia-induced atherosclerosis: elevated oxidative stress levels and reduced levels of endogenous NO metabolites [168]. Hypercholesteremia-induced atherosclerosis in FBM pigs mirrors a chronic cardiovascular co-morbidity, which can be also elicited e.g. by ELS in critical care patients and is therefore highly clinically relevant, since it reflects one physical component of ELS.

2.2.2 Experimental Procedures

Porcine Intensive Care Unit

The porcine intensive care unit (PICU), used in all projects of this thesis, provides: constant online-monitoring of hemodynamics, temperature and pH control, anesthesia, "lung-protective" mechanical ventilation, organ protection, fluid-resuscitation (and blood transfusion) and vasoactive support (noradrenaline) based on need [55, 56]. This is in accordance with principles of human clinical ICU procedures, which consists of specialized therapeutic care for critically ill patients during life-threatening organ failure, providing adequate organ support to preserve vital parameters [160].

Acute Subdural Hematoma Experiment

The experimental procedure of the ASDH project is taken from Denoix *et al.* [61]. Only abbreviations and references are adapted for compatibility with this thesis. This manuscript was published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

The study was approved by the Federal Authorities for Animal Research and the local Animal Care Committee (Reg.-Nr. 1316, date of approval June 20, 2016). All experiments were conducted in adherence with the National Institute of Health Guidelines on the Use of Laboratory Animals and the European Union "Directive 2010/63/EU on the protection of animals used for scientific purposes." This part of the thesis is an immunohistochemical study performed on available brain sections from a recently published porcine model of ASDH [56]: 5 female and 4 male (castrated) anesthetized BMW pigs (age 11 months, 65 kg). Anesthesia, surgical procedures and resuscitation were described in detail previously [56]. ASDH was induced by injection of 20ml of autologous blood *via* a subdural catheter above the left parietal cortex. Therefore, the skull was exposed and a craniotomy was performed by drilling a hole over the left and

right parietal cortex and a small incision of the dura was made. According to the 3R principle the right hemisphere was also instrumented to avoid the need for additional sham experiments. Microdialysis catheters and multimodal brain monitoring probes were inserted in both hemispheres of brain parenchyma [56]. Two hours after ASDH induction, resuscitation was initiated: CPP was titrated to baseline pressure (if not sufficient norepinephrine was used), neuro-intensive care measures were in accordance with the current guidelines of TBI management in an effort to further improve clinical translation of novel therapeutic interventions [44]. After 54h of intensive care, anesthesia was further deepened and pigs were sacrificed *via* injection of potassium chloride.

Septic Shock Experiment

The experimental procedure of the septic shock project is taken from Merz *et al.* [178]. Only abbreviations and references are adapted for compatibility with this thesis. This manuscript was published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

The study was approved by the University of Ulm Animal Care Committee and the Federal Authorities for Animal Research. The experiments were performed in adherence to the National Institute of Health Guidelines on the Use of Laboratory Animals and the European Union "Directive 2010/63/EU on the protection of animals used for scientific purposes" and authorized by the federal authorities for animal research of the Regierungspräsidium Tübingen (approved animal experimentation number: 1024), Baden-Württemberg, Germany, and the Animal Care Committee of the University of Ulm, Baden-Württemberg, Germany. This part of the thesis is a post hoc study performed on available material from the vehicle-treated group of a previous study [276] and sham-operated animals studied simultaneously under the same protocol [182, 199]. The underlying atherosclerosis in the pig strain has previously been characterized in the coronary vasculature by our group [181, 199]. Briefly, male castrated FBM pigs (age 15-30 months, 69kg (65-73kg)) with a high-fat diet-induced hypercholesterolemia and atherosclerosis [249] underwent polymicrobial septic shock (n = 8) induced by

inoculation of autologous feces into the abdominal cavity, or sham procedure, i.e., abdominal saline injection (n = 5), and subsequently received intensive care therapy for 24h. Anesthesia and surgical instrumentation have been previously described in detail [276]. All pigs had the right jugular vein and left carotid artery exposed for the insertion of a central venous catheter sheath and the placement of a balloon-tipped pulmonary artery catheter to measure central venous pressure, a thermistor-tipped arterial catheter for blood pressure (mean arterial pressure) recording and transpulmonary single indicator thermodilution-cardiac output measurement and placement of a left-ventricular catheter for the assessment of left-ventricular function [199, 276]. Animals were allowed to recover for 12h before the induction of sepsis. Mean arterial pressure was maintained at baseline target values by the continuous infusion of Ringer's solution, hydroxyethyl starch infusion and administration of noradrenaline based on need [276]. Noradrenaline infusion was not further increased, if the heart rate was higher than 160/min to avoid tachycardia-induced myocardial ischemia. The physiological data obtained in this model were all published previously [181, 182, 199] and are summarized in the Table S1: from Merz *et al.* [178]. Sepsis was confirmed by the presence of hyperlactatemia (>2 mmol/l) and significant hypotension in spite of adequate fluid and catecholamine resuscitation in the septic animals (see Table S1: from Merz *et al.* [178]). Furthermore, septic animals had left-ventricular dysfunction in that the stroke volume and ejection fraction could only be maintained by noradrenaline administration, concomitant with disturbed diastolic relaxation, as evidenced by the unchanged left-ventricular end-diastolic volume even though the pulmonary artery occlusion pressure was even higher in sepsis (Table S1: Merz *et al.* [178], [199]). Twenty-four hours after the induction of fecal peritonitis, anesthesia was further deepened and animals were sacrificed with potassium chloride [182].

Hemorrhagic Shock Experiment

The HS study is a post hoc analysis of post mortem brain tissue sections, not previously analyzed, obtained from the previous study of Datzmann *et al.* [55]. Experiments were conducted after approval by the Federal Authorities for Animal

Research (Regierungspräsidium Tübingen; Reg.-Nr. 1341, date of approval May 2, 2017), and the local University of Ulm Animal Care Committee, in adherence to the National Institute of Health Guidelines on the Use of Laboratory Animals and the European Union "Directive 2010/63/EU on the protection of animals used for scientific purposes". Anesthesia, surgical instrumentation, as well as the protocol of hemorrhage and resuscitation have been described in detail previously [55]. 7 female and 6 castrated male FBM pigs with median (interquartile range) body weight of 58 (49;66)kg and age of 24 (23;28) months, were investigated. These FBM pigs are a model for 'human- like coronary atherosclerosis' [104, 164, 181]. Briefly, induction of HS was performed by passive blood withdrawal of 30% of the calculated total blood volume. Hemorrhage was titrated to a targeted mean arterial pressure of 40±5mmHg for 3h. Resuscitation of the animals included shed blood re-transfusion, fluid administration (balanced electrolyte solutions), vasopressor support (norepinephrine) based on need to restore mean arterial pressure to baseline values, and lung-protective mechanical ventilation. $Na_2S_2O_3$ (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany, 25%, diluted in 500ml NaCl 0.9%; infusion rate 0.1 g kg^{-1} h^{-1}) (N=8) or the vehicle solution (2ml kg^{-1} h^{-1}) (N=5) was administered during the first 24h of re-transfusion. After 72h of intensive care treatment or premature termination of the experiment due to pre-defined termination criteria (acute respiratory distress syndrome, refractory hypotension), pigs were sacrificed under further deepened anesthesia, by injection of potassium chloride, and the brain was immediately removed [55].

2.2.3 Tissue Processing

The tissue processing of the ASDH and septic shock projects are taken from Denoix *et al.* [61] and Merz *et al.* [178]. Only abbreviations and references are adapted for compatibility with this thesis. These manuscripts were published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

Immediately after termination of the ICU experiments, the organs were removed by an experienced veterinarian (Dr. Andrea Hoffmann, Institute for Anesthesiological Pathophysiology and Process Engineering, Ulm University Medical Center). In the septic shock project, post-mortem, left-ventricular cardiac samples were fixed in 4% formalin [178]. In the HS project the brain was removed and fixed as a whole in 4% formalin. In the ASDH project, the brain was cut sagittally to separate the hemispheres and then fixed in 4% formalin [61]. After 6 days of fixation (fixation identical for all samples) the brain was cut from frontal to occipital into consecutive 4mm thick macroscopic sections (13-17 sections in total). The macroscopic sections were fixed in 4% formalin for one more day [56]. Available brain sections from Datzmann *et al.* [56] for further immunohistochemistry (IHC) included frontal, medial and occipital (limited, brainstem and cerebellum) regions from the ipsi- and contralateral side. If the macroscopic section was too large to fit as a whole into the embedding cassette (plastic, 26x31x4mm), they were laid flat and further dissected in up to five pieces in a manner which allows for reconstruction of the entire brain section [61]. For the purpose of the HS project, the macroscopic section, which included the hypothalamus was selected for analysis. Mapping and region identification was carried out in adherance to two different porcine brain atlases: Félix *et al.* [72] and Wouterlood *et al.* [283].

All formalin fixed tissue samples were further processed (at room temperature, if not other indicated) at the 7th day of fixation: The tissue was rinsed with H_2O bidest. (distilled water), dehydrated (40% Ethanol (45min), 70% Ethanol (50min), 70% Ethanol (60min), 80% Ethanol (60min), 90% Ethanol (60min), 100% Ethanol (60min), 100% Ethanol (60min), xylene (90min, 40°C), xylene (90min, 45°C)) using a fully automated "Gewebeinfiltrationsvollautomat MTM I & II" and transferred to 62°C warm paraffin. The embedding in paraffin worked by use of the "Dispensiereinheit MPS/P". The brain/heart tissue was placed on a preheated (approximately 60°C) metal embedding cassette. The cassette was pre-filled with drop of liquid paraffin (62°C). The tissue was transferred with warm forceps, which prevents the forceps from sticking to the tissue. Brief freezing (on the "Cooling Plate MPS/C" (-11°C)) of the metal embedding cassette prevents slippage from the organ. The labeled bottom of the plastic embedding cassette was placed on the tissue and filled with paraffin to the upper edge.

Air bubbles were avoided. Complete liquefaction of the paraffin prevented two-phased layers, which can affect subsequent cutting. The metal embedding cassette was then transferred to the "Cooling Plate MPS/C" (-11°C) where the paraffin hardens and the paraffin tissue blocks can be used for subsequent cutting with the microtome.

The paraffin tissue block was clamped in the semi-automatic microtome. To facilitate cutting, the blocks were placed on ice so that the paraffin hardens slightly. Approximately 100μm of the excess paraffin was removed in 10μm increments until the tissue was completely cut. Tissue sections were cut 3-5μm thick with consecutive sections obtained for high quality evaluations. Using cotton swabs, the sections were placed on a 42°C heated distilled water bath. The paraffin section expands and wrinkles level out. By placing the slide steeply against the section, the tissue was transferred to the glass slide. The sections dry in the incubator (heated to 42°C) for at least 24h before further processing.

2.2.4 Histopathology and Immunohistochemisty

Hematoxylin and eosin (HE) staining was performed for general neuro-histopathological evaluation, according to the routine protocol, in the laboratory of Dr. Angelika Scheuerle, Division of Neuropathology, Institute for Pathology, Ulm University Medical Center. In collaboration with Dr. Angelika Scheuerle, we focused on determining if hypoxic events led to brain tissue injury in cortico-subcortical brain regions after HS. Criteria to evaluate brain tissue injury included: morphology of nerve and glia cells, white matter and perivascular edema formation, necrosis of parenchyma and appearance of eosinophilic cells. Due to minor injury, descriptive evaluation was performed without quantification.

IHC Protocol

The IHC protocol of the ASDH and septic shock projects are taken from Denoix *et al.* [61] and Merz *et al.* [178]. Only abbreviations and references are adapted for compatibility with this thesis. This manuscript was published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

IHC allowed us to study and visualize the nuclear or cytoplasmic protein localization in the global landscape of the brain. In contrast to techniques which only use homogenized tissue, e.g., western blot, IHC allowed for detecting the spatial expression patterns and distinguishing between tissue and blood, a common confounding factor in homogenized tissue [191]. We used a colorimetric detection system which allows us to visualize the tissue architecture and cellular morphology (limited in immunofluorescence), e.g., vacuolization or necrosis [61].

All steps were performed at room temperature if not otherwise indicated. The following solutions were prepared for the IHC protocol:

- Citrate buffer (10mM): 1.92g of the citric acid (99%) was weighed out and dissolved in 1l of H_2O bidest. with constant stirring by a magnetic stirrer. A pH of 6 is adjusted by adding 2mol/l sodium hydroxide. The pH measurement is carried out with a "pH-Indikatorstäbchen".

- TBS solution: the TBS powder (already weighed in by the manufacturer) is inverted in 1l H_2O bidest. This yields 0.05M TBS.

- TBS Tween solution for washing: 100μl of Tween are added to 100ml TBS and poured in a dye box.

- TBS Tween solution for the blocking solution and antibody dilutions: 60μl of Tween and 10μl normal goat serum are added to 10ml TBS.

- The blocking solution consisted of 2700μl TBS Tween and 300μl normal goat serum.

The IHC protocol started with deparaffinization (4x in xylene-filled dye boxes *á* 2min, 3min, 4min, 5min) and rehydration of the samples with a graded series of ethanol (100% Ethanol (1min), 100% Ethanol (5min), 90% Ethanol (3min), 70% Ethanol (5min)) and H_2O bidest. (1min). Heat-induced antigen retrieval was performed by bringing the samples to a boil in a microwave oven (90sec) in a 10mM citrate solution (pH6) and cooling back to room temperature (10min). The samples were blocked with normal goat serum (10%) for 20-30min before 1h primary antibody incubation with: endogenous H_2S producing enzymes anti-CSE (Protein Tech, 12217-1-AP) and anti-CBS (Protein Tech, 14787- 1-AP), anti-OT (Millipore, AB911), anti-OTR (Protein Tech, 23045-1-AP [173]) and anti-GR (D8H2, Cell Signaling). Further mediators of injury were investigated by using the following primary antibodies: anti-GFAP (Abcam, ab7260) as a marker of cerebral injury; anti-nitrotyrosine (Merck Millipore, ab5411), as a marker of oxidative and nitrosative stress, since NO can react with superoxide to peroxynitrite, leading to nitrotyrosine formation due to nitration of protein tyrosine residues [199]; anti-pig albumin (Abcam, ab79960); anti-aquaporin 4 (AQP4) (Protein Tech, 16473-1-AP); anti-neuronal nuclei (NeuN) (Cell signaling, 24307) as a marker for neurons; anti-complement component 5a receptor (C5aR) (Protein Tech, 21316-1-AP); anti-induced complement component 3 (iC3) (Hycult biotech, HM2168); anti-complement component 1 Q subcomponent-binding protein (C1QBP) (Protein Tech, 24474-1-AP); anti-interleukin-6 receptor (IL-6R) (Protein Tech, 23457-1-AP); anti-tumor necrosis factor receptor 1 (TNFR1) (Protein Tech, 21574-1-AP) and tumor necrosis factor receptor 2 (TNFR2) (Protein Tech, 19272-1-AP). The respective dilutions of the primary antibodies are depicted in Table 7.

In the ASDH project, sections of ipsi- and contralateral brain specimens were analyzed concurrently, and negative controls were performed (examples see figure 11). Since the IHC detection of OTR in the brain has proven to be a particular challenge in the past, the specificity of the OTR antibody used in this study was validated by both negative controls (knock out [281]) and positive controls, western blot and immunoprecipitation (IP) (OTR Proteintech, methods

description in section 2.2.4, results in section 9 and figure 12). The specificity of the antibodies has been additionally confirmed in NCBI BLAST searches (courtesy of the U.S. National Library of Medicine). The BLAST 'finds regions of similarities between biological sequences, and compares protein sequences to sequence databases.' We compared immunogen sequences of the used antibodies to the sus scrofa database (see Table 7). When the immunogen sequence was not available we used the full length protein sequence of the species the antibody was made to, in this case human. A query cover above 90% and homology above 60% were investigated, when there was no significant homology to any other protein.

Detection of primary antibodies was performed according to the manufacturer's instructions using the Dako REAL™ Detection System (based on alkaline phosphatase conjugated secondary antibodies: anti-mouse, anti-rabbit). Washing steps (TBS 1min, TBS Tween 3min, TBS 1min) were performed after primary antibody incubation (1h), Dako REAL™ Link, Biotinylated Secondary Antibodies (AB2) incubation (30min), and Dako REAL™ Streptavidin Alkaline Phosphatase (AP) incubation (30min). Red chromogen was used for visualization according to the manufacturer's instructions, followed by counterstaining with Mayer's hematoxylin solution [56, 181, 182]. Therefore, the samples were immersed in approximately 100ml of Mayer's hematoxylin solution for 30sec, dipped in two consecutive dye boxed filled with tap water and a third one with H_2O bidest. Samples are then dehydrated in a graded series of ethanol and xylene: 70% Ethanol (3min), 90% Ethanol (3min), 100% Ethanol (5min), xylene (3min), xylene (5min). The attachment of the cover glasses and the long-term fixation of the tissue sections were carried out with Neo-Mount®.

Control Experiments IHC

The IHC control experiments of the ASDH project are taken from the supplement material from Denoix *et al.* [61]. Only abbreviations and references are adapted for compatibility with this thesis. This manuscript was published under the Cre-

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All primary antibodies were titrated to their optimal dilutions within the range recommended by the manufacturer. To check the specificity of IHC, we routinely perform control experiments. Whenever the immunogen peptide is available, we use primary antibody pre-incubated with the immunogen peptide 10X excess (3h at room temperature or overnight at 4°C) and incubate the tissue with this pre-absorbed antibody instead of primary antibody alone. A second negative control is performed *via* incubation with diluent instead of primary antibody to control for the specificity of the secondary system. Exemplary pictures of the performed negative controls for the CSE antibody are shown in figure 11. Blocking of the slides with 10% serum of the secondary antibody species before the primary antibody incubation prevents non-specific protein-protein interactions and cross-reactions with tissue Fc receptors. Non-specific activity of the alkaline phosphatase detection system is prevented by the addition of levamisole, which blocks all endogenous alkaline phosphatases except the intestinal isoform, which the detection is based on.

OTR IP and Western Blot Protocol

The specificity of the used OTR antibody from Proteintech was confirmed in protein lysates from Caco2 cells, since this cell line was easily accessible to us and its expression and activity of OTR is reported in the literature [134]. The cells were bred to 100% confluency in T25 cell culture flasks, washed with PBS, and lysed with 100μl Pierce^{TM} RIPA buffer + protease inhibitor cocktail (Thermo Scientific) per flask. Cells were broken up with two freeze-thaw cycles in liquid nitrogen. After 90min incubation on ice, the lysates were centrifuged for 20min at 12000rpm (4°C) and the pellet was discarded. The samples for IP were prepared according to the manufacturer's instructions for the Pierce^{TM} Protein-A/G-Agarose Kit (Thermo Scientific). Briefly, the lysate supernatant was diluted 1:1 in binding buffer, OTR antibody was added at a concentration of 5μg/ml and incubated overnight at 4°C with gentle rocking. We used two different

antibodies for the IP, rabbit polyclonal (Proteintech) and mouse monoclonal (C-4, Santa Cruz). The IP samples were purified using the Pierce™ Protein-A/G-Agarose Kit (Thermo Scientific) and a buffer exchange to PBS was performed with Amicon® Ultra 15ml Centrifugal Filters (Merck Millipore) according to the manufacturer's instructions.

The samples were analyzed *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot detection was performed as described below, with the rabbit polyclonal OTR antibody (1:1000, Proteintech). The protein concentration of the IP samples was determined according to the Bradford method and diluted with PBS to obtain either 2.5 or 5μg protein of the IP samples respectively. IP samples (12μl) were mixed with sample buffer (4μl leammli buffer and 1:10 β-mercaptoethanol) and heated to 95°C for 5min. For the SDS-PAGE method, a collection gel was added upstream of the separation gel to achieve a sharper delineation of protein bands. A comb was used to create pockets for the samples. After one hour, polymerization of the gels was complete, the comb was removed, and the pockets were washed three times with 10X electrophoresis buffer to remove any contaminants. The gel was loaded with the samples (max. 30μl each) and 5μl color marker (Precision Plus Protein™ Kaleidoscope™ standards). The color marker separates the different molecular weights in a color-coded manner. After loading, the gel was placed in the electrophoresis chamber, which was filled with 1X electrophoresis buffer. Initially, a current of 60V was applied for 20min, which was then increased to 110V for approximately 90min. The first 15min were used to collect the protein mixture before subsequent separation in the separation gel. During electrophoresis, the nitrocellulose membrane was labeled and then blotted with transfer buffer.

The blotting process took place in Bio-Rad's Trans-Blot® SD Semi-Dry Transfer Cell. For this purpose, the nitrucellulose membrane and the gel, surrounded on both sides by eight Whatman filter papers, each impregnated with transfer buffer, were placed in the chamber, where any air pockets were removed by rolling. Now the stack was placed under a vertical voltage of 15V for 45min. This resulted in the transfer of proteins to the nitrocellulose membrane, where they attached due to hydrophobic interactions. Blotting was followed by washing with

TBS and 0.1% Tween for 5min. For blocking of non-specific binding sites, the membrane was incubated with the blocking buffer (TBS with 5% milk powder and 0.1% Tween) for one hour. The primary antibody (rabbit polyclonal OTR antibody Proteintech) was added (diluted 1:1000 in TBS with 0.1% Tween and 5% milk powder) to the membrane and incubated overnight at 4°C with gentle swirling and then washed three times with TBS with 0.1% Tween for 10min each under panning. Then, the secondary antibody (anti-rabbit antibody prepared in swine coupled with horseradish peroxidase diluted 1:2000 in TBS with 0.1% Tween and 5% milk powder) was applied to the nitrocellulose membrane and incubated for one hour, after which the membrane was washed three times for 10min with 15ml of TBS and 0.1% Tween and 5min with TBS. To visualize the protein bands, the nitrocellulose membrane was incubated in a detection solution Clarity™ Western ECL Substrate according to the manufacturer protocol for 1min. Picture was taken with the ChemiDoc MP imaging system.

2.2.5 Microscopic Analysis and Statistics

The microscopic analysis and statistics of the ASDH and septic shock projects are taken from Denoix *et al.* [61] and Merz *et al.* [178]. Only abbreviations and references are adapted for compatibility with this thesis. These manuscripts were published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

Slides were visualized using a Zeiss Axio Imager A1 microscope with 2.5X, 10X, 20X and 40X objectives. In the septic shock project, quantification for intensity was performed on multiple 800,000-μm^2 sections using the AxioVision 4.8 software (Zeiss) [182]. Data are presented as densitometric sum red. In the HS project, multiple 800,000-μm^2 sections were used for quantification of % positive area using the Zen Image Analysis Software (Zeiss). Data are presented as % positive area.

In the septic shock project, statistical analysis were performed with GraphPad Prism Version 4. In the HS project, Graph Pad Prism Version 8 was used for

statistical analysis. The data are presented as median (quartiles) with interquartile ranges. Inter-group differences were analyzed with the Mann-Whitney rank sum test after exclusion of normal distribution using the Kolmogorov-Smirnov test.

3 Results

Antibodies for immunohistochemical detection of: CSE, CBS, OT, OTR, GR and markers of injury, targeting inflammatory processes and pathophysiological changes: albumin, AQP4, NeuN, C5aR, iC3, C1QBP, IL-6R, TNFR1, and TNFR2 were validated for their specificity on porcine heart and brain tissue [61, 178].

3.1 Acute Subdural Hematoma

The results of the ASDH project below are taken from Denoix *et al.* [61]. Only abbreviations and figure references are adapted for compatibility with this thesis. This manuscript was published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

The pictures in the following figures are representative examples of all the IHC experiments for each protein of interest, performed on tissue from various animals. The exact number of animals are given in the figure legends.

Cytoplasmic OTR expression was found in cortical neurons in both hemispheres of the frontal cortex (see figures 8 A1,B1). OTR and OT expression was present in the parenchyma around the sulci of the ipsilateral side (see figures 8 A,C). Specifically, we observed an OTR positive lining around the nucleus of small cells in the white matter. Sections from the ASDH induction site showed OTR expression in and around the parenchyma of necrotic areas (associated with loss of cellular architecture/vacuolization). Hippocampal neurons and granular neurons showed OTR and OT expression, primarily in the cytoplasm. Perivascular

swelling was associated with more parenchymal OTR expression (see figure 8 A1). CSE was expressed in cortical neurons (cytoplasmic) of the frontal cortex in the contralateral side (see figure 8 F1) and was reduced in the ipsilateral side (see figure 8 E) confined to the sulci. CSE was also expressed in hippocampal neurons and granular neurons. At the injury site, CSE was present in necrotic areas where parenchymal bleeding was present and tissue architecture was disrupted (CSE potentially coming from the circulation/blood). There was little CBS expression in the parenchyma of the contralateral side (see figure 8 H). In the ipsilateral side CBS was strongly expressed around the sulcus (see figure 8 G) and not expressed in cortical neurons (see figure 8 G1).

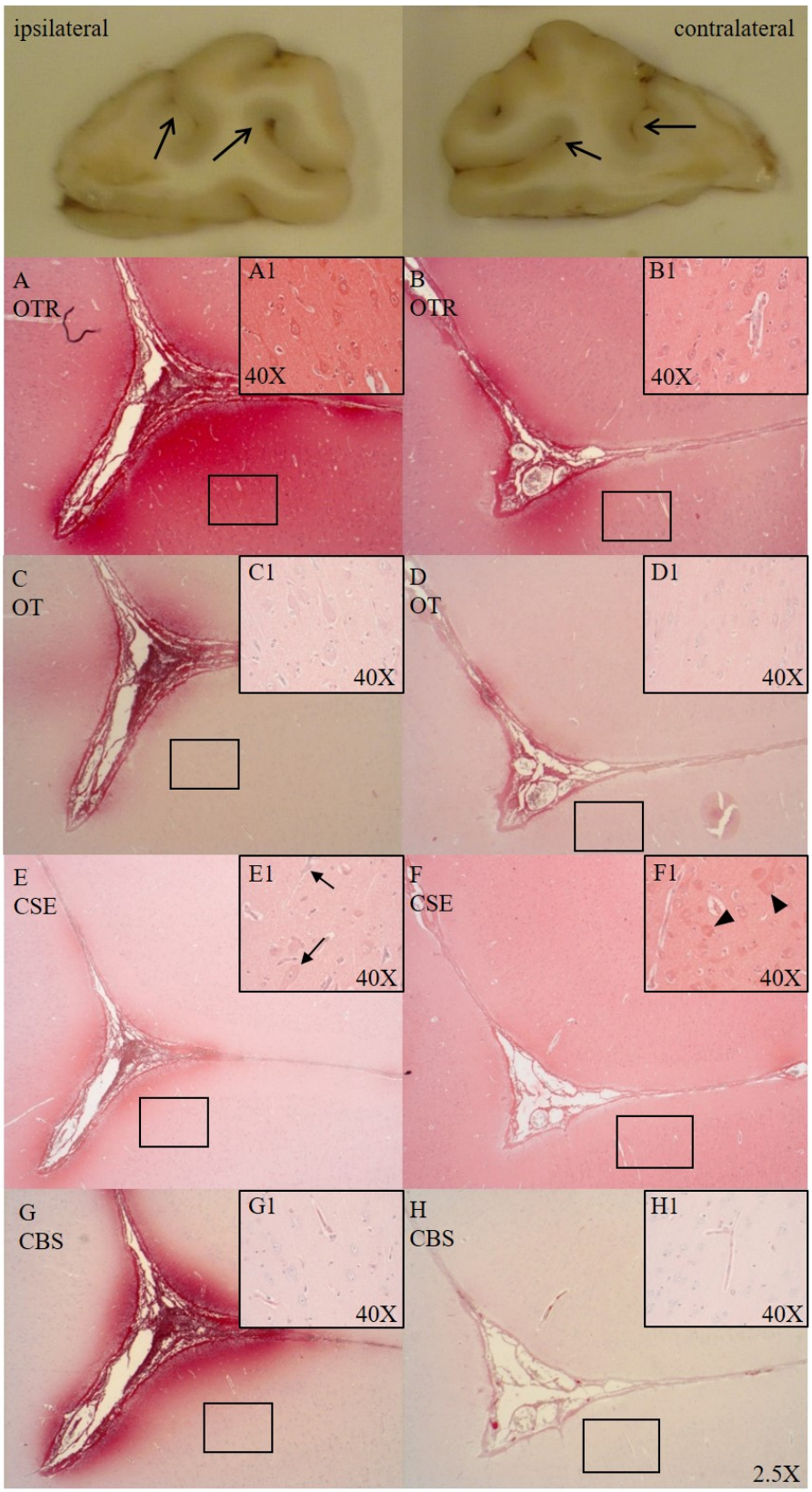

Figure 8: Comparison of OTR (A,B, n = 6), OT (C,D, n = 7), CSE (E,F, n = 7), and CBS (G,H, n = 6) expression in matching consecutive sections of a sulcus of the frontal cortex (ipsilateral (A,C,E,G) vs. contralateral side (B,D,F,H)). Macroscopic exemplary sections are shown at the top (open arrows pointing to sulci)...

... Ipsilateral OTR expression in the pial vasculature and glia limitans with blushing effect in the parenchyma surrounding the base of the sulcus (A), cortical neurons magnified in (A1), less pronounced OTR expression on the contralateral site (B), cortical neurons magnified in (B1). OT expression is more limited than OTR expression but shows the same expression pattern as OTR in the ipsi- (C, magnified in C1) and contralateral side (D, magnified in D1). In the ipsilateral side CSE is pronouncedly expressed surrounding the sulcus and negative in the parenchyma moving away from the sulcus (E) and shows little to no expression in cortical neurons, with nuclear counter-staining with hematoxylin (black arrows magnified in E1). CSE is expressed in the pial vasculature and is evenly distributed in parenchyma of the contralateral side around the sulcus (F) with (red) CSE-positive cortical neurons (black arrow heads magnified in F1). In the ipsilateral side CBS shows pronounced expression surrounding the sulcus (G) and is not expressed in cortical neurons (magnified in G1). CBS expression in the contralateral side is weak (H) and present in the microvasculature (magnified in H1). The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

At the ASDH site, the parenchyma of necrotic areas with vacuolization (see figure 4 A), and singular neurons (see figure 4 B) were positive for albumin. However, the majority of the neurons were negative for albumin (see figure 4 C). Distant to the injury in midbrain subcortical regions, neurons were positive for albumin expression (see figures 4 D-F). Interestingly, in the hippocampus we found nuclear localization of Alb (see figures 4 G,H).

We established the staining of AQP4, a water channel protein, in the pig brain (parietal cortex). AQP4 shows variable expression as we move away from the injury site to the morphologically intact tissue (see figures 5 A-C).

In the frontal cortex, NeuN was strongly expressed in the intact regions (see figure 6 B), and its expression was lower in neurons near the injury site (see figure 6 A).

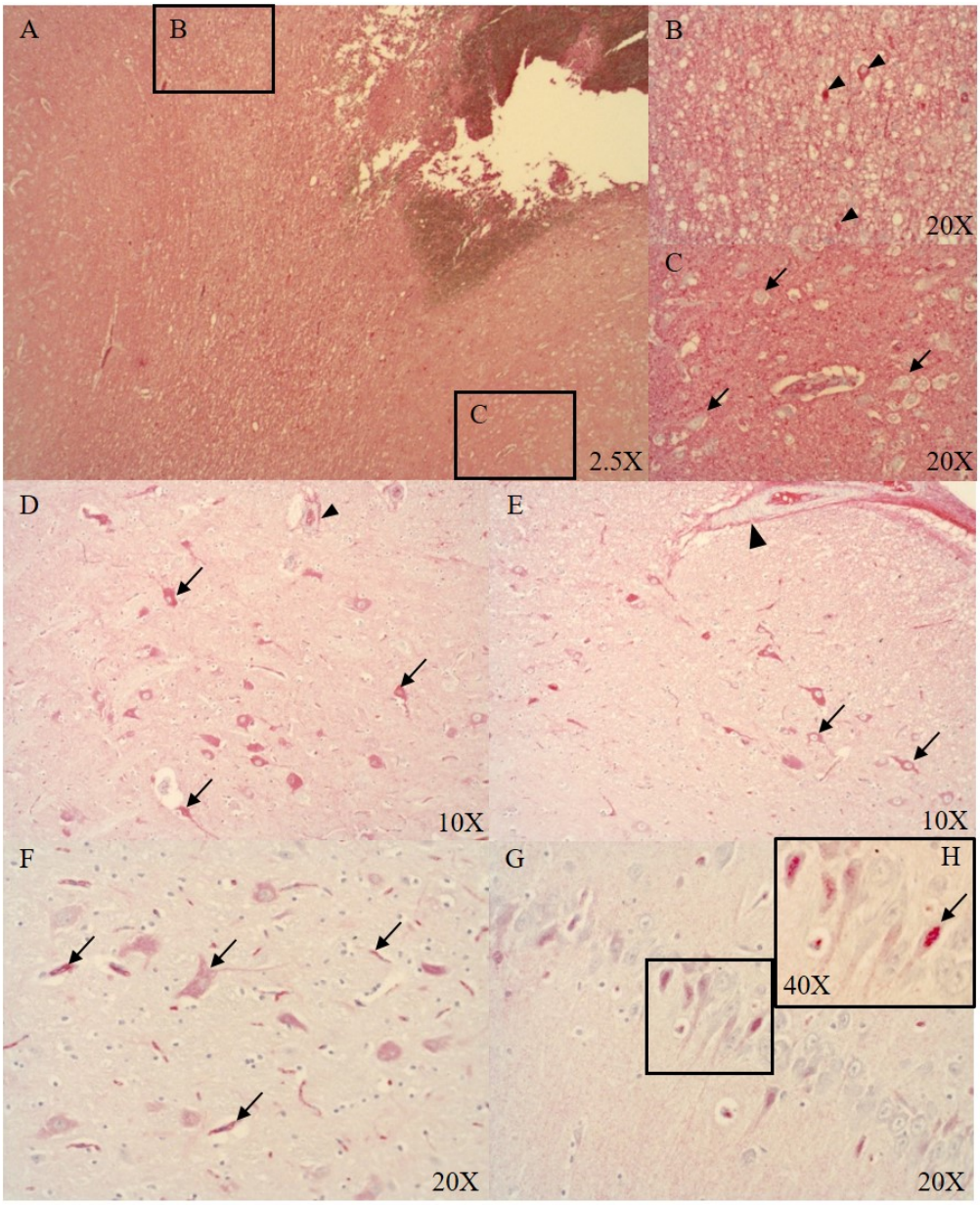

Figure 4: Albumin expression in the ipsi- (A-C, midbrain cortical region) and contralateral (D-G, midbrain subcortical region) side (n = 9). Albumin expression at the ASDH site (ipsilateral): extensive albumin extravasation (A), singular neurons around the ASDH site are positive for albumin (black arrow heads B), most cortical neurons were negative for albumin (black arrows C). Albumin expression in the contralateral hemisphere (distant to the ASDH site): minor albumin extravasation from microvasculature (indicated by positive adventitia of microvasculature (arrow head) and parenchyma), cytoplasmic positive neurons around vacuolization (black arrows D), pial artery SMCs negative, positive adventitia (arrow head), positive neurons associated with swelling (black arrows E), albumin contained in neurons and inside the microvasculature (black arrows F), hippocampal neurons show nuclear albumin expression (G) magnified in (H), see black arrow. The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

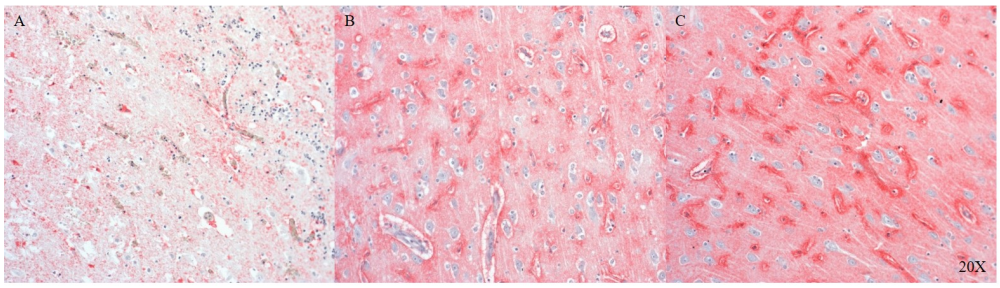

Figure 5: AQP4 expression in the parietal cortex at the injury site (A), in neighboring areas (B) and distant to the injury site (C) (n = 6). At the injury site cellular architecture is impaired and AQP4 expression is weak (A), moving away from the injury there is more AQP4 expression (B), maximal AQP4 expression is present distant to the injury (C). The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

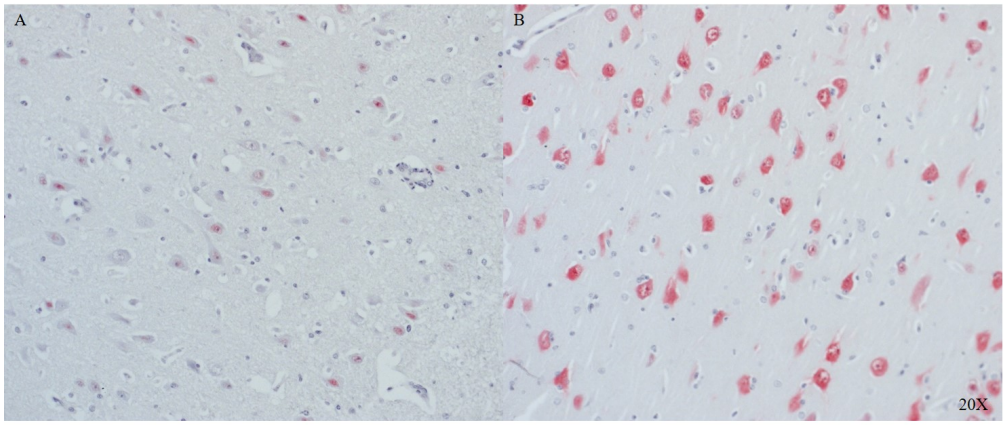

Figure 6: NeuN expression in cortical neurons of the frontal cortex close to (A) and further away (B) from the injury site (n = 6). Cortical neurons show little to no expression of NeuN in proximity to the injury (A), in distance to the injury cortical neurons show NeuN expression (B). The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

Vasculature

Pial arteries expressed OTR and OT in the SMCs and in the endothelium (see figures 8 A-D). Around pial and microvascular arteries there was OTR and OT positive 'blushing' expression pattern around sulci of the frontal cortex in the ipsilateral hemisphere (see figures 8 A,C). CSE was expressed in the SMCs of the pial arteries. In the microvasculature, CSE was expressed in vessel walls, mostly in the SMCs and the endothelium (see figure 8 B). In the microvasculature and pial arteries albumin was localized in the adventitia (see figures 4 D,E).

Inflammation

CSE positive immune cells were present in and around the microvasculature and pial arteries (see figure 8 B). OTR positive immune cells were present near the ruptured microvasculature. Cortical neurons of the parietal cortex showed nuclear positivity for C5aR in proximity and distant to the ASDH site (see figure 7 A4). Close to the injury site there was an infiltration of C5aR-expressing immune cells in areas of intraparenchymal bleeding (see figure 7 A1-3). In the brainstem, C5aR was mostly present in the cytoplasm, in contrast to the cortical region. Expression of iC3 was more limited in the parietal cortex in comparison to C5aR but also found in infiltrating immune cells and parenchyma (see figure 7 B1-3). In the hippocampus, granular neurons also expressed C5aR.

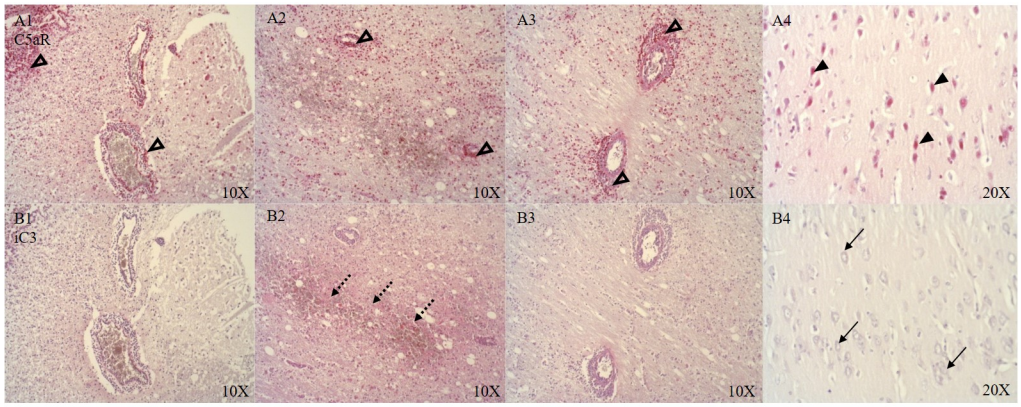

Figure 7: C5aR (A, n = 8) and iC3 (B, n = 7) expression around the ASDH site in four anatomical levels of the parietal cortex (A/B 1-3) and distant to the ASDH site (A/B 4). Around the ASDH site perivascular infiltration of C5aR positive immune cells (unfilled arrow heads A1-3), immune cell and parenchymal localization of iC3 (punctuated arrows B1-3), distant to the ASDH site nuclear expression of C5aR in cortical neurons (black arrow heads A4), no neuronal iC3 detection distant to the ASDH site (black arrows B4). The figure and- the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

C1QBP was positive in the cytoplasm of neurons around the ASDH site in the frontal cortex (see figure 8 A). C1QBP was detected associated with the microvasculature, strongly expressed in immune cells within the vessel wall and in areas of discrete bleeding (see figure 8 A,A1).

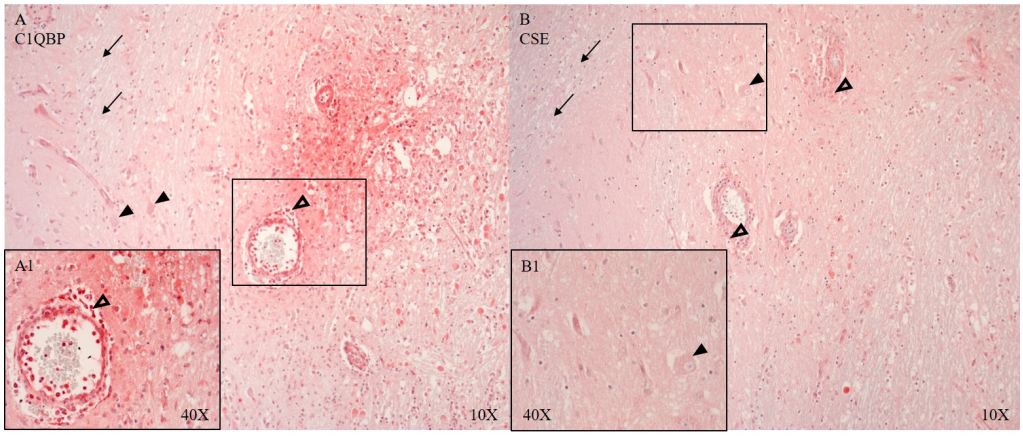

Figure 8: C1QBP (A, n = 5) and CSE (B, n = 7) expression in matching consecutive sections of the frontal cortex at the border zone of gray and white matter. Perivascular infiltration of C1QBP and CSE positive immune cells (unfilled arrow heads A,A1,B), C1QBP expression in the endothelium and SMCs of the microvascular artery (A) (vessel magnified in A1) and in the parenchyma with interstitial extravasated erythrocytes (A,A1). C1QBP and CSE in neurons (black arrow heads) (A,B magnified in B1). CSE expression in the arterial SMCs. White matter is negative for both: C1QBP and CSE (black arrows) (A,B). The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

IL-6R is predominantly expressed in the parenchyma and in the cytoplasm of neurons in areas with perivascular swelling in the parietal cortex (see figure 9 A). Close to the injury, immune cells in the parenchyma and in the microvasculature were IL-6R positive (see figure 9 A). Further away from the injury IL-6R expression was limited to neuronal cytoplasmic expression (see figure 9 B).
We only looked for TNFR1 and TNFR2 expression in the brainstem and the cerebellum, where the neurons showed cytoplasmic and nuclear TNFR1 and TNFR2 expression (see figures 10 A1,2,B1,2).

IHC control experiments

The pictures in figure 11 B/B1 and C/C1 show exemplary IHC negative control experiments for CSE. A positive signal could be prevented by either preincubation of the primary antibody with the immunogen peptide (see figure 11 B/B1) or performing the IHC with diluent without the addition of primary

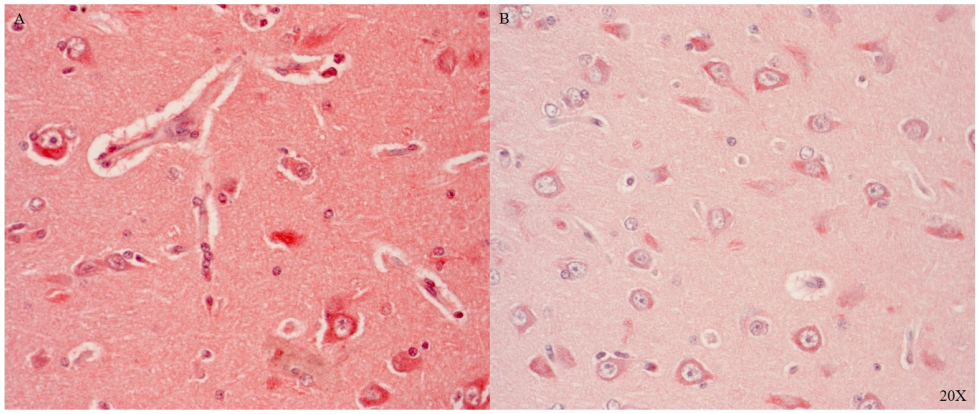

Figure 9: IL-6R expression in the parietal cortex in proximity (A) and distant to the injury (B) (n = 1). IL-6R expression in neurons, immune cells, parenchyma and the microvasculature close to the injury (A), and limited neuronal cytoplasmic expression distant to the injury (B). The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

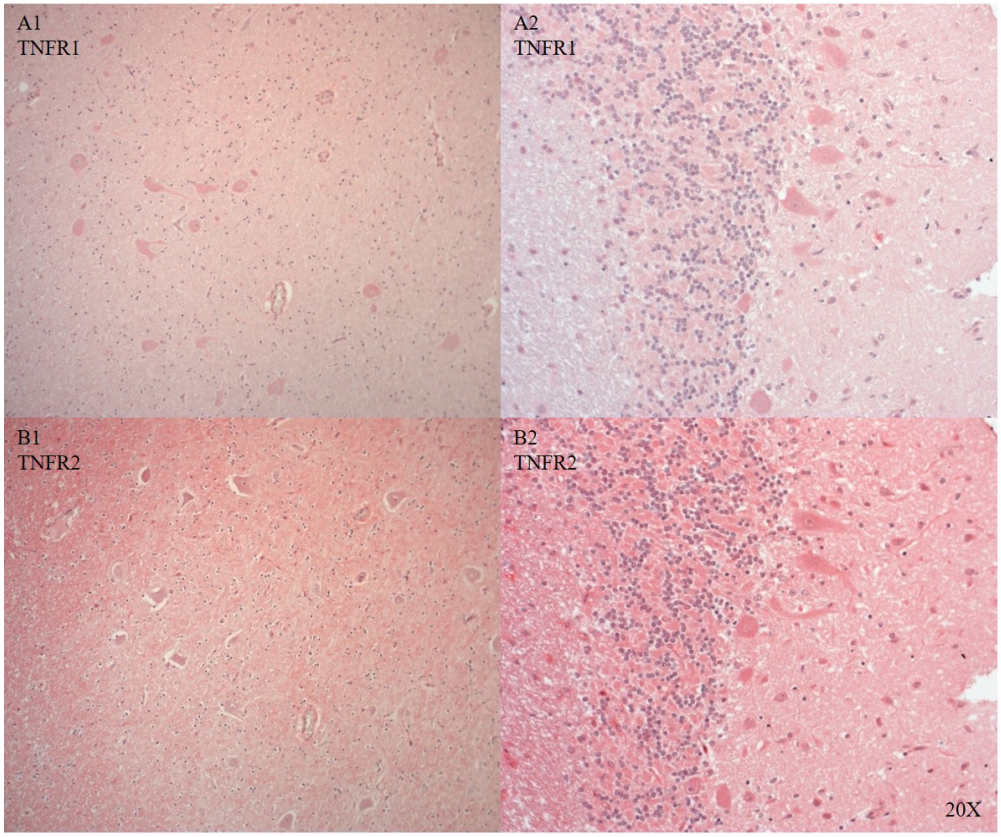

Figure 10: TNFR1 (A, n = 1) and TNFR2 (B, n = 1) in the brainstem (A/B 1) and in the cerebellum (A/B 2). In the brainstem TNFR1 (A1) and TNFR2 (B1) were expressed in neurons and microvasculature, and TNFR2 was also expressed in the parenchyma (B). In the cerebellum purkinje cells expressed TNFR1 (A2) and TNFR2 was expressed in purkinje, granular cells and parenchyma (B2). The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

antibody (see figure 11 C/C1), suggesting that the positive red signal in figure 11 A/A1 (replicated from figure 8 E/E1) is specific for CSE.

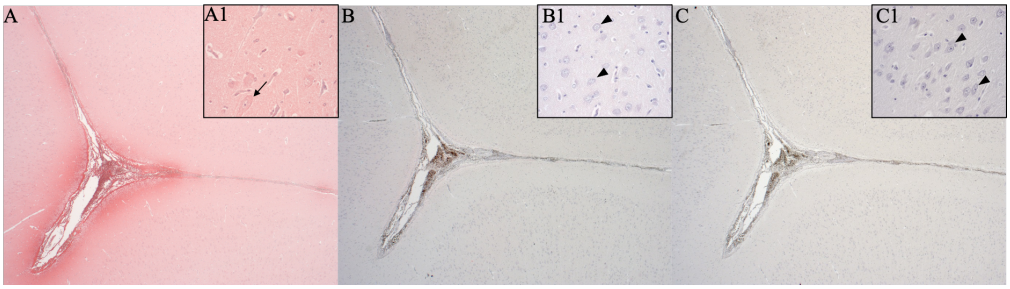

Figure 11: Consecutive tissue sections of CSE IHC and negative controls, (A, B, C) 2.5X overview pictures, inserts (A1, B1, C1) 40X magnification. (A/A1) are a copy of figure 8 E/E1 as an example of positive CSE (CSE ab: 12217-1-AP Proteintech) IHC, with the arrow in A1 indicating a neuron with little CSE expression (pink) and nuclear hematoxylin counter-staining. (B/B1) are examples of IHC with pre-absorbed (CSE fusion protein: Ag2872 Proteintech) primary antibody, arrows in B1 pointing to negative neurons with nuclear hematoxylin counter-staining. (C/C1) are examples of IHC with diluent without primary antibody, arrows in C1 pointing to negative neurons with nuclear hematoxylin counter-staining. The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

OTR Immunoprecipitation

As can be seen in figure 12, the IP of OTR from Caco2 cells with either the monoclonal or polyclonal OTR antibody was detected by the polyclonal OTR antibody and revealed a single band for each set of samples. Thus, we concluded that both antibodies specifically detect OTR. We used the polyclonal antibody for our experiments, since the monoclonal antibody neither was validated nor did it work for IHC on paraffin embedded tissue.

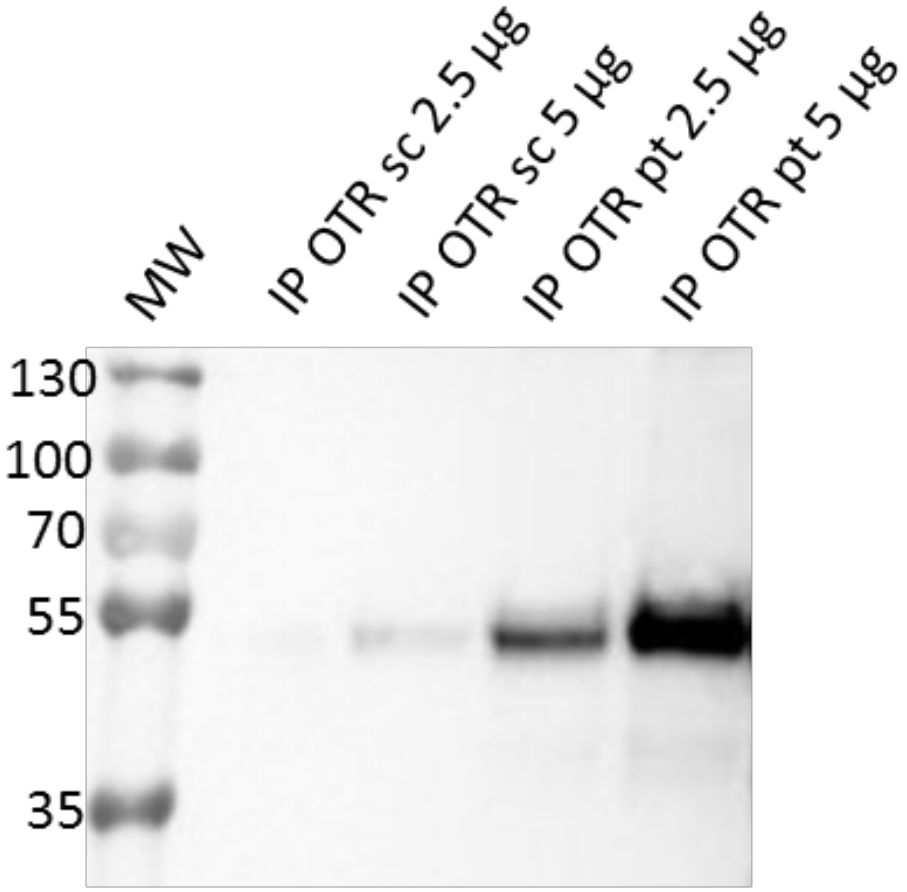

Figure 12: Western blot of OTR IP of Caco2 cells. Sample loading is indicated above the blots: antibody used for IP and amount of protein loaded. MW = molecular weight marker, sc = Santa Cruz, pt = Proteintech. The blot was detected with polyclonal OTR antibody (1:1000, Proteintech).The figure and the figure legend are taken from Denoix *et al.* [61] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

Taken together, the pathophysiological changes of the porcine brain after ASDH were: perivascular swelling in both hemispheres, barrier dysfunction and oxidative stress in the injured brain hemisphere and in particular at the bases of the sulci. CSE, CBS, OT and the OTR were localized (i) in the parenchyma at the depth of the sulci, (ii) in cortical neurons within the gyri, (iii) in pial arteries and the microvasculature, and (iv) in infiltrating immune cells [61].

3.2 Hemorrhagic Shock

Neuro-Histopathological Damage

The histopathological findings were signs of discrete swelling and minor perivascular edema in cortico-subcortical brain regions (in the same macroscopic sectional plane as the PVN, see figure 13), otherwise the brain tissue looked histologically uninjured, presenting with normal appearing singular heterotopic ganglion cells, and intact parenchyma.

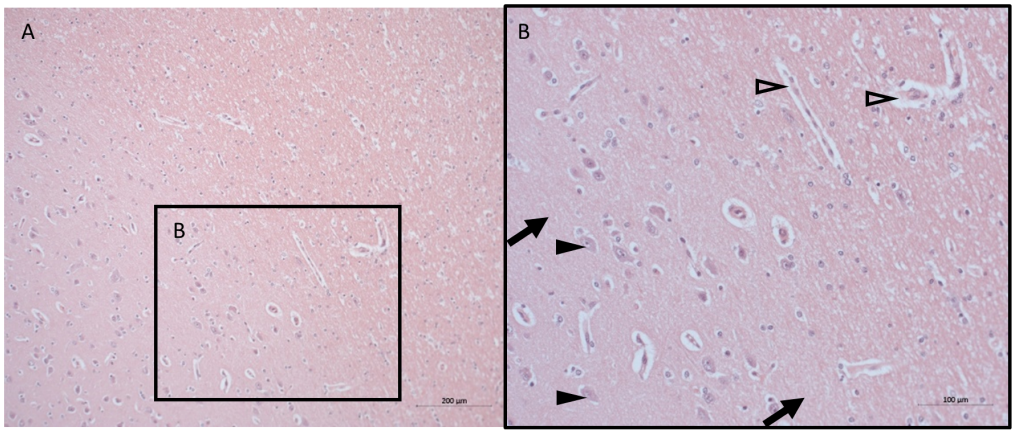

Figure 13: Neuro-histopathological evaluation. Exemplary pictures of HE staining in cortico- subcortical brain tissue with signs of minor perivascular edema in sections from the same macroscopic sectional plane as the PVN at 10X (A) and 20X (B). Black arrows point to intact parenchyma, black arrowheads depict nerve cells, open arrowheads point to minor perivascular edema.

Lack of Effect of $Na_2S_2O_3$ on CSE, CBS, OT, OTR, GFAP and Nitrotyrosine in the PVN

The endogenous H_2S producing enzymes CSE, CBS, and OT and its receptor were found to be present in the porcine hypothalamic PVN (figures 14-15). CSE, CBS, OT and the OTR were positively localized in the magnocellular neurons of the PVN in consecutive sections of the porcine hypothalamus (Denoix *et al.* [60]). The GR was also present in the PVN, mostly around the vasculature and to a lesser degree than the other proteins of interest (see figure 16). None of the proteins of interest showed any significant inter-group difference in the porcine hypothalamic PVN (figures 14-16). GFAP was also present in the PVN of both

groups (see figure 16) but, did not show a significant inter-group difference. Nitrotyrosine showed negligible to no expression in the analyzed tissue specimens (vehicle: n=5, 0.00[0.00; 0.00] %; $Na_2S_2O_3$: n=8, 0.01[0.00;0.03] %).

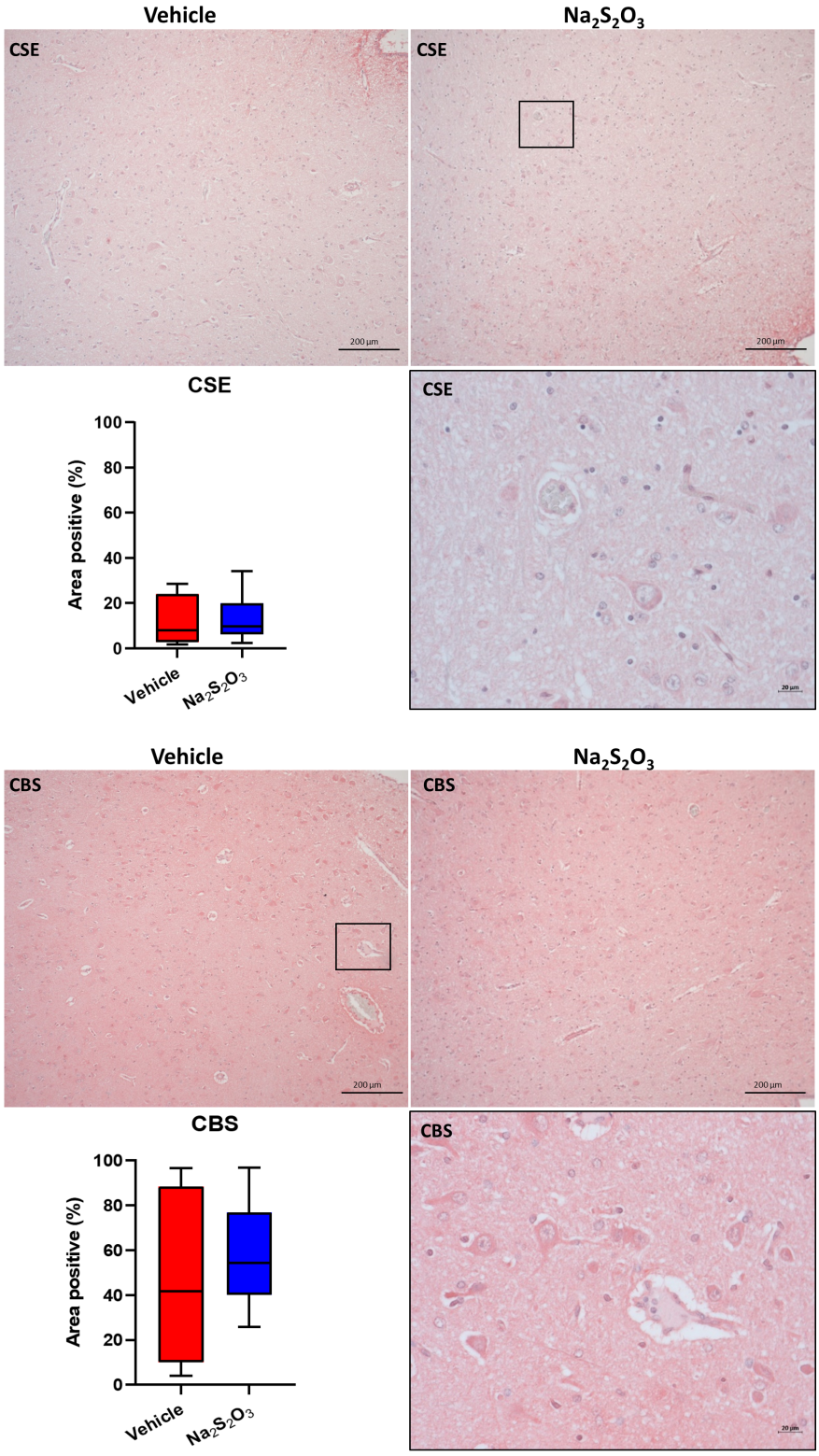

Figure 14: H_2S producing enzymes in the porcine PVN. Quantification of the immunohistochemical stainings as positive percentage area in the PVN for CSE (vehicle: n=4; $Na_2S_2O_3$: n=6) and CBS (vehicle: n=5; $Na_2S_2O_3$: n=7). Boxes represent the interquartile ranges with the median (black line), whiskers represent minimum and maximum values. Exemplary immunohistochemical pictures of CSE and CBS in the PVN of vehicle and $Na_2S_2O_3$ treated animals (10X). Higher magnification pictures of CSE and CBS originate from the black box in the respective 10X picture.

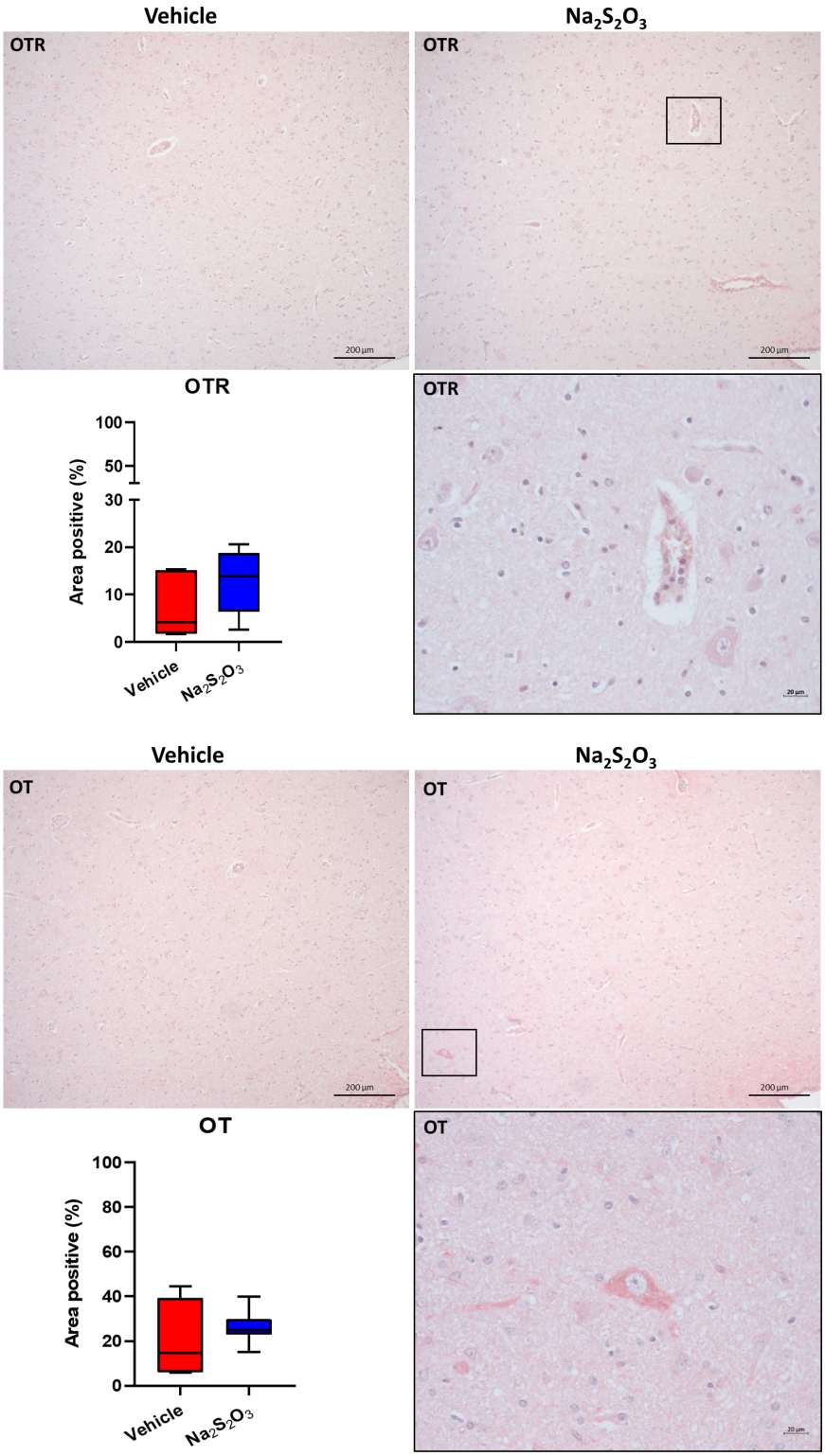

Figure 15: OTR and OT in the porcine PVN. Quantification of the immunohistochemical stainings as positive percentage area in the PVN for the OTR (vehicle: n=5; $Na_2S_2O_3$: n=7) and OT (vehicle: n=5; $Na_2S_2O_3$: n=7). Boxes represent the interquartile ranges with the median (black line), whiskers represent minimum and maximum values. Exemplary immunohistochemical pictures of the OTR and OT in the PVN of vehicle and $Na_2S_2O_3$ treated animals (10X). Higher magnification pictures of the OTR and OT originate from the black box in the respective 10X picture.

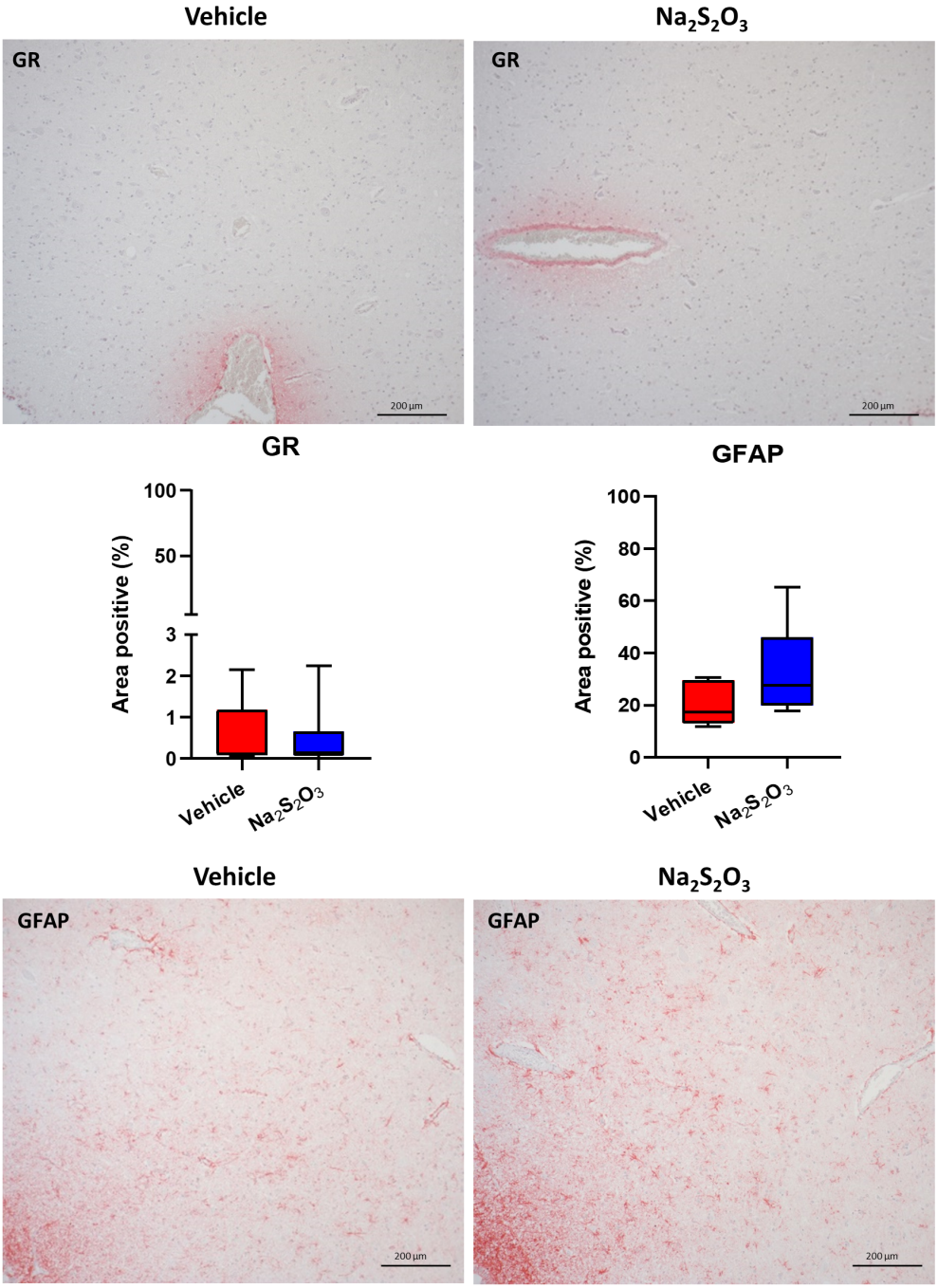

Figure 16: GR and GFAP in the porcine PVN. Quantification of the immunohistochemical stainings as positive percentage area in the PVN for the GR (vehicle: n=5; $Na_2S_2O_3$: n=7) and GFAP (vehicle: n=5; $Na_2S_2O_3$: n=8). Boxes represent the interquartile ranges with the median (black line), whiskers represent minimum and maximum values. Exemplary immunohistochemical pictures of the GR in the PVN of vehicle and $Na_2S_2O_3$ treated animals (10X).

Taken together, neuro-histopathological analysis showed mostly uninjured brain tissue with only minor perivascular edema in cortico-subcortical areas after resuscitated HS in pigs with underlying atherosclerosis. Immunohistochemistry showed that (i) CSE, CBS, OT, OTR and the GR were present in the PVN and that (ii) $Na_2S_2O_3$ administration did not lead to significant differences in neuro-histopathological findings and protein levels of CSE, CBS, OT, OTR, GR, GFAP nor nitrotyrosine in the PVN compared to vehicle treated animals.

3.3 Septic Shock

The results of the septic shock project below are taken from Merz *et al.* [178]. Only abbreviations and figure references are adapted for compatibility with this thesis. This manuscript was published under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).
IHC revealed the presence of the OTR protein and its expression in septic hearts was significantly reduced in comparison to sham animals ($p = 0.001$) (see figure 17). GR protein was also expressed in the heart and reduced in sepsis ($p = 0.059$) (see figure 18).

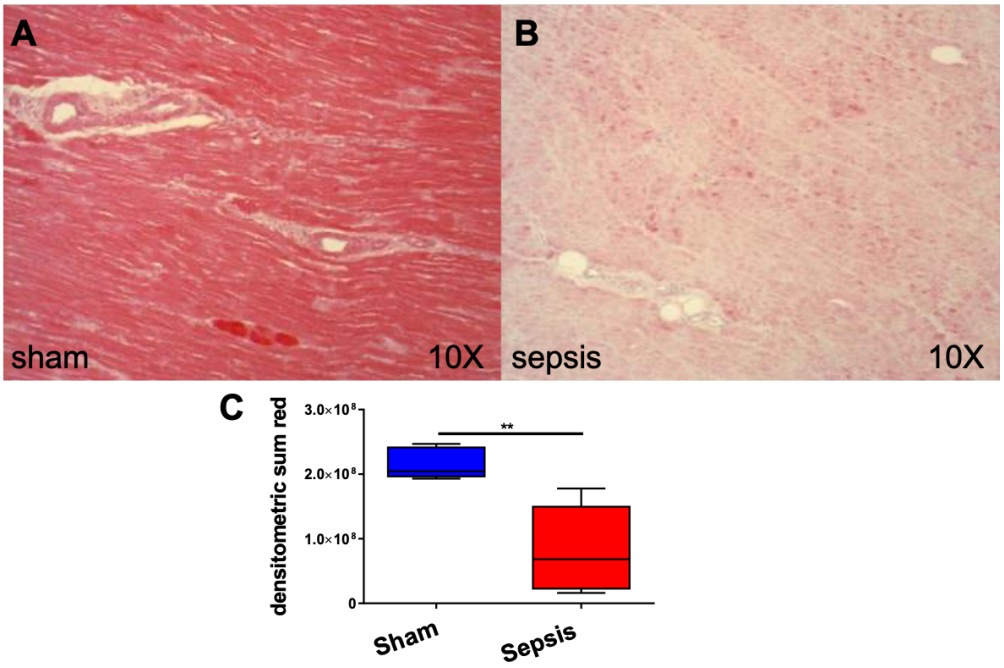

Figure 17: Myocardial OTR expression. (A) shows an example of immunohistochemical staining of OTR in a sham animal and (B) shows an example of immunohistochemical staining of OTR in a septic animal. (C) displays the quantification of the immunohistochemical stainings as densitometric sum (red), sham: n = 5, sepsis: n = 8. Boxes represent the interquartile ranges with the median indicated by a black line, whiskers represent minimum and maximum values. **p<0.01. The figure and the figure legend are taken from Merz *et al.* [178] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

Taken together, in the atherosclerotic model of septic shock (i) the OTR and GR were present in the porcine heart, (ii) septic shock lead to a significant loss of OTR and GR in cardiac tissue [178].

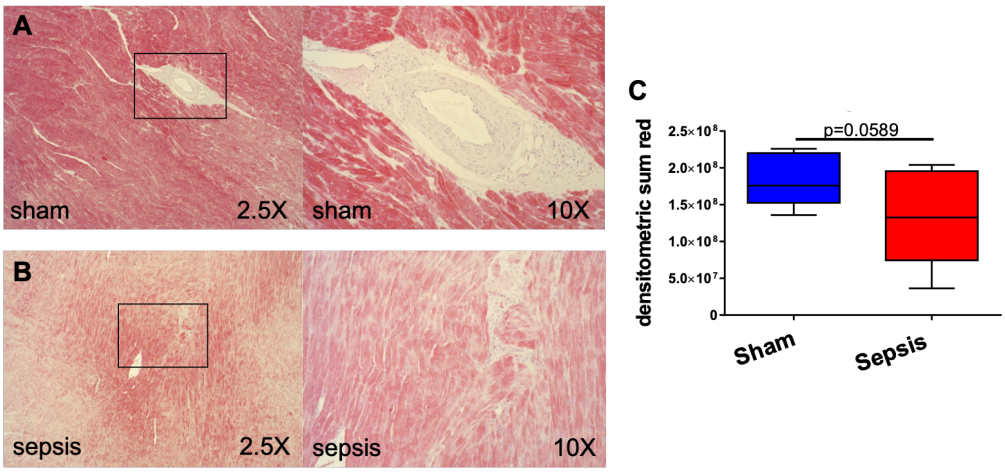

Figure 18: Myocardial GR expression. (A) shows examples of immunohistochemical staining of GR in a sham animal at 2.5X and a higher magnification of an arteriole. (B) shows examples of immunohistochemical staining of GR in a septic animal in two different magnifications. The black boxes indicate the location where the higher magnification picture was taken. (C) displays the quantification of the immunohistochemical stainings as densitometric sum (red), sham: n = 5, sepsis: n = 8. Boxes represent the interquartile ranges with the median indicated by a black line, whiskers represent minimum and maximum values. The figure and the figure legend are taken from Merz *et al.* [178] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

4 Discussion

4.1 Antibody Validation

This thesis aimed to identify the H_2S and OT systems in the porcine heart and brain. Compared to rodents, there is a lack of established antibodies for the pig in general and in particular for the porcine brain [264]. Thus, within the scope of this thesis, antibodies made to human immunogen sequences, targeting the H_2S and the OT systems, injury markers with relevance to pathophysiological changes, inflammation and edema, were validated successfully for immunohistochemistry on porcine tissue (CSE, CBS, OT, OTR, GR, C5aR, iC3, C1QBP, IL-6R, TNFR1, TNFR2, NeuN and AQP4) [61].
In order to confirm the specific reactivity of the antibodies, the human immunogen sequences of the antibodies were compared to the sus scrofa target protein sequences by NCBI BLAST searches (courtesy of the U.S. National Library of Medicine) [61]. Antibodies with a homology above 60% and a query cover above 90% were considered for investigation [61]. For further validation of the specific reactivity of the antibodies, negative controls were performed: pre-absorbing of the antigenic site with the respective peptide when available or incubation without primary antibody to control the secondary system (for example CSE see [61], supplement). The specific detection of the OTR in the brain *via* immunohistochemistry has been a particular challenge in the past. Therefore, the specific reactivity of the OTR was validated within the scope of the ASDH project by different negative (Wigger *et al.* OTR knock out mice [281]) and positive controls: the immunoprecipitated OTR was detected by the OTR antibody in western blot analysis and showed one explicit single band, which confirmed the specificity of the OTR antibody (see [61], supplement). Immunohistochemistry allowed for detailed detection of region and cell-specific expression patterns in the global landscape of the brain, which would have not been possible by analysis that require homogenization of tissue specimens.

4.2 Interaction of Hydrogen Sulfide and Oxytocin in the Heart and the Brain

After this extensive validation of antibodies, it was possible to determine that the H_2S and the OT systems were expressed in a variety of cell types and regions of the porcine brain. CSE and the OTR were present in similar cell types as in the human brain [59, 61], which is salient since there are significant differences in expression patterns of CSE and the OTR between rodent and large animal models/humans. Analysis of the human brain show higher CSE activity than murine CSE activity [64]. In the porcine brain, CSE was expressed in cortical neurons, in hippocampal neurons and granular neurons [61] and in purkinje cells of the cerebellum [59]. These findings are in line with the literature on human CSE expression: analysis of the human brain show CSE messenger ribonucleic acid (mRNA) in human cortical and midbrain neurons, pyramidal neurons of the CA3 region in the hippocampus and in cerebellar purkinje cells [64, 209]. Furthermore, CSE was present in the SMCs of pial arteries and in the endothelium of the microvasculature [61]. In piglets, immunohistochemical expression of CSE is reported in penetrating vessels and pial arterioles, located in endothelial cells of the cerebral vasculature [141, 208]. In human brain specimens, CSE was expressed in SMCs of the cerebral arterioles [140], supporting the findings above.

There are differences in cerebral OTR expression patterns comparing rodents and humans; even among rodent species, there are marked deviations in OTR localization in the forebrain comparing mice, rats and rabbits [84, 118, 289]. In the hippocampus for example, the OTR is present in rodents [289], whereas hippocampal OTR could not be detected in the human brain [4, 26]. In the porcine brain, the OTR was expressed in cortical neurons [61] and magnocellular neurons of the hypothalamus [59, 60]. In the vasculature, the OTR was expressed in SMCs and the endothelium of the pial arteries [61]. Characteristically, the OTR showed a 'blushing' expression pattern right around the microvasculature in the porcine brain in injured areas [61]. These findings are in line with reports on human expression patterns of the OTR: Boccia *et al.* reports OTR expression in cortical neurons and in the hypothalamus [26] and McKay *et al.* shows OTR expression in vascular profiles [173]. Furthermore, the OTR was expressed in

neurons and granular neurons of the hippocampus [61] in the porcine brain. This is in contrast to the literature: hippocampal OTR could not be detected in the human brain [4, 26]. This discrepancy of result might be due to the fact that Boccia *et al.* only analyzed two female human (adult) brains of which very little information was provided regarding the quality of the autopsy material [26]. In the present study, five female and four male (castrated) adult pig brains, which were properly fixed and processed, have been analyzed [59–61].
CBS was absent in cortical neurons [61], in contrast to the literature, where it is reported that CBS is expressed in neurons of uninjured newborn piglets (up to five days old) [141, 208]. However, in this ASDH project, adult swine were examined, and thus, the findings from Leffler *et al.* and Patel *et al.* might not be representative for an adult, fully developed porcine brain, since protein expression patterns can change during development [128, 141, 208]. Analysis of CBS expression in the human brain underscore that its expression pattern changes with age and injury [128]. Taken together, this thesis identified that CSE and the OTR were present in similar cell types as in the human brain, underscoring the relevance of the pig as an animal model that mirrors the cerebral human expression patterns of the H_2S and OT systems [59, 61, 169]. With regards to the expression of the endogenous H_2S producing enzymes in peripheral organs, it has been shown that the pig is closer to humans than mice [29, 169, 181].
Based on the findings from Merz *et al.* [179] and Wigger *et al.* [281], in that CSE and the OTR were reduced in the murine heart after physical and psychological trauma, their expression patterns were analyzed in the heart of the porcine model of septic shock with underlying atherosclerosis, since septic shock can develop after physical trauma and is often accompanied by myocardial dysfunction [15, 77]. The parallel decrease in cardiac CSE and OTR after trauma in rodent models [179, 281] was confirmed to be also the case in porcine septic cardiomyopathy [178, 199], which is of greater translational value to the human clinical situation. Reduced cardiac CSE and OTR expression coincided with reduced GR expression in comparison to sham-operated animals [178, 199]. This study was the first to show cardiac GR expression in relation to sepsis-induced myocardial dysfunction [178] and suggests that CSE, OTR and the GR might interact in the pathophysiology of cardiac dysfunction in response to sepsis [178]. In fact,

GR signaling has been reported to play an important role in anti-inflammatory responses to sepsis and proper heart function and is therefore crucial for survival [133, 178, 201, 260, 262]. Impaired GR signaling has been reported to worsen organ dysfunction and hemodynamic instability in a rodent model of endotoxic shock [278]. In the liver for example, a downregulation of the GR during sepsis contributes to organ dysfunction [125]. These findings support that the downregulation of the GR in the heart after septic shock contributed to myocardial dysfunction [178]. Interestingly, CSE has been reported as a regulator for GC/GR signaling [255], which is also important for heart development and functioning [201]. Furthermore, CSE and CBS are important for adequate GC production in the adrenal gland in the course of sepsis [265] underscoring their important intercommunication in sepsis-induced heart injury [178].

Wang *et al.* proposed that CSE regulates the OTR expression in the heart in function- and tissue-dependent ways *via* the RISK pathway in atherosclerosis [267]. In the septic heart, parallel reduction of CSE, OTR and GR expression was associated with lower signaling of GR related genes, and lower nuclear factor (erythroid-derived 2)-like 2 (Nrf2) mRNA [178]. Nrf2 is a common antioxidant downstream signaling molecule of the RISK pathway, which can be activated by both, CSE and the OTR through pro-survival kinases of the RISK pathway (see figure 19 [60]) [48, 60, 147, 267, 270, 286, 294]. Among them phosphoinositide 3-kinases (PI3K)/protein kinase B serine/threonine-specific protein kinase (Akt) and extracellular regulated kinase 1/2 (ERK1/2) [48, 106, 138, 267, 290]. PI3K/Akt and ERK1/2 can activate eNOS, which endogenously produces NO in the endothelium, which can in turn stimulate CSE activation [267, 270, 294]. Cardiac CSE was reduced in protein level, but CSE mRNA was upregulated [178]. CSE mRNA upregulation might be an effort to restore the loss of CSE protein and could be a compensatory mechanism to restore local H_2S levels to act on vascular tone.

In fact, nitrotyrosine, a marker of nitrosative and oxidative stress, was upregulated in the septic heart, indicating increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) and dysfunctional NO signaling [178, 181, 199], which is in line with the findings of reduced eNOS expression in the coronary arteries of atherosclerotic swine after sepsis [181] and reduced blood levels of NO in the atherosclerotic pigs and patients [113, 168, 181]. The dysfunctional

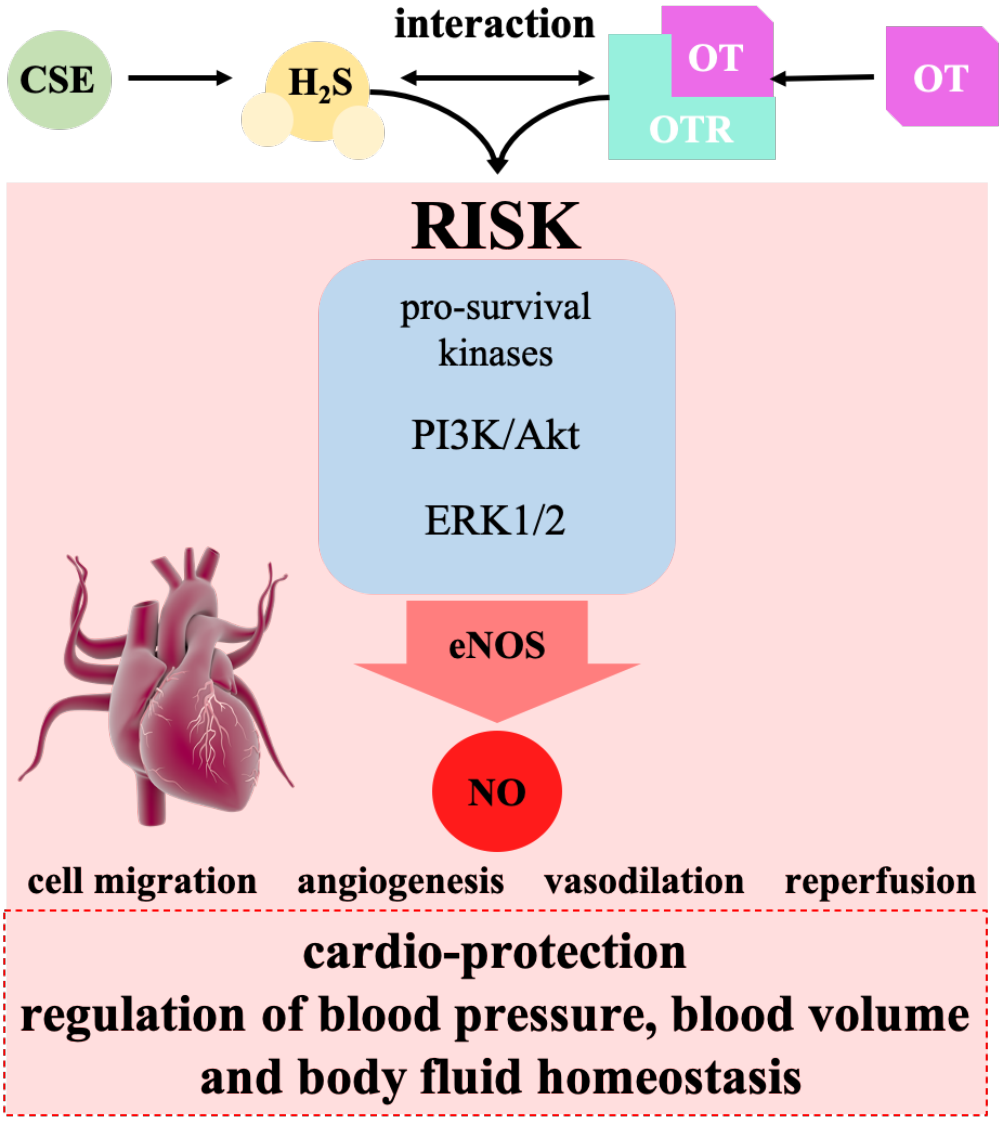

Figure 19: Cardio-Protective Interaction of H_2S and OT *via* the RISK Pathway. Immediate with reperfusion after ischemic events, H_2S, mainly produced by CSE in the heart, and binding of OT to the OTR can activate pro-survival kinases of the RISK pathway: PI3K/Akt and ERK1/2. Both can stimulate NO production trough eNOS. NO release initiates: cell migration, angiogenesis, vasodilation, reperfusion leading to a series of cardio-protective events as, regulation of blood pressure, blood volume and body fluid homeostasis. The figure is modified from Denoix *et al.* [60] under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).

NO signaling and reduced CSE levels in concert with reduced OTR, GR expression and decreased Nrf2 mRNA indicates failing antioxidant mechanisms and impaired regulation of vascular tone in small and large vessels contributing to the pathophysiology of septic cardiomyopathy in patients with underlying atherosclerosis [178, 181, 269, 270, 281, 285]. Rodents, and in particular rats, have high levels of circulating NO, which would distort the translation of pre-clinical results into the clinics, especially when analyzing interactions with gaseous mediators [222]. Rodent studies of lipopolysaccharide-induced sepsis report NO metabolite levels of up to 400-800 μMol [222]. Septic patients can have a concentration of NO metabolites ranging between 20 and maximal 187 μMol [222, 280]. Thus, results from septic rat models will probably only result in benefits for a rat. The FBM pigs, resemble a biomarker pattern that is similar to patients with hypercholesterolemia-induced atherosclerosis, including reduced blood levels of NO [168, 181]. Therefore, the comparable circulating NO levels in atherosclerotic patients and FBM pigs are of clinical relevance, considering NO-mediated vasoactive effects of the H_2S and OT systems. In the septic shock project, full-scale intensive care was implemented in the experimental set-up and provided an often missing component in pre-clinical research. Hollenberg *et al.* demonstrated in 2001 that standard intensive care procedures as fluid resuscitation and administration of antibiotics elevated survival to 46% in contrast to 0% survival in non-treated septic mice [112]. The human-like intensive care measures add significant translational value to the potential interaction of CSE, OTR and the GR systems in septic shock-induced inflammation, oxidative and nitrosative stress and finally myocardial dysfunction [178].

In the HS project, full-scale intensive care [55] apparently prevented neuro-histopathological injury after HS in atherosclerotic swine. Neuro-histopathological evaluation on hematoxylin and eosin stained brain tissue samples was performed in order to detect potential HS-induced hypoxic events in cortico-subcortical areas, in collaboration with an experienced neuro-histopathologist (Dr. Angelika Scheuerle, Division of Neuropathology, Institute for Pathology, Ulm University Medical Center). Main criteria of evaluation were: presence of eosinophilic cells, morphology of neuronal and glial cells, signs of edema, and necrosis. The evaluation of cortico-subcortical brain regions revealed only minor neuro-histopathological injury with minor perivascular edema after

HS, which is in line with the few reports on comparable experiments [36, 282, 296]. Studies of HS with a particular focus on neuro-histopathology and cerebral protein expression changes after profound systemic hypoxia are limited in large animals. The existing studies on large animals with HS-induced brain damage either lack advanced intensive care [36] or combine HS with either TBI or cardiac arrest [196, 233, 282, 296]. Cardiac arrest creates a transitory 'no flow' compared to a 'low-flow' situation in HS. A combination of cardiac arrest and HS is thus not comparable to the results of HS alone [196, 233]. A study from Bronshvag *et al.*, comparing un-anesthetized sheep with HS to control animals, showed no anoxic neurons, no pathological changes and minimal to no vacuolization (as a measure for neuronal damage) after HS either [36]. Wisner *et al.* and Zhuang *et al.* performed combined experiments but respectively analyzed a subgroup of sheep and swine that only underwent HS under anesthesia [282, 296]. Wisner *et al.* found no increase in brain water content, a measure for brain swelling, in sheep subjected to HS [282]. Zhuang *et al.* found minor accumulation of polymorphonuclear neutrophils (PMNs) and injured neurons in the cerebral cortex and no increase in brain water content compared to controls [296]. Hence, reports on HS alone in large animals describe no major pathophysiological brain injury and swelling [36, 282, 296] and are in line with our findings of minor neuro-histopathological injury in the HS model.

In vitro and *in vivo* studies in rodents suggest that H_2S, OT and the GR systems may interact on hypothalamic level in response to systemic fluid shifts [50, 51, 225, 226]. Therefore, CSE, CBS, OT, and the OTR were analyzed in our model and were found to co-localize in the magnocellular neurons and to be present in the microvasculature and parenchyma of the hypothalamus [59, 60]. The endogenous H_2S producing enzymes within the hypothalamus are more active after water deprivation in rats, leading to more secretion of OT [50]. Another study in hypothalamic explants from rats showed that in response to an acute osmotic stimulus (hypertonic solution), H_2S positively regulates OT [51]. Hence, the co-localization of CSE, CBS, OT, and the OTR in the magnocellular neurons might be indicative for their interaction in response to systemic hypovolemia, hypotension and fluid tonicity changes, regulating systemic fluid homeostasis [60]. Moreover, there is evidence that OT and H_2S can both induce the release of adrenocorticotropic hormone (ACTH) (from the pituitary gland), a precursor

of GR signaling [16, 185, 190]. Furthermore, GCs can decrease OT release in response to systemic osmotic pressure and volume alterations [225] and CSE can regulate GC signaling [255]. As expected, the GR was also present in the hypothalamus, although in low levels, after resuscitated HS in atherosclerotic swine. Thus, the H_2S and OT systems might act in concert with the GR/GC system to restore systemic blood volume after HS [60].

FBM pigs and atherosclerotic patients have a vascular CSE deficiency and thus, may particularly benefit from exogenous H_2S administration [158, 181, 271]. Since the purpose of this HS study was to investigate the effects of $Na_2S_2O_3$ treatment, no control animals without HS were included, thus no conclusions on the regulation of endogenous H_2S producing enzymes, OT and the OTR compared to the basal state can be drawn. In the porcine HS model, there was no effect of intravenous $Na_2S_2O_3$ administration, neither on tissue morphology in cortical and subcortical regions, nor on CSE, CBS, OT, OTR, GR GFAP and nitrotyrosine expression within the PVN. The previously validated antibodies served as valuable tools, to answer whether $Na_2S_2O_3$ had an effect in the porcine hypothalamus [61]. Nevertheless, these findings suggest that either the analyzed brain regions did not reveal any of the cerebral $Na_2S_2O_3$-mediated effects, which were previously shown in rodents [50, 162], or that $Na_2S_2O_3$ might have not crossed the BBB. These findings are in contrast to the results in un-resuscitated rodent studies: in young, healthy, male, un-resuscitated rodents, intraperitoneal $Na_2S_2O_3$ administration has been reported to be neuro-protective after cerebral I/R injury [162]. Nevertheless, the obtained results are in line with results from clinical studies, which report a lack of effect of systemic $Na_2S_2O_3$ administration within the brain (with intact BBB) [193, 194]. A five-fold higher dose of $Na_2S_2O_3$ compared to the applied dose in the HS model (0.1 g kg-1 h-1) could not cross the intact human BBB [193, 194].

In humans, $Na_2S_2O_3$ only showed an effect on the brain when the BBB was chemically disrupted before $Na_2S_2O_3$ administration [193, 194]. Regardless of the $Na_2S_2O_3$ administration, blood transfusion, fluid resuscitation and vasopressor support prevented, HS-induced brain tissue damage, BBB disruption and alterations on protein level. Furthermore, the neuro-histopathologic evaluation found no evidence of BBB disruption. In agreement with our findings, Meybohm *et al.* reports that resuscitation preserved the BBB integrity in mice and pigs even

after cardiac arrest [183]. The implementation of resuscitative measures in rodent studies would therefore most likely preserve BBB integrity and might thus result in impermeability of the BBB for $Na_2S_2O_3$ as well. It is known that pathophysiology differs between injury models without and with full-scale intensive care [251] and ICU treatment already belongs to the standard first-line therapy in humans. Thus, the lack of implementing intensive care management in most rodent brain injury models might contribute to the failure of translation into the clinic [66, 200].

The pig brain is similar in size and anatomy to the human brain, which allowed specialized neuro-intensive care in the ASDH model, according to the current guidelines of clinical TBI treatment without requiring specialized miniaturized equipment, and prevented neurological dysfunction after acute brain injury [44, 56]. The similar brain anatomy of pigs and humans, makes the adult pig a more clinically relevant animal model in pre-clinical ASDH research compared to rodents. It was particularly salient that the gyrencephalic anatomy allowed to show for the first time an injury pattern at the base of the sulci, which has been so far only identified in humans [32, 87]. The spatial injury pattern at the base of the sulci in our ASDH model resembled the human injury pattern in response to ICP increase: perivascular swelling in both hemispheres, barrier dysfunction and oxidative stress in the injured brain hemisphere and in particular at the base of the sulci [56, 61, 261]. Furthermore, the neuronal marker for post-mitotic neurons (NeuN) showed a similar expression pattern as in human TBI: high NeuN signal was observed in areas where cytoarchitecture was intact and low expression of NeuN was associated with loss of cytoarchitecture [61]. The cerebral water channel protein AQP4 showed a similar expression pattern as NeuN. AQP4 was most abundant where tissue integrity was intact and its expression declined with impaired cytoarchitecture [61]. The porcine ASDH model might thus be a clinically relevant pre-clinical animal model to further examine acute brain injury and potential treatment strategies [61, 261].

The gyrencephalic brain of pigs and humans obviously differs fundamentally from the lissencephalic rodent brain (see figure 7) [66, 170, 264]. This is particular salient with regards to ICP increase after ASDH, since rodent lissencephalic brains, lacking gyri and sulci, present with even distribution of pressure, whereas in the gyrencephalic brain, the bases of the sulci are most vulnerable to ICP

increase, since this is the area where the maximal mechanical pressure increase first occurs (see figure 20) [132, 264].

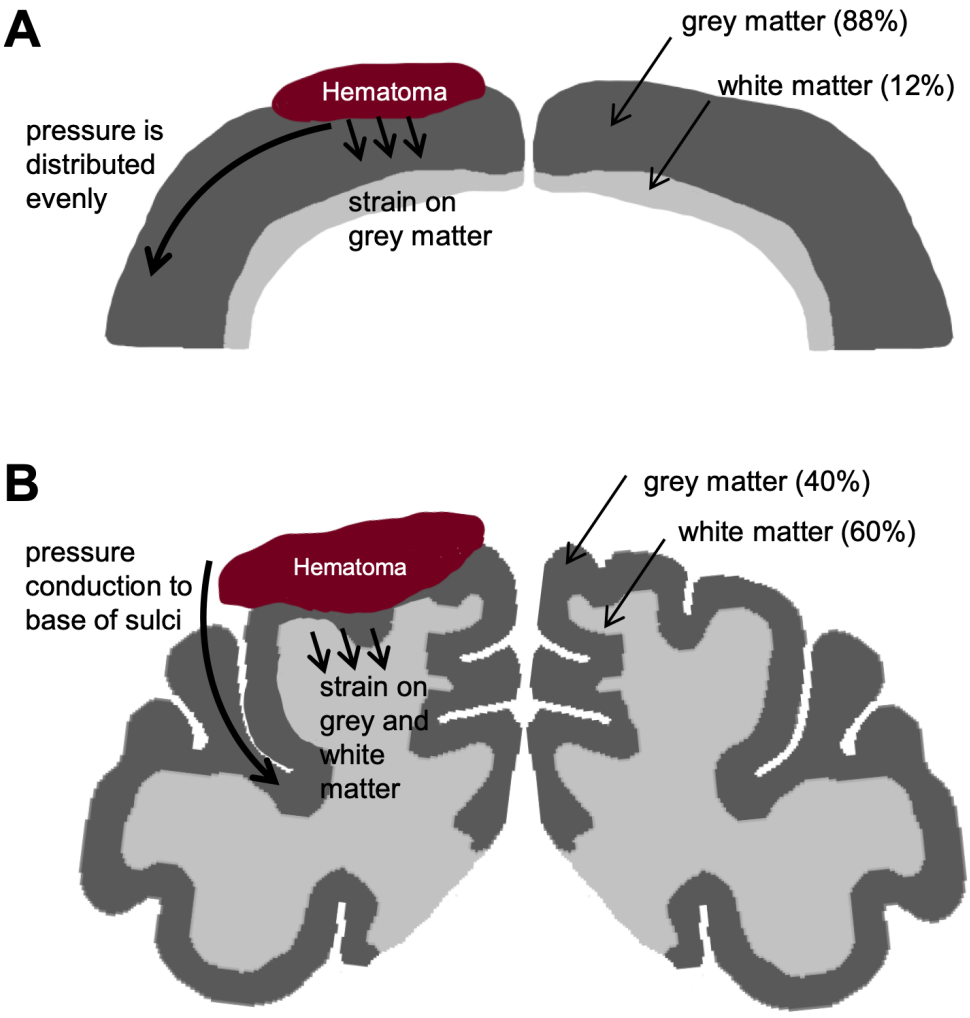

Figure 20: Pressure distribution in the cerebral cortex after ASDH. (A) In the lissencephalic cerebral cortex, pressure increase in response to ASDH is distributed evenly. (B) In the gyrencephalic cerebral cortex, pressure increase in response to ASDH is conducted along the sulci, with maximum pressure at the base of the sulci. The size of the lissencephalic brain in comparison to the gyrencephalic brain is overestimated. The figure and the figure legend is taken from McCook *et al.* [170] under the Creative Commons Attribution- NonCommercial-ShareAlike 4.0 License (CC BY-NC-SA 4.0) (https://creativecommons.org/licenses/by-nc-sa/4.0/).

The pressure distribution is also affected by the structures surrounding the brain. Pigs and humans share a very rigid tentorium cerebelli, which separates the cerebrum from the cerebellum [264]. In contrast, in rodents, the tentorium cerebelli is an expansible tissue membrane, which allows the pressure to distribute among cerebrum and cerebellum, whereas in the gyrencephalic brain, pressure increase

is confined to the cerebrum (see figure 21) [61, 264] and leads to hypoxia-induced injury at the base of the sulci [32, 87].

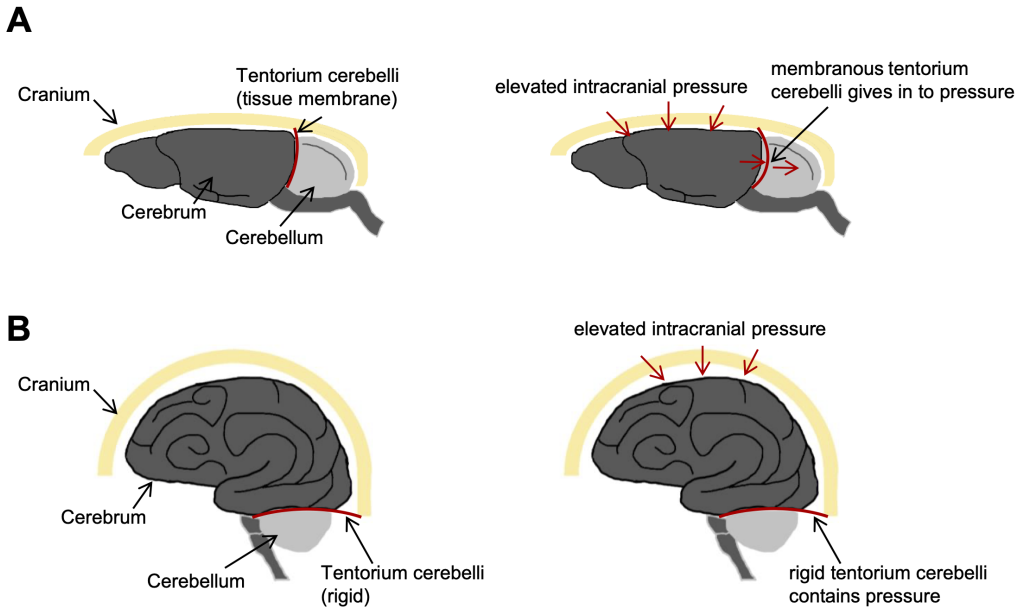

Figure 21: Schematic depiction of the brain within the skull. (A) In the rodent brain, the vestigial tentorium cerebelli can mitigate an elevation of intracranial pressure. (B) In the human (and porcine) brain, elevated intracranial pressure is confined to the cerebrum by the rigid tentorium cerebelli. The size of the rodent brain in comparison to the human brain is overestimated. The figure and the figure legend is taken from McCook *et al.* [170] under the Creative Commons Attribution- NonCommercial-ShareAlike 4.0 License (CC BY-NC-SA 4.0) (https://creativecommons.org/licenses/by-nc-sa/4.0/).

Hence, brain injury due to a space-occupying lesion and ICP increase is closer reflected in the porcine gyrencephalic brain compared to the lissencephalic rodent brain. Furthermore, adults rats have approximately only 12% white matter in relation to total brain matter, whereas adult pigs and humans share a proportion of 60% white matter [132]. This is not trivial for translation, inasmuch as the white matter is more prone to develop edema along axonal tracts due to lower interstitial flow resistance [207]. Thus, edema formation in response to ASDH might be closer mimicked in the pig compared to rodents [264] and allows for observations that closely mirror human edema formation along white matter tracts and around the cerebral vasculature [66, 264].

Exactly where pressure-induced injury is expected at the base of the sulci of the human brain [32, 87, 264], CSE, CBS, OT and its receptor were expressed in the porcine ASDH model [61]. The detection of the pressure-induced injury and the

expression patterns of the H_2S and the OT systems at the base of the sulci would not have been possible in a rodent due to their lissencephalic brains [61]. The perivascular swelling, barrier dysfunction and oxidative stress at the base of the sulci was accompanied by reduced CSE and high OTR, OT and CBS expression in the injured brain hemisphere [61]. In the uninjured brain hemisphere, CSE showed constitutive expression in the parenchyma, cerebral vasculature and in cortical neurons within the gyri [61]. The presence of CSE in the parenchyma is not commonly reported, wherefore it was essential to thoroughly validate the anti-CSE antibody (see [61], supplement) to assure specific detection [61]. The constitutive expression of CSE in the uninjured hemisphere is in accordance with the literature, since it is reported that H_2S has anti-oxidant properties and CSE expression has positive effects on barrier integrity [126, 240, 274, 275], suggesting that constitutive CSE expression is associated with normal barrier function and intact brain tissue [61], as it is reported for the human brain [140].
Interestingly enough, reduced CSE expression in the injured brain hemisphere of the ASDH model [61] was associated with albumin extravasation, indicating barrier dysfunction and nitrotyrosine formation, indicating oxidative and nitrosative stress [56]. Under normal conditions, albumin is contained within the vascular compartment and is absent in brain tissue and cerebro-spinal fluid. When the BBB breaks down, large blood-borne molecules as albumin can invade into the brain parenchyma and change the osmolarity between compartments. Albumin extravasation is therefore associated with electrolyte and water movements in the cerebral fluid environment and leads to edema formation [233]. Thus, the absence of CSE [61] and the concurrent albumin extravasation in the injured hemisphere [56] indicate barrier dysfunction and subsequent fluid shifts from the vascular compartment into the interstitial and intracellular compartment leading to cerebral edema formation and swelling [56, 61, 233, 280]. In addition to massive albumin extravasation around the site of injury, albumin was present further away from the injury in the cytoplasm and in nuclei of morphologically uninjured neurons [61]. This has not been described in ASDH before, and could be a protective mechanism, since endogenous cerebral albumin production in reaction to brain injury has been described before as being neuro-protective [6, 218]. Moreover, it is reported by Tabernero *et al.* and Johnson *et al.* that neurons and astrocytes can take up blood-borne albumin in the case of BBB

breakdown, leading to increased glutamate release [127, 246]. In turn, the release of glutamate stimulates astrocytes to clear out excess glucose, which can be elevated as a consequence of brain injury. Thereby, the albumin-induced glutamate release and the subsequent astrocytic glucose uptake can contribute to the regeneration of a normal extracellular environment after trauma [63, 137, 213]. Sharma *et al.* studied piglets after cardiac arrest and thoroughly analyzed albumin expression patterns in the brain [233]. In line with our findings, these authors not only detected leaked albumin from vessels, but also showed distinct albumin-positive neurons in different brain areas [233]. The spatial localization of albumin is significant in starting to understand the role of albumin in the pathophysiological changes after brain injury, and indicates that albumin plays a far more important role than previously assumed, not only serving as a marker for BBB dysfunction.

The bases of the sulci in the injured hemisphere showed significantly higher GFAP [56] and more pronounced OTR expression, especially in, and right around the the vessels, suggesting that the OTR plays an important role in the cerebral vasculature when it comes to ischemic events at the base of the sulci [32, 61, 87, 264]. These findings are in line with the findings from McKay *et al.*, who reported an upregulation of the OTR and GFAP in and around vascular profiles in the prefrontal cortex due to reduced cerebral blood perfusion in humans [173]. The OTR was also present in pial arteries and the microvasculature, suggesting that the OTR might also contribute to BBB integrity [173] and regulation of local cerebral blood flow [61]. Higher expression of CBS, OT and the OTR in the injured hemisphere and in particular in the vasculature, might indicate a compensatory mechanism for the lack of CSE in response to ASDH-induced barrier dysfunction and fluid shifts, which cause edema formation and swelling [61]. The presence of the OTR in close association with ischemic events and CSE expression in the uninjured cerebral vasculature in the porcine brain, is similar to the human brain and thus further strengthens the translational value of these novel findings. Moreover, underscoring the clinical relevance of this porcine ASDH model to study therapeutic interventions.

When characterizing the immune response in the brain after ASDH, identical inflammatory patterns were present in the pig brain compared to what is reported in humans regarding the adaptive immune system and the complement system

[61]. It is known from humans that IL-6 plasma levels rise in the initial course after closed head trauma and that the IL-6 plasma levels correlate with the lesion severity assessed by computer-tomography scans [13]. Trautz *et al.* and Holmin *et al.* showed by immunohistochemistry and and *in situ* hybridization that an increase in IL-6 and GFAP in pericontusional zones was associated with fatal traumatic changes in the human brain [253]. In the porcine ASDH study, we showed for the first time that the IL-6R was expressed in the parenchyma, in neurons, the vasculature and in immune cells in close proximity to injury [61]. Interestingly, moving away from the areas of ASDH-induced injury, the expression of the IL-6R is more limited and confined to the cytoplasm of neurons [61]. Reports on human IL-6R expression in the brain are limited to results from Dame *et al.* and Hampel *et al.* [53, 99]. Dame *et al.* found IL-6R immunoreactivity in neurons and in the neuropil of fetal brain tissue and in pyramidal neurons in the cerebral cortex in the adult human brain [53]. Using immunohistochemistry, Hampel *et al.* showed the IL-6R and its ligand expressed in normal brain tissue in all regions of the human brain in autopsies from Alzheimer's patients [99]. The neuronal IL-6R expression is in line with reports from Dame *et al.* and Hampel *et al.* [53, 99]; presence of the IL-6R in the vasculature and in immune cells is in line with the literature on rodent IL-6R expression patterns [25, 163, 250]. The porcine ASDH model allowed to identify the spatial expression pattern of the IL-6R, which appears to closely mimic the human situation and widens the knowledge of the pathophysiological changes after ASDH. It is also known that levels of the pro-inflammatory cytokine TNFα are elevated in the brain after human TBI [102]. In the ASDH model, both receptors of TNFα: TNFR1 and TNFR2 were successfully detected after ASDH in the porcine cerebellum and brainstem, differentially expressed in the cytoplasm and nuclei of neurons [61].
In concert with the adaptive immune system, activation of the complement system is reported after TBI and contributes to the neuro-inflammation mediated neuropathology and potential BBB dysfunction [98, 224, 239]. The C5aR showed positivity in infiltrating immune cells and surrounding the microvasculature in areas of intraparenchymal bleeding [61], which supports that the C5aR is involved in TBI-induced barrier dysfunction [149, 159]. It remains speculative, whether the C5aR positive immune cells originated from the intraparenchymal bleeding or endogenously from the brain. In line with our findings, Gasque *et*

al. showed in the human brain that the C5aR is localized to inflammatory cells and around the blood vessel wall and is up-regulated in endothelial cells of the cerebral vasculature [81]. These authors analyzed human brains and showed complement activation without traumatic injury but due to neurodegenerative diseases, meaning that there could be an endogenous component of complement activation separately from the intraparenchymal bleeding due to ASDH [81]. Cortical neurons, further away from the injured brain tissue in our study, were also C5aR positive [61], which is in agreement with reports on normal human brain, showing constitutive C5aR mRNA expression in cortical neurons by *in situ* hybridization [202]. The complement component iC3 showed a different pattern compared to the C5aR. In close neighborhood to the ASDH, where intraparenchymal bleeding was present, iC3 was positive in the parenchyma, reflecting injury-associated complement activation [61]. C1QBP was detected in the endothelium and the SMCs of the microvasculature, in infiltrating immune cells and the parenchyma in areas of bleeding and in neurons in close association to the ASDH induction [61]. Interestingly enough, in the very same region, CSE was also positive in infiltrating immune cells and neurons in areas of bleeding, but almost absent in the microvasculature and the surrounding parenchyma [61]. H_2S derived from L-cyteine, could downregulate complement component 1q (C1q) mRNA expression in the cortex of neonatal mice after hypoxic-ischemia *in vivo* and prevented neuronal apoptosis *in vitro* by promoting a shift of microglia to the M2-phenotype *via* the adenosinmonophosphat-activated protein kinase (AMPK) pathway [295]. The absence of CSE in the microvasculature and the parenchyma in an area where C1QBP is markedly expressed may indicate a lack of inhibitory function of H_2S on C1QBP [61, 295]. Taken together, the neuro-inflammatory and complement activation in response to ASDH showed similar patterns compared to humans [61, 156], which further underscores the clinical relevance of this pre-clinical model.

Human post-mortem immunohistochemical studies on protein expression changes after human brain injury are scarce due to multiple simple reasons: human brain tissue from critically ill patients is rare since brain surgeries must be as minimally invasive as possible, patient characteristics and reasons of death are very diverse, immediate and adequate fixation of tissue can not be guaranteed in many cases, and the effort to get ethical approval and informed consent to

process human post-mortem brain tissue is a hurdle. Therefore, the pig represents a significant animal model, close to humans, to perform reproducible imunohistochemical analysis of pathophysiological changes in the brain after injury.

4.3 Future Directions

In general, there is a dearth of biological understanding in relevant interdependencies of body and mind. The H_2S and OT systems could be of great impact in tackling some of the unresolved connections of body and mind due to their parallel roles in physical and psychological trauma [60, 179, 281].
The atherosclerosis in the FBM model originates from hypercholesterolemia and feeding a high fat diet [55]. Nevertheless, the FBM model could be a meaningful 'tool' to study psychological trauma-induced chronic cardiovascular diseases, due to the fact that ELS and psychological diseases in adulthood are considered as significant risk factors for the development of chronic cardiovascular co-morbidities in humans as recently reviewed in McCook *et al.* [9, 17, 54, 71, 73, 110, 152, 167, 171, 176, 257, 267]. For example, child maltreatment led to a downregulation of the OTR in peripheral blood mononuclear cells (PBMCs) of women short after giving birth [136]. A downregulation of the OT/OTR system is associated with dilated cardiomyopathy [94], cardiovascular dysfunction [121, 122], and may complicate the treatment when it comes to ICU admission of such patients due to physical trauma. Pre-clinical studies on the effect of exogenous H_2S administration in myocardial infarction revealed positive results as: attenuated myocardial inflammation and oxidative stress, preserved cardiac function, reduced infarct size and better cardiac function [47, 67, 79, 148, 243, 277]. In connection to the findings of this thesis, the loss of cardiac CSE/OTR in sepsis-induced myocardial dysfunction might be compensated by the supplementation of either one system (i.e. $Na_2S_2O_3$ or OT) to restitute endogenous H_2S/thiosulfate or OT availability and could potentially bring a benefit for cardiac function, especially for patients with underlying atherosclerosis [178]. Protein and gene expression analysis from the septic shock model contributed to the necessary understanding of underlying pathophysiological changes [178].

A further potential site of interaction of the H_2S and OT systems, connecting the heart and the brain, seems to be the parasympathetic vagus nerve, which fine-tunes the blood pressure regulation *via* the aortic baroreceptors (see figure 22) [167]. The vagus nerve bidirectionally connects the heart and the brain. Aortic baroreceptors sense the arterial pressure and signal to the dorsal motor nucleus (DMN), which is located in the brainstem and expresses CBS and the OTR [167]. Interestingly, the DMN is also innervated by the PVN, which is fundamental in maintaining the volume and osmolarity of body fluids and is a site for H_2S and OT interaction: OT, the OTR, CSE and CBS co-localize in the porcine PVN (looking at pigs from the HS project of this thesis) [59, 167].

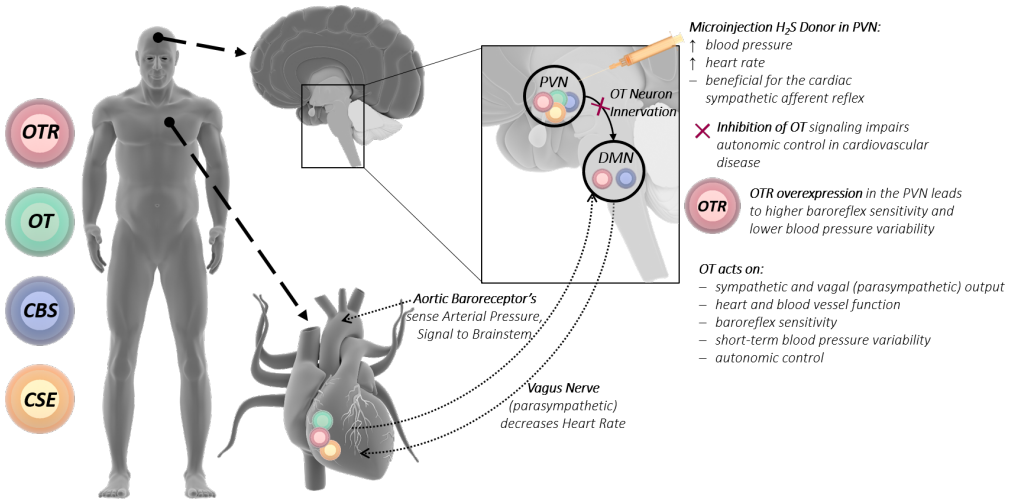

Figure 22: H_2S and OT directly influence the vagus nerve, connecting heart and brain. Baroreceptors in large arteries sense the arterial pressure and signal to the DMN of the brainstem. The DMN also receives input from OT neurons of the hypothalamic PVN, which centrally regulates cardiovascular homeostasis. The vagus nerve (parasympathetic) signals from the DMN to the heart and decreases the heart rate. Notably, the OTR, OT, CBS, and CSE are expressed within the PVN. The OTR and CBS are expressed in the DMN, while cardiac tissue expresses the OTR, OT, and CSE. These expression patterns suggest a mutual interplay of the OT and H_2S systems, connecting the heart and the brain. Pre-clinical studies show that acute trauma and/or chronic stress affects cardiac endogenous OT/R and CSE levels, thereby affecting physiological responses that influence the development of cardiovascular disease. The figure and the figure legend is taken from McCook *et al.* [167] under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license (https:// creativecommons.org/licenses/by/ 4.0/).

Baroreceptor sensitivity is promoted by OTR stimulation [24, 154] and Xiao *et al.* showed that exogenous H_2S could activate the carotid baroreceptor activity [284]. Moreover, Makarenko *et al.* and Xiao *et al.* showed that both pharmacological and genetic CSE inhibition leads to inhibition of carotid baroreceptor activity [157, 284].

Moreover, Medland *et al.* could show that early weaning stress in piglets (mirroring early life adversity in humans) induces long lasting pathophysiological

changes in the gastrointestinal tract, which increase the susceptibility to develop chronic gastrointestinal diseases in adulthood [174]. In rodents, Li *et al.* reported that exogenous H_2S administration protected the epithelium of the colon from maternal separated mice *via* antioxidative and anti-inflammatory actions [145]. Significantly, the above-mentioned stressed piglets, mimicked key pathophysiological responses that are known to be affected in human chronic gastrointestinal disorders [215]. Thus, it is likely that the effects of early life adversity would elicit a cardiovascular response in pigs, which closely reflects the human cardiovascular pathophysiology. Hence, such a porcine early weaning stress model could be of use to investigate biological mechanisms between ELS and the development of chronic cardiovascular diseases, as well as their contribution to patient outcome after circulatory shock and ASDH under ICU treatment, the significance of which was recently reviewed by Merz *et al.* [180].

In the HS project, OTR and CSE expression were reported for the first time in the porcine cerebellum and showed a reciprocal expression pattern [59]. In the human brain, the cerebellum is particularly responsive to strong emotions [91] and displays OTR expression alterations in psychological disorders [254]. Additionally, CSE was expressed in the porcine hypothalamus, where it co-localized with CBS, OTR and OT [60]. The fact that CSE and the OTR co-localize exactly in the hypothalamus where the stress response is regulated in the brain [60], suggests their relevance in biological mediation of psychological- and physical-induced stress responses [225, 278]. The reciprocal expression pattern of CSE and OTR in the cerebellum and the co-localizing expression pattern of CSE and OTR in the hypothalamus, suggests that the H_2S and OT systems may have varying interaction patterns in distinct brain regions (either acting in concert or in a compensatory mechanism). The localization of the H_2S and the OT systems in the prefrontal cortex of the ASDH model is salient with regards to TBI-induced psychological diseases and PTSD, since both TBI and PTSD are often associated with prefrontal cortex dysfunction [78, 166]. The H_2S and OT systems may contribute to a molecular biological environment in the prefrontal cortex for normal brain function [78], which may be altered in ASDH-induced PTSD. Mice with a deletion of 3MST reveal anxious behavior [192], and schizophrenic patients presented with an elevation in 3MST (which correlated with disease severity) and CBS gene expression in post-mortem brain tissue [117]. Ide *et al.*

suggests that a tissue-specific dysregulation of the endogenous H_2S producing enzymes manifests in "sulfide stress" and is associated with severe schizophrenic symptoms [117]. As mentioned in section 1.2, ICU admission of critically ill patients by itself can elicit PTSD symptoms [40, 223]. However, little is known about the underlying biological mechanisms and factors that contribute to the susceptibility to develop PTSD after ICU admission due to trauma. Therefore, early weaning stress in combination with ASDH in pigs might be an adequate experimental setup to identify how ELS influences the immediate post-traumatic response after acute brain damage and how it may predispose someone to develop PTSD symptoms after an ICU admission due to TBI. $Na_2S_2O_3$ did not affect the analyzed brain regions in the HS model, but might still be an attractive treatment in ASDH: firstly, since it might exert its neuro-protective effects on the brain due a mechanical disrupted BBB allowing $Na_2S_2O_3$ to enter the brain and, secondly due to its potential impact to re-establish the cerebral sulfide homeostasis [162, 193, 194]. Interestingly, clinical testing of intranasal OT has already been carried out in a pilot study in PTSD patients (Clinical Trials Registrary NCT03238924: 'Prolonged Exposure and Oxytocin') [76] and is being checked in a consecutive multi-center study (Clinical Trials Registrary NCT04228289: 'Oxytocin to Treat PTSD') [75].
There is growing awareness that the susceptibility to develop chronic cardiovascular diseases after ELS or PTSD after TBI is gender specific, shown in pigs and humans [174, 184, 215]. Hence, gender differences in these research fields make the data situation even more complex and warrant further investigation.

4.4 Conclusion

In conclusion, the long-term, resuscitated, porcine intensive care models evaluated in this thesis reflected well the human pathophysiology after circulatory shock and ASDH. The close homology of pigs and humans made it possible to validate the specific reactivity of antibodies for immunohistochemistry on porcine tissue, which were originally made to human immunogen sequences. These validated antibodies provide valuable tools for future in-depth injury characterization and therapeutic target identification. The porcine H_2S and OT system expression patterns in the heart and brain resembled human expression

patterns and extended the knowledge about their spatial changes in relation to barrier dysfunction, inflammation, and oxidative stress. This thesis confirmed that the pig is of greater translational value to the human clinical situation compared to rodents, in particular with regards to brain injury, to ask research questions, which are unobtainable in humans and allows to explore potential targets for improved therapies in circulatory shock and ASDH. Based on the findings of this thesis, the H_2S and OT systems may be implicated as molecular mediators in so far unrecognized converging biological mechanisms, connecting psychological trauma with chronic cardiovascular co-morbidities and long-term psychological consequences with acute brain injury.

5 Summary

Physical trauma, like circulatory shock and acute brain injury, are the most frequent causes for intensive care unit (ICU) admission and they share key pathophysiological events like barrier dysfunction, inflammation, and oxidative stress. ICU patients with chronic cardiovascular co-morbidities, often need prolonged ICU treatment and hospitalization and have a higher morbidity and mortality. There is growing awareness that psychological trauma is a risk factor for developing cardiovascular co-morbidities, and that the ICU admission of critically ill patients, can induce long-term psychological impairments. However, pre-clinical short-term studies in young, healthy, un-resuscitated rodents often do not reflect the pathophysiology of comorbid ICU patients, and thus, often fail to translate beneficial findings into the clinics. Therefore, in this thesis, long-term studies were performed in clinically relevant pig models, two of which had pre-existng atherosclerosis. The pig, as a model, is well established in the ICU and additionally presents with a variety of advantages, as e.g. human-like: size, anatomy, inflammatory- and redox profile.

Hydrogen sulfide (H_2S) and oxytocin (OT) assume importance in both, physical and psychological trauma, inasmuch through mediating antioxidant, anti-inflammatory, neuro- and vasculo-protective effects and regulating heart rate, blood pressure and blood volume. Therefore, the role of the cardiac and cerebral H_2S and OT systems in circulatory shock and acute subdural hematoma (ASDH) were elucidated.

Since there is a lack of readily available antibodies for immunohistochemical detection of pathophysiological changes on porcine tissue, thirteen antibodies, targeting the H_2S and OT systems and markers of injury, were validated for their specific reactivity on porcine heart and brain tissue.

In the septic heart, parallel reduction of cystathionine-γ-lyase (CSE), oxytocin receptor (OTR) and glucocorticoid receptor (GR) expression was associated with lower signaling of GR related genes and lower nuclear factor (erythroid-derived

2)-like 2 (Nrf2) messenger ribonucleic acid (mRNA). This study was the first to show cardiac GR expression in relation to septic cardiomyopathy in pigs with atherosclerosis.
In the paraventricular nuclei (PVN), CSE, cystathionine-β-synthase (CBS), OT, and the OTR co-localized in the magnocellular neurons after resuscitated hemorrhagic shock (HS). HS lead to only minor perivascular edema, revealed by neuro-histopathological evaluation of cortico-subcortical areas. We could not detect an effect of sodium thiosulfate ($Na_2S_2O_3$), more than likely, due to an inability to cross the blood brain barrier (BBB), as observed in humans.
In the ASDH project: the H_2S and OT systems were shown, for the first time, to be expressed at the base of the sulci. In the injured prefrontal cortex, CSE was downregulated at the base of the sulci and associated with barrier dysfunction, edema, perivascular swelling, oxidative and nitrosative stress, in contrast to the OTR, OT and CBS, which were upregulated in comparison to the uninjured. The translational value of the findings is strengthened by the fact that CSE and the OTR were localized in the same cell types as in the human brain. Traumatic brain injury (TBI) and admission to the ICU are associated with the risk of developing post traumatic stress disorder (PTSD). Both, TBI and PTSD are associated with prefrontal cortex dysfunction. The cerebral dysregulation of the OTR and altered sulfide homeostasis in the prefrontal cortex are associated with psychological disorders. Thus, the localization of the H_2S and the OT systems in the prefrontal cortex of the ASDH model is significant with regards to TBI-induced PTSD. $Na_2S_2O_3$ might be a viable treatment for ASDH, since a disrupted BBB would allow it to enter the brain and the site of injury. In the ASDH model, both the spatial injury pattern, at the base of the sulci, and the immune reaction, reflected the human response to injury. In conclusion, it was particularly significant that the gyrencephalic anatomy of the pig brain allowed to show for the first time an injury pattern at the base of the sulci, which has been so far only shown in humans.

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Curriculum Vitae and Publications

The curriculum vitae has been listed in a restricted form for reasons of data protection.

Personal Details

Name	Nicole Denoix
Year of Birth	1992
Place of Birth	Starnberg

Education

since 10/2018	PhD student within the International Graduate School in Molecular Medicine Ulm
since 04/2018	PhD thesis "Interaction of Hydrogen Sulfide and the Oxytocin System in Trauma and Shock" at the Institute of Anesthesiological Pathophysiology and Process Development and Clinic for Psychosomatic Medicine and Psychotherapy, Ulm University Medical Center, Ulm University
10/2015–10/2017	Master in Molecular and Translational Neuroscience, Ulm University "Inhibition of FKBP51 to treat psychiatric disorders: Tool compounds to test inhibition of FKBP51"
10/2012–12/2015	Bachelor in Biology, Ulm University "Aberrant expression of VENTX induces a leukemia"

List of Publications

Poster Presentations

- **Nicole Denoix** et al. (**2019**): "Oxytocin and hydrogen sulfide expression in porcine brain after hemorrhagic shock" *International Symposium on Intensive Care and Emergency Medicine (ISICEM), Brüssel*

Oral Presentations

- **Nicole Denoix** et al. (**2018**): "Interaktion von Oxytocin- und H_2S-System bei Kresilaufschock" *Zentrum für Traumaforschung (ZTF) Workshop Brücken-projekte Somatik Psyche Trauma, Ulm*
- **Nicole Denoix** et al. (**2019**): "Zerebrales Oxytocin und Schwefelwasserstoff System bei hämorrhagischem Schock" *Deutsches Kollegium für Psychosomatische Medizin (DKPM), Berlin*
- **Nicole Denoix** et al. (**2019**): "Rolle des Oxytocin- Rezeptors und des Schwefelwasserstoff (H_2S)-Systems in der Präeklampsie und beim HELLP-Syndrom" *Kick-Off Meeting Klinische Fall-Kontroll-Studie an Patientinnen mit Präeklampsie bzw. HELLP- Syndrom an der Universitätsklinik Paracelsus Medizinischen Privatuniversität, Nürnberg*
- **Nicole Denoix** et al. (**2019**): "Interaction of Oxytocin with the H_2S System in Stress and Circulatory Shock" *Société de Réanimation de Langue Française (SRLF) Jeune Chercheurs, Paris*
- **Nicole Denoix** et al. (**2020**): "The H_2S and the oxytocin systems in the hypothalamus of a porcine model of hemorrhagic shock with administration of sodium thiosulfate" *Paris Redox World Conference 2020 Virtual Conference*

Publications

- **Nicole Denoix**, Tamara Merz, Sarah Unmuth, Andrea Hoffmann, Ester Nespoli, Angelika Scheuerle, Markus Huber-Lang, Harald Gündel, Christiane Waller, Peter Radermacher, Oscar McCook (2020). "Cerebral Immunohistochemical Characterization of the H_2S and the Oxytocin Systems in a Porcine Model of Acute Subdural Hematoma" *Frontiers in Neurology* 11:649.

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- Tamara Merz, **Nicole Denoix**, Daniela Wigger, Christiane Waller, Martin Wepler, Sabine Vettorazzi, Jan Tuckermann, Peter Radermacher, Oscar Mccook (2020). "The Role of Glucocorticoid Receptor and Oxytocin Receptor in the Septic Heart in a Clinically Relevant, Resuscitated Porcine Model With Underlying Atherosclerosis" *Frontiers in Endocrinology (Lausanne)* 11:299

- Tamara Merz, **Nicole Denoix**, Martin Wepler, Holger Gässler, David A.C. Messerer, Clair Hartmann, Thomas Datzmann, Peter Radermacher, Oscar McCook (2020). "H_2S in acute lung injury: A therapeutic dead end(?)" *Intensive Care Medicine Experimental* 8(Suppl 1):33.

- Tamara Merz, **Nicole Denoix**, Markus Huber-Lang, Mervyn Singer, Peter Radermacher, Oscar McCook (2020). "Microcirculation versus Mitochondria -What to target?" *Frontiers In Medicine* 7:416.

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- Britta Trautwein, Tamara Merz, **Nicole Denoix**, Csaba Szabo, Enrico Calzia, Peter Radermacher, Oscar McCook (2021). "ΔMST and the regulation of cardiac CSE and OTR expression in trauma and hemorrhage" *Antioxidants* 10(2):233.

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- Thomas Datzmann, Andrea Hoffmann, Oscar McCook, Tamara Merz, Ulrich Wachter, Jonathan Preuss, Sabine Vettorazzi, Enrico Calzia, Michael Gröger, Fabian Kohn, Andreas Schmid, **Nicole Denoix**, Peter Radermacher, Martin Wepler (2020). "Effects of sodium thiosulfate ($Na_2S_2O_3$) during resuscitation from hemorrhagic shock in swine with preexisting atherosclerosis" *Pharmacological Research* 151:104536.

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The acknowledgements have been removed for privacy reasons.

Statutory Declaration

I hereby declare that I wrote the present dissertation with the topic

"Interaction of Hydrogen Sulfide and the Oxytocin System in Trauma and Shock"

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.
I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis“ [Rules of the University of Ulm for Assuring Good Scientific Practice].

Ulm,

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Nicole Denoix