



**University of
Zurich^{UZH}**

Master Thesis
Master of Science in Biology / Neurosciences

The effects of acute stress on
Apold1 gene expression and blood-
brain barrier permeability

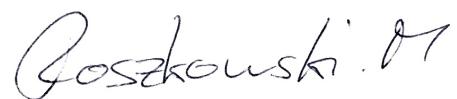
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Statement of Authorship

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A handwritten signature in black ink, appearing to read "Roszkowski. M".

Martin Roszkowski
11.11.2014, Zurich

Abstract

Stressful experiences can have a strong impact on health and psychological well-being. Stressors exert a powerful impact on the brain and activate fine-tuned circuit-level effects that involve multiple neurotransmitter and hormone systems. Blood supply to the brain is one of these tightly regulated mechanisms. Endothelial cells, pericytes and astrocytes form the blood-brain barrier that protects the sensitive neuronal tissues from pathogens, damaging compounds and neurotoxins. Evidence is emerging that stress can influence the permeability of the blood-brain barrier through adrenergic neurotransmitters epinephrine and norepinephrine. They form - together with other peptides, hormones and metabolites - an intricate regulatory mechanism that ensures adequate energy and oxygen supply through regulation of the cerebral blood flow and blood-brain barrier permeability.

In the present work we investigate how stress can alter gene transcription in the brain and what the functional consequences of such gene regulation may be. Specifically, we focus on the stress-induced up-regulation of a vascular immediate early response gene (*Apold1*), which we have identified in the hippocampus. We demonstrate that *Apold1* expression is increased shortly after stress exposure, peaks at 30-45 minutes after stress and rapidly returns to baseline levels. *Apold1* expression is also increased in the prefrontal cortex and cerebellum of stressed mice. Expression is regulated by the neurotransmitter norepinephrine via β -adrenergic receptors and can be blocked by the β_2 -adrenergic receptor antagonist ICI118,551.

Because *Apold1* is thought to play a role in regulating endothelial permeability, we then tested the hypothesis that there may be a functional link between stress and blood-brain barrier permeability. We first establish and validate an *in vivo* permeability assay based on the fluorescent dye sodium fluorescein. Using several acute stress paradigms, we demonstrate conclusively that acute stress does not induce changes in blood-brain barrier permeability. However, following cold-swim stress we find that cerebral extraction ratio for sodium fluorescein is decreased rapidly and transiently. The same effect is observed after cold restraint, suggesting that the decreased body temperature is a key factor in the observed reduction in brain perfusion.

These results are an important step forward in our understanding of *Apold1* regulation in response to acute stress, and also add to a growing body of literature that challenges the controversial idea that acute stress increases blood-brain barrier permeability. The function of *Apold1* up-regulation in response to acute stress remains to be determined, our data suggest a possible role for *Apold1* in counterbalancing the mismatch between increased neuronal activity in response to stress and decreased cerebral blood perfusion.

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1. Introduction

1.1. Nervous System and Stress

All animals, including humans, are part of a dynamic environment in which survival often depends on rapid adaptation to changing environmental conditions. In biological systems, stress is broadly defined as any severe perturbation of the physiological or psychological homeostasis and requires the organism to counteract this imbalance (Cannon 1915).

The evolution of the nervous system has endowed organisms with the ability to react quickly to such stressful situations. Sensory systems of the peripheral nervous system (PNS) enable organisms to collect information about their surrounding, which is then processed by the central nervous system (CNS). The motor system then translates any output into movement and other measurable behaviors. The autonomic nervous system (ANS) is part of the PNS and is involved in the control of critically important organ functions, such as respiration, heart rate and digestion. The ANS can be further distinguished into enteric nervous system, sympathetic and parasympathetic divisions. The parasympathetic division promotes a state of “rest-and-digest” while the sympathetic nervous system is attributed to the “fight-or-flight” state. They modulate together the state of arousal (Szabadi 2013). The two main systems that initiate and maintain the fight-or-flight state are the sympathoadrenomedullary (SAA) and hypothalamic-pituitary-adrenocortical (HPA) axis (Ulrich-Lai and Herman 2009).

1.1.1. Sympatho-Adrenomedullary Axis (SAA)

The SAA consists of the preganglionic sympathetic neurons in the spinal cord and the adrenal medulla, located on top of the kidneys. The preganglionic sympathetic neurons are activated by preganglionic nerve fibers, which originate in the locus coeruleus (LC) and the ventrolateral medulla (Kim and Diamond 2002). The preganglionic sympathetic neurons project to the adrenal medulla. Acetylcholine is released by these axons, which trigger the secretion of epinephrine and norepinephrine into the bloodstream (Ulrich-Lai and Herman 2009). Epinephrine acts mainly as a hormone that exerts a plethora of physiological responses: increase of

heart and respiratory rate, vasoconstriction in skin and gut, vasodilatation of brain and muscle blood vessels, inhibition of peristalsis and digestion, dilatation of bronchioles, increase of lipolysis and gluconeogenesis (Purves 2008).

Norepinephrine in the brain acts as a neurotransmitter and is released from neurons in the LC of the brainstem. These neurons project to all brain areas, most notably the neocortex, hippocampus, cerebellum and thalamus (Foote, Bloom, and Aston-Jones 1983; Mason and Fibiger 1979). In the CNS, norepinephrine release has wide spread effects, impacting anxiety, arousal, alertness, temperature control, decision-making, learning and memory processes (Carter et al. 2010; Hilakivi 1987; Itoi and Sugimoto 2010; Kramarczyk, Brown, and Thurmond 1984; McGaugh and Roozendaal 2002). Although norepinephrine is not able to cross the blood-brain barrier its signal can be relayed by the vagus nerve. Epinephrine from the blood stream can bind to β -adrenergic receptors located on the vagus nerve, which then promotes the release of cortical norepinephrine by neurons of the LC (Peña, Engineer, and McIntyre 2013; Tanaka et al. 2000).

1.1.2.The Hypothalamic-Pituitary-Adrenocortical Axis (HPA axis)

Neurons in the paraventricular nucleus of the hypothalamus form the start of the HPA axis, as they release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the surrounding median eminence (Ulrich-Lai and Herman 2009). The median eminence is a highly vascularized small swelling devoid of the blood-brain barrier, one of the few areas in the brain where neurons are in direct contact with blood vessels (Scott and Pepe 1987). The median eminence connects the hypothalamus with the pituitary gland. CRH promotes the secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland. ACTH is released into the blood and reaches the inner adrenal cortex. Its main function is the synthesis and release of glucocorticoid hormones like cortisol. ACTH triggers the release of glucocorticoids, which then circulate in the body and lead to several tissue specific physiological changes, including mobilization of stored energy (Akana et al. 2001), modulation of the immune response (Bellavance and Rivest 2014), maintenance of blood pressure and regulation of body fluid homeostasis (Ulrich-Lai and Herman 2009). Glucocorticoids do not only lead to global physiological changes but they also act directly on neurons. For example, they can strengthen and prime synaptic

connections in the hippocampus and improve hippocampus-dependent learning (Blank et al. 2002).

Both stress response axes are tightly regulated by other brain circuitry (most noticeably the parasympathetic) and also differentially activated by different stressors. One of the key nuclei involved in the stress response is the locus coeruleus composed of noradrenergic projection neurons. The locus coeruleus is linked to the HPA axis, as an interaction between prolactin-releasing peptide and norepinephrine can regulate neuroendocrine neurons in the hypothalamus (Uchida et al. 2010).

1.1.3. Adrenergic Neurotransmitters and Receptors

Acetylcholine is the main neurotransmitter in the sympathetic and parasympathetic divisions. It mediates the release of norepinephrine by the sympathetic division. The released norepinephrine then evokes different responses in the tissue and cells depending on their expression, distribution and composition of adrenergic receptors. Two receptor types of adrenergic receptors (α and β) can be distinguished that bind the neurotransmitters with different affinities and also lead to different intracellular responses (Hieble, Bondinell, and Ruffolo 1995).

α -adrenergic receptors are divided into α_1 - and α_2 -adrenergic receptors and lead to opposite responses in neurons. α_1 -adrenergic receptor stimulation by norepinephrine leads to an excitatory response in neurons and α_2 -adrenergic receptor stimulation to an inhibitory response (Szabadi 2013). α -adrenergic receptors can be also found in microvessels but they are mainly present in the presynaptic bouton of astrocytes where they trigger Ca^{2+} influx which in turn affected cellular metabolism, gene expression and signal transduction to other cells (Hertz et al. 2010; Yokoo et al. 2000).

β -adrenergic receptors are divided into β_1 -, β_2 - and β_3 -adrenergic receptors. In contrast to α -adrenergic receptors the norepinephrine-mediated response by the β -adrenergic receptors is highly dependent on the cell type (Szabadi 2013).

1.1.4. β -adrenergic Receptors and Intracellular Signaling

The β_1 - and β_2 -subtype have been extensively studied due to their role in the regulation of heart rate, vasoconstriction and vasodilation. The β_2 -adrenergic receptor subtype is of particular interest because it is expressed in endothelial cells

of the blood vessels (Galasso et al. 2013), microglia (Heneka et al. 2010), astrocytes (Laureys et al. 2010) as well as in neurons of the hippocampus where they mediate norepinephrine effects on learning and memory (Murchison et al. 2011; Schutsky et al. 2011).

β -adrenergic receptors are G-protein-coupled receptors. Upon binding of norepinephrine to the receptor the secondary messenger pathway cAMP/PKA is activated (Sibley and Lefkowitz 1987). PKA subunits then translocate to the nucleus where they phosphorylate CREB (Hagiwara et al. 1992). CREB is a potent transcription factor that is known to regulate many genes, which are also well studied in their role in memory and synaptic plasticity (Kandel 2001). Among them are also immediate early genes such as Fos and EGR-1 (Bing et al. 1991; Stone et al. 1991).

1.1.5. Stress-Induced Gene Expression

The profound impact of stress on brain function is reflected by the dramatic stress-induced transcriptional response in various brain regions. A brief exposure to acute swim stress, for example, can cause strong, transcriptome-wide changes involving hundreds of genes in the hippocampus (Gray et al. 2013). Expression of these genes is regulated by a wide array of hormones and neurotransmitters (Joëls and Baram 2009).

Previous work from our group (see Figure 8) has identified Apolipoprotein L domain containing 1 (Apold1) as a gene that is dramatically increased in the hippocampus of mice following acute cold swim stress. Apold1 (also known as vascular early response gene (Verge)) shares an amino acid sequence homology with Apolipoprotein L of approximately 30 %, which is relatively low when compared to other proteins in the Apolipoprotein family. The predicted motifs for Apold1 are an N-terminal leucine-zipper, two hydrophobic stretches, C-terminal coiled-coil domain and several predicted phosphorylation sites for PKC and PKA (Regard et al. 2004). Apold1 is expressed in developing and adult endothelial tissues. Furthermore overexpression of Apold1 in endothelial cell cultures lead to changes in the actin cytoskeleton, which increased permeability of the monolayer (Regard et al. 2004). Therefore, it is possible that Apold1 may regulate changes in blood-brain barrier permeability *in vivo*. Indeed, several studies have suggested that blood-brain barrier permeability may change in response to acute stressful situations (Esposito et al. 2001; Friedman et al. 1996; Sarmento, Borges, and Azevedo 1991; Sarmento,

Borges, and Lima 1994; Sharma, Cervós-Navarro, and Dey 1991) and we will review this literature further below. In order to understand the potential role of Apold1, norepinephrine and stress in blood-brain-barrier permeability, we will first provide some background information on blood-brain barrier function.

1.2. Blood-brain barrier

From an energetic point of view, the human brain is very expensive. Neuronal activity increases the demand for energy (Attwell and Laughlin 2001) and especially for oxygen (Thompson, Peterson, and Freeman 2003). A reliable and tightly controlled energy supply is therefore important in order to avoid damage to the CNS.

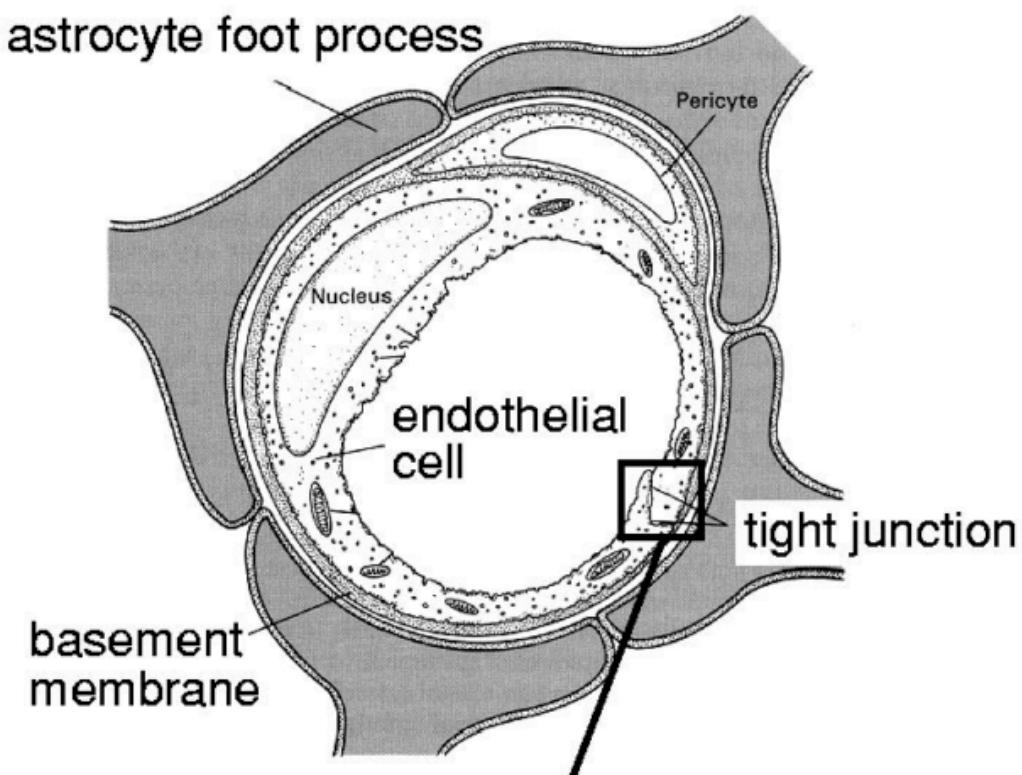


Figure 1: Endothelial cells, pericytes and astrocytes form the blood-brain barrier. Endothelial cells are the main interface between the blood and cortical tissue. Specialized tight junctions between endothelial cells ensure that most molecules can't pass through. Pericytes are wrapped around the endothelial cell layer. Pericytes are involved in the structural stability and regulation of blood vessels. Astrocytes transport nutrients and molecules from the blood through the endothelial cells deeper in to the cortical tissue to neurons and oligodendrocytes. (Gloer et al. 2001)

The blood-brain barrier (BBB) is the regulatory interface between the central nervous system (CNS) and the circulating blood. With very few exceptions, the BBB is present along all brain capillaries, and it is comprised of endothelial cells and tight junctions, astroglia, pericytes and perivascular macrophages (Bradbury 1985; Gloer

et al. 2001; Wolburg and Lippoldt 2002) (see Figure 1). While the BBB acts as a physiological barrier protecting the brain from many unwanted compounds and molecules (e.g. neurotoxins), it is selectively permeable for essential molecules such as water, gases, nutrients and metabolites (Gloor et al. 2001; Purves 2008). This strict selectivity, which prevents the uptake of almost all large-molecule drugs and more than 98% of all small-molecule drugs, has led to increased efforts by the scientific community and drug manufacturers to further elucidate the properties of the BBB (Pardridge 2005).

Certain diseases like cerebral edema, inflammations, cerebral ischemia or injuries can lead to altered blood-brain barrier permeability (BBBP) or even a complete breakdown of the BBB (Ballabh, Braun, and Nedergaard 2004; de Vries et al. 1997). However, other rather mild physiological changes in the CNS such as increased glucose or oxygen demand due to neuronal activity can also alter the BBBP in a reversible manner (Pardridge 2003).

1.2.1. Endothelial Cells in the Blood-Brain Barrier

Endothelial cells form the inner layer of blood vessels. They are the primary interface between the circulating blood and the brain tissue. As opposed to most other endothelial cells in other tissues their transcellular trafficking is very limited and highly selective. The tight junctions between the endothelial cell surfaces prevent most molecules from entering the cortical tissue (Gloor et al. 2001).

While their transport mechanisms have been studied extensively, surprisingly little is known about the effects of norepinephrine on endothelial cells. Endothelial cells express adrenergic receptors and they appear to be involved in increased pinocytosis (Borges et al. 1994; Sarmento et al. 1991), and ion pumping (Harik 1986) when exposed to epinephrine and norepinephrine (Iaccarino et al. 2005). The β_2 -adrenergic receptor pathway seems to be also involved in the regulation of cell proliferation and angiogenesis (Iaccarino et al. 2005; Lamy et al. 2010; Steinle, Cappoccia, and Jiang 2008).

1.2.2. Pericytes in the Blood-Brain Barrier

The contribution of pericytes to the regulation of BBBP is still poorly understood, mainly because they are usually intertwined with endothelial cells and smooth muscle cells, thus complicating the interpretation of many studies (Krueger and Bechmann

2010). Pericytes are wrapped around the endothelial cells (see Figure 1). In the BBB they provide structural integrity, regulate the vascular tone, provide a second line of defense against unwanted compounds or molecules that might pass through the tight junction (Broadwell and Salcman 1981), and play an important role in angiogenesis (Balabanov and Dore-Duffy 1998). The contribution of pericytes to the regulation of cerebral blood flow (CBF) is even less well understood, but it appears that they can relax and contract around the blood vessels they surround. Neuronal activity has been shown to cause relaxation, which in turn allows the blood vessels to dilate and increase CBF. On the other hand, low oxygen supply that occurs during ischemia can lead to cell death of pericytes. The dying cells contract around the endothelial cells, damage the BBB and reduce CBF (Hall et al. 2014).

1.2.3.Neurovascular coupling

Another important regulatory mechanisms that ensures adequate supply of blood (and therefore the supply of essential molecules) is the cerebral vascular tone. Arterioles consist of endothelial cells wrapped in smooth muscle cells so that they can contract and dilate (Koehler, Roman, and Harder 2009). This way the cerebral blood flow (CBF) can be fine-tuned. Regardless of the precise mechanisms of how the CBF is regulated it is apparent that increased neuronal activity coincides with increased CBF. Initially, neurovascular coupling was thought to be regulated by metabolic products that would reflect the neuronal activity but in the last decade it became apparent that astrocytes play a crucial role. They can sense the increased neuronal activity and propagate the signal accordingly to the pericytes, smooth muscle and endothelial cells (Lovick, Brown, and Key 2005).

1.2.4.Astrocytes in the Blood-Brain Barrier

The signal transmission from neurons to blood vessels is not unidirectional. Astrocytes can also relay signals in both directions (Fellin et al. 2006) and potentiate the release of neurotransmitters at synapses (Perea and Araque 2007). Astrocytes are not only able to modulate the cerebral vascular tone and therefore the CBF but their end feet also directly reach the endothelial cells. Therefore, astrocytes have the ability to directly modulate the transcellular transport of nutrients through endothelial cells (Huber, Egleton, and Thomas 2001), which in turn means that they can influence the BBBP.

1.2.5.Norepinephrine and Cerebral Blood Flow

Cerebral norepinephrine is produced mainly by the neurons of the locus coeruleus (LC). Approximately 8 % of all nerve projections from the LC end in the immediate vicinity of blood vessels. Further electron microscopy imaging revealed that the nerve terminals in the vicinity of blood vessels are mainly present at astrocyte processes (Cohen, Molinatti, and Hamel 1997). Stimulation of the LC causes a widespread increase of cortical norepinephrine that will target a wide range of brain areas and cell types (Florin-Lechner et al. 1996). The increased neuronal activity stemming from the norepinephrine stimulation also leads to increased CBF through neurovascular coupling (Toussay et al. 2013).

1.2.6.Stress, Norepinephrine and Blood-Brain Barrier Permeability

Several studies have raised the intriguing possibility that the BBB may become more permeable in response to severe stressors. In rats and mice, several studies have reported stress-induced increases in BBBP using various tracer molecules (Esposito et al. 2001; Sarmento et al. 1991, 1994; Sharma et al. 1991). In agreement with these findings, stimulation of the adrenergic system, which becomes rapidly activated by stress, has been shown to alter BBBP. The most prominent study suggested that chronic stress-induced BBBP may have caused/contributed to long-term illness in Gulf War veterans (referred to as “Gulf-War Syndrome”). During the gulf war, soldiers were administered the acetylcholinesterase inhibitor pyridostigmine as pretreatment against potential chemical warfare. Under normal circumstances, pyridostigmine does not cross the BBB, but the study reported an increased brain penetrance of the drug in mice exposed to stress (Friedman et al. 1996). However, several subsequent studies failed to replicate the findings that stress promotes entry of pyridostigmine into the brain or that stress increased BBBP for the used dye (Ovadia et al. 2001; Park et al. 2008) while others report that pyridostigmine and stress induce effects on the CNS (Kant et al. 2001; Shaikh et al. 2003). More recent studies found that these conflicting results could be explained by an indirect mechanism of pyridostigmine effects on the CNS that is not mediated by a stress induced BBB opening (Amourette et al. 2009; Barbier et al. 2009; Lamproglou et al. 2009). Taken together, it still remains unclear whether stress indeed alters BBBP, or whether other factors and mechanisms may explain these conflicting results.

1.3. Aim

The aim of the studies presented in this manuscript is to first characterize the expression of Apold1 levels in response to acute stress and the mechanisms underlying its regulation. Thereafter, we assess whether different stressors can impact BBBP. Further we test which variables other than stress may regulate the brain uptake of the fluorescent dye in our stress experiments.

2. Methods

2.1. Animals

C57Bl/6J female and male mice (2.5 months old) were obtained from Elevage Janvier (Le Genest Saint Isle, France) and maintained in a temperature and humidity controlled facility on a 12-hour reversed light–dark cycle with food and water *ad libitum* unless stated otherwise. Mice were bred in-house and their offspring were used in the experiments. Mice were kept in groups of 3-5 animals per cage. For acute stress experiments animals were transferred to single housing 24 hours prior to the experiments in order to minimize the additional stress of removal of cage mates on the test day. For each experiment mice of the same age or cohort were used and they were usually between 2 and 6 months old. All procedures were carried out in accordance to regulations for animal experiments by the veterinary office of the canton Zurich. Experiments and procedures were approved under license number 175/2013 and experimenters were licensed and trained. In accordance with the 3R guidelines an effort was made to reduce the numbers of used animals in each experiment.

2.2. Animal Experiments

Whenever possible we attempted to reduce noise, light, handling and odors in order to avoid stress reactions in the animals. Experiments and injections were carried out in a dark behavior room. In case of waiting periods between the stress paradigm, handling or injection and tissue collection the cage with the animal was returned to the racks in the animal colony room. In experiments where sodium fluorescein was injected, mice did not have access to water between the injection and sacrifice. Behavior, surgery and animal colony rooms were adjacent to each other. Tissue collection and perfusion were done under the fume hood in the surgery room to avoid the odor of blood and urine in the room. Whenever a cage had to be transferred an effort was made to avoid noise and abrupt movements. All rooms were only illuminated by red light (40 lux) when the animals were handled or transferred between rooms. For tissue collection, perfusion and other procedures normal light

was only turned on once the animal became unconscious. Gloves were changed between handling of different animals and surfaces briefly cleaned with 70% EtOH.

2.3. Forced Swim

All forced swim experiments were carried out in a dark room dimly lit by red light. A plastic cylinder (24 cm high and 21 cm diameter) was filled with 18 ± 1 °C tap water up to a water depth of 15 cm. The mouse was lifted by the tail out of the cage and gently lowered into the water. Animals were closely monitored and returned to their cage after 6min or if they struggled to keep their head above water. Water was changed and the cylinder briefly rinsed after every animal. Control animals were picked up by their tail, briefly held above the plastic cylinder and then returned to their cage.

For the warm forced swim experiment water with the temperature of 39 ± 1 °C was used and the animals returned to their cage after 30min. The plastic cylinders were not insulated and the water was loosing temperature. The temperature was measured every 5 minutes and animals were transferred to another cylinder with fresh warm water if the water temperature dropped below 36 °C. This was usually the case after 10min. Therefore the water was changed twice during a 30min forced swim.

2.4. Restraint

Mice were placed for 30min in a 50 ml Falcon tube and the tube placed in their homecage. The Falcon tube had the tip cut off to allow breathing and a hole in the cap so that the tail could stick out. In case of cold restraint the tube was partially immersed in 18 ± 1 °C water for 6min. Control animals were briefly lifted by their tail and then put back in their cage.

2.5. Drugs

All drug injections were administered i.p. 45min before the stress paradigm unless stated otherwise. Drugs were dissolved in 0.9% saline and the injection volume was 10µl per gram body mass. Injected drugs were at room temperature. In experiments where a drug was used control animals were injected with vehicle. The following drugs were administered in concentrations according to Table 1.

Table 1: List of used drugs for adrenergic receptor blocking and anesthesia.

Drug name	Short description	Concentration [mg/kg]	Manufacturer	Cat.#
MK-801	NMDA receptor antagonist	0.3	Sigma-Aldrich	M107
Volinanserin	5-HT _{2A} receptor antagonist	1	Sigma-Aldrich	M100907
Propranolol	β-AR antagonist	10	Sigma-Aldrich	P0884
Betaxolol	β1-AR antagonist	1	Sigma-Aldrich	B5683
ICI-118,551	β2-AR antagonist	1	Sigma-Aldrich	I127
Esconarkon	Pentobarbital	200	Streuli Pharma	1270147AA

2.6. Anesthesia

For i.v. injections and transcranial cold lesion animals were anesthetized with 1.5-3% isofluran inhalation in oxygen (600 ml/min). Isofluran concentration was adjusted during the whole procedure to ensure a breathing frequency of roughly 1 breath per second. Mice were first placed in a box until they lost consciousness and then transferred to the stereotactic frame for the transcranial cold lesion or a mouth piece for i.v. injections. The mouthpiece for i.v. injections was made out of a 50 ml Falcon tube and 2 tubing adapters for in and out flow of the anesthesia gas. The cap was hollowed out in the middle and a piece of latex or nitril glove was placed between the tube and the cap (see Figure 2). Mice were placed on a heating pad and the rectal temperature measured with a probe. The setup ensured that the body temperature of the mice stayed at 37 °C. Prior to any procedure successful anesthesia was confirmed by pinching the skin of the hind paws with tweezers.

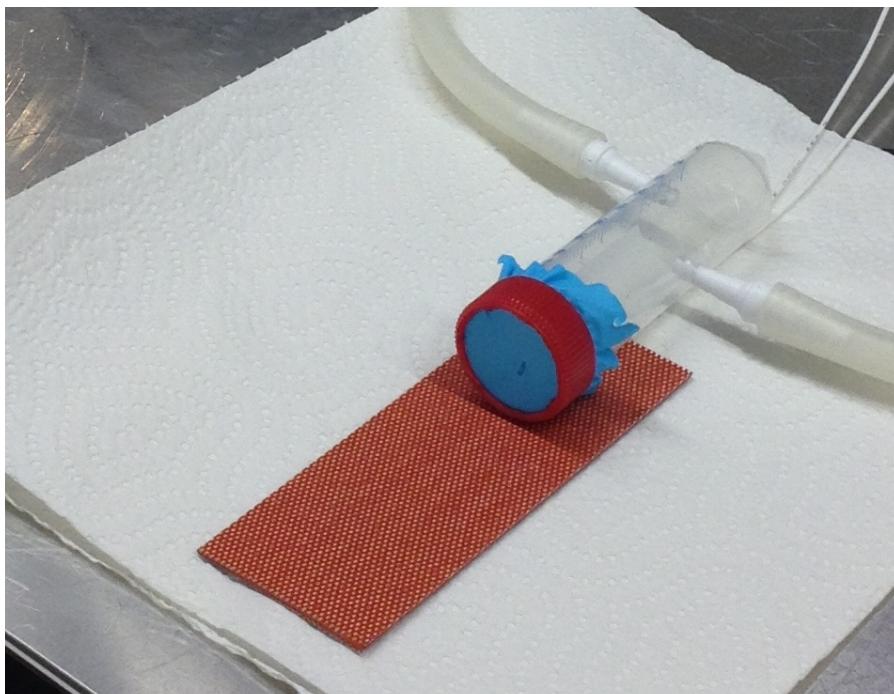


Figure 2: Anesthesia mask for mice i.v. injections made out of a 50 ml Falcon tube, nitril gloves, and tubing adapters.

2.7. Cold Injury

Female mice were used for this experiment. They were kept under isofluran anesthesia for the whole procedure until sacrifice. The head was fixed in the stereotactic frame using earbars. Skin tissue on the head was removed with scissors and the exposed skull treated with 30% hydrogen peroxide (Sigma, Cat.# H1009). Bregma was used as the reference point to place the cold metal rod on the right hemisphere of the exposed skull for 30s (see Figure 3). The metal rod (4 mm diameter, 30 cm long) was cooled in liquid nitrogen for 5min. After the cold treatment mice were i.p. injected with sodium fluorescein (80 mg/kg body weight with a concentration of 10 mg/ml) in 0.9% saline. 30min after sodium fluorescein administration animals were i.p. injected with Esconarkon. Animals were transferred to the fume hood for perfusion and tissue collection.

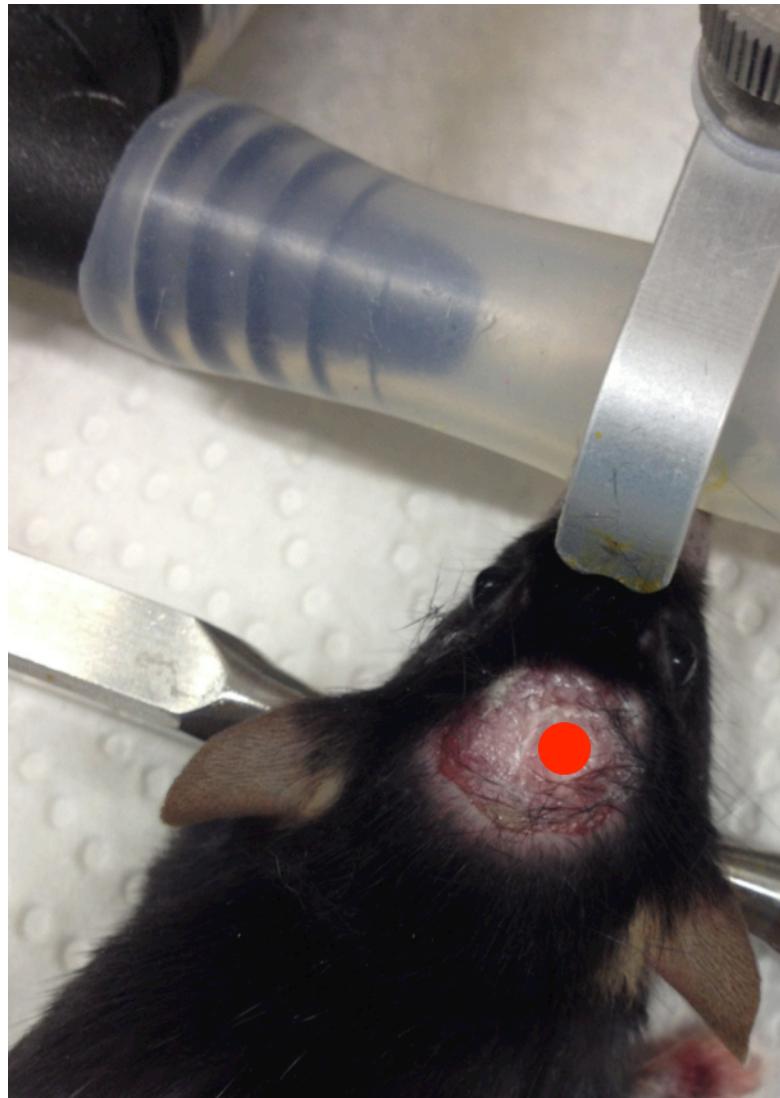


Figure 3: Anesthetized mouse in the stereotactic frame with exposed skull. Cold metal rod was held against the red marked area for 30s.

2.8. i.v. Injection

Animals were anesthetized with isoflurane. After the mice were stably anesthetized the tail was warmed in 45 °C warm water in a 25 ml tube for 30 s to dilate the tail vein. Before injection the tail was wiped with 70% ethanol. For easier intravenous injection a 0.3 ml insulin syringe (BD Micro-Fine Demi, Cat.# 324626) was connected by a 15 cm long PE tube (Plastics One, Pe10, 0.28 x 0.61 mm, Cat.# C314CT) to a broken off cannula tip (BD Microlance 3, 30G, 0.3 x 13 mm, Cat.# 304000). The tubing was flushed with saline. Successful cannula placement was ensured by drawing a bit of blood in to the tubing (see Figure 4). The syringe was exchanged and the appropriate amount of sodium fluorescein in 0.9% saline (40 mg/kg body weight with a concentration of 10 mg/ml) administered. The syringe was then

exchanged again to the previous syringe filled with 0.9% saline and the remaining sodium fluorescein in the tube was flushed. The cannula was then removed and bleeding stopped by gently pressing a piece of paper tissue against the tail. Animals were then returned to their cage and monitored until they woke up. It usually took several attempts until successful cannulation was achieved. We aimed to achieve a successful i.v. injection within 8min.

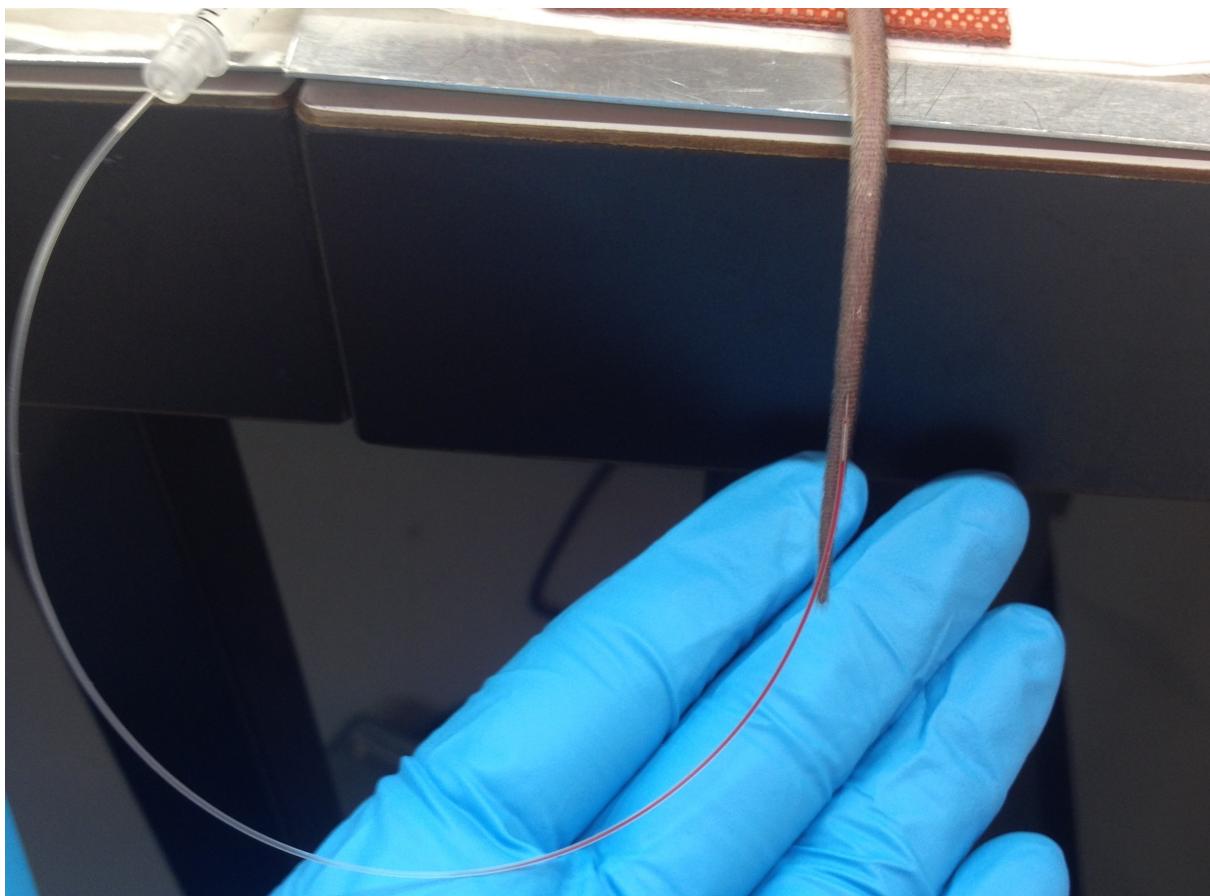


Figure 4: Example of a successful cannulation of the tail vein: blood can be drawn in to the tube which is filled with saline. As a next step the syringe is exchanged against the syringe containing the fluorescein dye for the injection.

2.9. Tissue Collection

For experiments where no sodium fluorescein was used animals were sacrificed by cervical dislocation. Immediately after sacrifice by cervical dislocation or after successful perfusion the brain was removed. Different brain regions were rapidly dissected on a cold glass surface cooled by liquid nitrogen: olfactory bulb, prefrontal cortex, cerebellum, hippocampus. Additionally, cortex was collected, considered the remaining brain tissue without brain stem. The dissected samples were snap frozen in liquid nitrogen and stored in -80 °C freezer until further processing.

2.10. Perfusion, Blood collection, Temperature

In experiments where blood-brain barrier permeability was assessed animals were perfused with saline. Mice were i.p. injected with Esconarkon. Once the mice were unconscious and unresponsive to pinching of the paws with tweezers they were placed on their back. Limbs were spread and fixed with needles. After the rectal temperature was measured the skin was removed in the area of the sternum. The chest was opened with a pair of surgical scissors. Once the heart was exposed blood was collected from the right heart ventricle twice with a 0.3 ml insulin syringe (BD Micro-Fine 0.3 ml, Cat.# 324626) for a total of approx. 700 ml of blood (see Figure 5). Blood samples were stored in 1.5 ml tubes on ice until further processing. A 22G butterfly cannula was inserted in to the left heart ventricle (see Figure 6) and the animal was perfused with 50 ml 0.9% saline for 10min. The solution was pumped through with a peristaltic pump.



Figure 5: Mouse with opened chest and intracardiac blood collection from the right heart ventricle. A yellow or orange discoloration of the abdomen, paws, ears and snout is apparent in animals that were successfully injected with sodium fluorescein.



Figure 6: A mouse with a cannulized left heart ventricle for perfusion.

2.11. Serum Processing

Blood samples were processed on the same day as the experiment. Blood was centrifuged (2'000 rcf, 10min, 4 °C) and the supernatant transferred to a fresh 1.5 ml tube. Serum samples were stored at -80 °C.

2.12. Tissue Homogenization and RNA Extraction

1 ml of ice-cold TRIzol reagent (Invitrogen, Cat.# 15596-026) and 1 steel bead were added to each tissue sample. Samples were homogenized using a TissueLyserII (QIAGEN) set to 20 Hz for 1-2min until a sufficient homogenization was reached. Samples then incubated at room temperature for 5min. After a brief centrifugation (to settle the foam) 200 µl chloroform (Sigma-Aldrich, EC#154733) were added, shaken by hand for 15s and then incubated at room temperature for 3min. Samples were then centrifuged (12'000 rcf, 4 °C, 15min) and the aqueous phase transferred into fresh tubes. Another 500 µl of chloroform were added and again as before shaken, incubated, centrifuged and the aqueous part transferred into fresh tubes. To aid in the recovery of the RNA 10 µl of Glycogen (Invitrogen, Cat.# AM9510) were added and the tubes 4 times inverted. 500 µl of isopropanol (Sigma-Aldrich, EC#200-661-7) were added, tubes 4 times inverted and then incubated at room temperature for 10min. After centrifugation (12'000 rcf, 4 °C, 10min.) the supernatant was removed. To wash the pellet 1 ml of 75 % Ethanol (Merck Millipore, Cat.# 100983500) was added, vortexed and then centrifuged (7'500 rcf, 4 °C, 5min.). The washing step was repeated once and the tubes were then placed with open lids in a heat block at 55 °C for 5-10min to evaporate remains of ethanol. The pellet was then dissolved in 20 µl H₂O (Roche Diagnostics, RNase free) and the RNA concentration measured with a Nanodrop 1000 (Thermo Fisher Scientific). Samples were stored at -80 °C.

2.13. RNA Cleaning

The extracted RNA was DNase treated and cleaned with a “DNA-Free RNA Kit” from Zymo Research (Catalog#R1014). A 20 µl aliquot of the extracted RNA was diluted with H₂O (Roche Diagnostics, RNase free) to a final concentration of 200 ng/µl and 30 µl of master mix (25 µl H₂O and 5 µl DNase I buffer) added. After the addition of 2 µl DNase I the samples for incubated in a heat block at 37 °C for 30min. 100 µl RNA binding buffer and 150 µl abs. ethanol (Merck Millipore, Cat.# 100983500) were added and the solution then transferred onto spin columns. The spin columns were

centrifuged (12'000 rcf, 4 °C, 2min.) and the flow through discarded. 400 µl RNA Prep Buffer were added to the column, centrifuged (12'000 rcf, 4 °C, 2min.) and the flow through discarded. The step was repeated with 800 µl RNA Wash Buffer and once more with 400 µl RNA Wash Buffer. After an additional centrifugation (to get rid of remaining RNA Wash Buffer) the spin columns were placed in fresh tubes and 20 µl H₂O were added to the column matrix and incubated at room temperature for 1min. The columns were centrifuged (10'000 rcf, 4 °C, 2min.). The RNA concentration was measured with a Nanodrop 1000 (Thermo Fisher Scientific) and the samples stored at -80 °C.

2.14. RNA to cDNA Conversion

The cleaned RNA was converted to cDNA with the “SuperScript III First-Strand Synthesis System for RT-PCR” kit from Invitrogen (Catalog#18080-051). A 5 µl aliquot of the cleaned RNA was diluted with H₂O (Roche Diagnostics, RNase free) to a final concentration of 100-200 ng/µl in PCR strips. 8.5 µl of Master mix 1 (7 µl H₂O, 0.5 µl oligo dT Primer (500 ng/µl, Promega, Catalog#C1101), 1 µl dNTPs (10mM, Promega)) were added to each well and then incubated in a PCR machine at 65 °C for 5min. Then 6.5 µl of Master mix 2 (4 µl 5X First-Strand Buffer, 1 µl DTT (0.1 M), 1 µl RNaseOUT, 0.5 µl SuperScript III RT) were added and incubated with the following PCR program: 50 °C for 50min, 70 °C for 15min. Samples were then stored at -20 °C.

In addition two negative controls were added: one control “noRT” did not contain any SuperScript III RT (the RNA for this sample was pooled from 5 different samples) and one control (noInput) did not contain any RNA.

2.15. Primers for qPCR

Primers were designed with Primer3Plus (Untergasser et al. 2007) or Quantprime (Arvidsson et al. 2008). Custom made primers were supplied by Microsynth (Switzerland) (see Table 2). The quality of the used primers was assessed by melting curves and gel electrophoresis to ensure single PCR products of the right size (data not shown).

Table 2: Sequences (5' -> 3') used in forward and reverse primers for qPCR of different gene transcripts.

Gene	Forward primer sequence	Reverse primer sequence
Apold1	ACCTCAGGCTCTCCTCCATCATC	ACCCGAGACAAAGCACCAATGC
Tubd1	TCTCTTGCTAACTTGGTGGTCCTC	GCTGGGTCTTAAATCCCTCTACG
Fos	ACAGATACACTCCAAGCGGAGAC	TGGCAATCTCAGTCTGCAACGC
Cyr61	AGCTCACTGAAGAGGGCTCCTGTC	ACTCGTGTGGAGATGCCAGTTC
Per1	GAGGGATTGGCAGATGAA	GGGACAAGGGGGTTATTGT
Sgk1	GCCGGTGCCACCCCTGGATCTAT	AGGTGCTTGGAGTTCAGGAGCAA

2.16. Gene Expression Analysis by qPCR

The qPCR was done according to the instructions of the LightCycler 480 II from Roche Diagnostics. Samples were diluted 1:10 in new PCR strips. 3x7 µl of Master mixes were prepared for the genes and endogenous control (Tubd1) as follows: 3.75 µl Roche SybrGreen MasterMix, 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM), 2.85 µl H₂O (Roche Diagnostics, RNase free). 2 µl of diluted cDNA and 22 µl of Master mix were added to wells in an 80-well PCR plate and centrifuged (2'000 rcf, 4 °C, 2min). The solution was mixed with a multi pipette and again centrifuged. The samples were then pipetted in triplicates of 7 µl in a white 364-well plate. The plate was sealed with a foil and centrifuged (2'000 rcf, 4 °C, 2min). Cycling conditions were 5min at 95 °C, then 45 cycles with denaturation (10s at 95 °C), annealing (10s at 60 °C), and elongation (10s at 72 °C) for a total of 45 cycles. Melting curves were acquired by heating from 65 °C to 95 °C with a ramp rate of 0.2 °C per second.

A first quick evaluation of the qPCR quality was done by taking a look at the amplification and melting curves. At this step it was also possible to assess the RNA to cDNA conversion quality based on the noRT and noInput controls mentioned earlier. The results from the LightCycler were exporter as a text file. For data analysis Excel was used and the Livak method applied (Livak and Schmittgen 2001).

2.17. Gel Electrophoresis of cDNA

In addition to analysis of the melting curves the amplified cDNA was analyzed on a 2 % agarose gel to check if the amplicon size was in the range of the expected fragment size. 2 g agarose powder was dissolved in 100 ml TBE buffer and heated in a microwave until no fibers were visible anymore and 4 µl of GelRed (Biotium,

#41003) was added. The gel was casted in a Plexiglas chamber with combs for small wells. After the gel had cooled it was transferred in to the electrophoresis bath. 8 µl of cDNA was mixed with 1.4 µl loading buffer and then loaded in to the gel wells. 2 µl of size marker (Promega, BenchTop 100 bp DNA ladder, #G8291) were loaded on both sides. The gel was run at 125 mV for 30min. A picture of the gel was acquired with an UV imager (Alpha Innotech).

2.18. Protein Processing for Western Blot

Protein extraction was done using an extraction buffer consisting of the following compounds (amounts for one sample and extraction): 100 µl RIPA (1 % TritonX, 50 mM TRIS base, 0.1% SDS, 150 mM NaCl, 0.5 % sodium deoxycholate,), 1:500 PMSF (Sigma, Cat. #P5726), 1:200 phosphatase inhibitor cocktail 2 and 3 (Sigma, Cat. #P5726 and #P0044), 1:200 protease inhibitor cocktail (Sigma, Cat. #P9599). 100 µl of RIPA buffer was added to one hemisphere of a hippocampus tissue sample. The samples were homogenized with a 26G cannula (Braun) and a 1 ml syringe (Primo) passing it 15 times through the cannula and centrifuged (13 000 rcf, 4 °C, 30min). The supernatant was transferred in to a fresh 1.5 ml tube and the remaining solution once more homogenized. Supernatants were then combined in to one tube.

The obtained protein concentrations were quantified with a Bradford assay. 1 µl of 1:10 in H₂O diluted sample solution was added to 99 µl of 1:5 BioRad Mix (BioRad Protein Assay Dye Reagent Concentrate, Cat. #500-0006) in each well. Standard curves were prepared with 1 µl of BSA in different concentrations (2 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.1 ng/µl, 0.0 ng/µl in RIPA buffer) in 99 µl of 1:5 BioRad Mix. In addition 3 wells contained 1 µl of extraction buffer in 99 µl of 1:5 BioRad Mix to assess the background. Samples were loaded in triplicates on a clear ELISA plate (BD Falcon, Microtest M 96-well plate, Cat.# 353072). Plate was scanned with a Multiskan Ascent instrument (measurement filter 595 nm). Average values of the standard curve were used for a linear regression and the protein amounts in the samples were calculated accordingly.

Homogenate brain tissue from Apold1 knockout mice for antibody testing was generously provided by Dr. Paul Worley.

2.19. Western blot

An 8 % polyacrylamide gel was used to run protein samples for Apold1. The gel casting chamber, glass plates and combs were cleaned with ethanol and then assembled. Running gel was prepared in a 50 ml Falcon tube (13.9 ml H₂O, 8 ml 30% acrylamide mix (Bio-Rad, Cat.# 161-0154), 7.5ml of 1.5 M Tris (pH8.8), 300 µl 10% SDS (Bio-Rad, Cat.# 161-0416), 300 µl 10 % ammonium persulfate, 18 µl TEMED (Bio-Rad, Cat.# 161-0801)). The casted gel was overlaid with ethanol to ensure a smooth surface. Gel was allowed to polymerize for 30min. Ethanol was removed and stacking gel added (10.2 ml H₂O, 2.4 ml 30 % acrylamide mix (Bio-Rad, Cat.# 161-0154), 1.89 ml of 1 M Tris (pH 6.8), 150 µl 10 % ammonium persulfate, 15µl TEMED (Bio-Rad, Cat.# 161-0801)). After 30min the gel was either directly used to run a gel or wrapped in paper towels soaked with running buffer in a plastic box and stored at 4 °C.

Protein samples were diluted with water to ensure equal protein amounts between samples to a final volume of 20 µl with 4x loading buffer (200mM Trizma Base pH 6.8, 8 % SDS, 0.4 % Bromophenol blue, 40 % glycerol) and 10 % Beta-Mercaptoethanol. Samples were vortexed, spinned down and incubated at 80 °C for 10min. The wells of the gel were flushed before loading with electrophoresis buffer. 20 µl of the samples and 3 µl of size marker (BioRad, Precision Plus Protein Dual Color Standards, Cat. #161-0374) were then loaded on the 8% polyacrylamide gel. Electrophoresis was done with ice-cold buffer (25 Mm Trizma base, 250 mM Glycine, 0,1 % SDS, pH 8.3) at 90 V (constant voltage) for 20min until a sharp line was reached between the stacking and separation gel. Voltage was changed to 120 V for 150min and stopped once the bands reached the bottom of the gel. The proteins were transferred to a nitrocellulose membrane (BioRad, Trans-Blot Turbo Mini Nitrocellulose Transfer Pack, Cat.# 170-4158) using the TransBlot Turbo (BioRad, default protocol High MW, 1.3 A, 25 V, 10min). The membrane was then blocked in 5% non-fat milk in TBS-T, shacking at room temperature for 60 minutes.

Membrane was transferred in to a 50 ml Falcon tube that contained 4 ml of the primary antibody (4 ml blocking solution, 20 µl primary antibody) and incubated over night at 4 °C on a rotator (see Table 3). The membrane was washed 3 times for 10min in TBS-T and then incubated with the second antibody (15 ml blocking solution, 1.5 µl Goat anti rabbit IRDye 800CW (LI-COR, Cat.# 926-32211)) shacking for 1 hour at room temperature. The membrane was again washed 3 times for 10min

in TBS-T and then scanned using an Odyssey infrared imaging scanner (LI-COR). Scanned membranes were quantified with Image Studio Lite (LI-COR).

Table 3: Antibodies used for Western Blot.

Antibody	Cat. #	Manufacturer	Dilution in 5% milk
Anti-APOLD1	ab105079	abcam	1:1000
Anti-APOLD1 C-terminal	ab179740	abcam	1:0000
GAPDH	21185	Cell Signal	1:10000
IRDye 800CW	926-32211	Li-Cor	1:10000

2.20. Processing of sodium fluorescein samples

Tubes with tissue samples were weighted before and after to determine the exact weight of the collected tissue. Tissue was then homogenized in PBS (hippocampus 250 µl, olfactory bulb 250 µl, prefrontal cortex 250 µl, cortex 750 µl, cerebellum 400 µl) with 1 steel bead added to each tissue sample. A TissueLyserII (QIAGEN) then shook samples at 20 Hz for 4min. Homogenized samples were centrifuged (1250 rcf, 5min, 4 °C) and the supernatant transferred in to a fresh 1.5 ml tube. 100 µl of the supernatant was mixed with 300 µl of 20 % trichloroacetic acid (Acros organics, Cat. #421455000) in a fresh 1.5 ml tube and incubated over night at 4 °C. The samples were then centrifuged (10'000 rcf, 15min, 4 °C) and the supernatant transferred to a new 1.5 ml tube. Processed samples were stored at -80 °C.

10 µl of serum was diluted in 90 µl of PBS and 900 µl 20% trichloroacetic acid (Acros organics, Cat. #421455000) added. Serum samples were incubated over night at 4 °C. They were then centrifuged (10'000 rcf, 15min, 4 °C) and the supernatant transferred to a new 1.5 ml tube. Processed serum samples were stored at -80 °C.

2.21. Quantification sodium fluorescein

Processed samples were quantified using a standard curve. 15 µl of processed sample were added in triplicates to a well that contained 90 µl 0.1 M borax buffer (Sigma, anhydrous sodium tetraborate, Cat. #B-0127) with an adjusted pH 10. This ensured for the mix to be at pH 10 where sodium fluorescein has the highest excitability (see Figure 7). Standard curves were prepared from a sodium fluorescein

mother stock (10'000 ng/ml). 12 standard curve points (700 ng, 600 ng, 500 ng, 400 ng, 300 ng, 200 ng, 100 ng, 75 ng, 50 ng, 25 ng, 10 ng, 0 ng) with different dilutions (1:10 (serum), 1:100 (serum), 1:1000 (brain tissue), 1:10'000 (brain tissue)) were used. 15 µl standard curve, 15 µl 20 % trichloroacetic acid and 75 µl 0.1 M borax buffer were mixed in a well. Black 96-well plates were used (Greiner, black polypropylene wells flat bottom, Cat. #M9685-100EA) for fluorescence measurements. Plates were scanned with a NovoStar (BMG Labtech) using 485 nm excitation and 520 nm emission filters. The standard curve data points were used for a linear regression and the sodium fluorescein amounts in the samples were calculated accordingly.

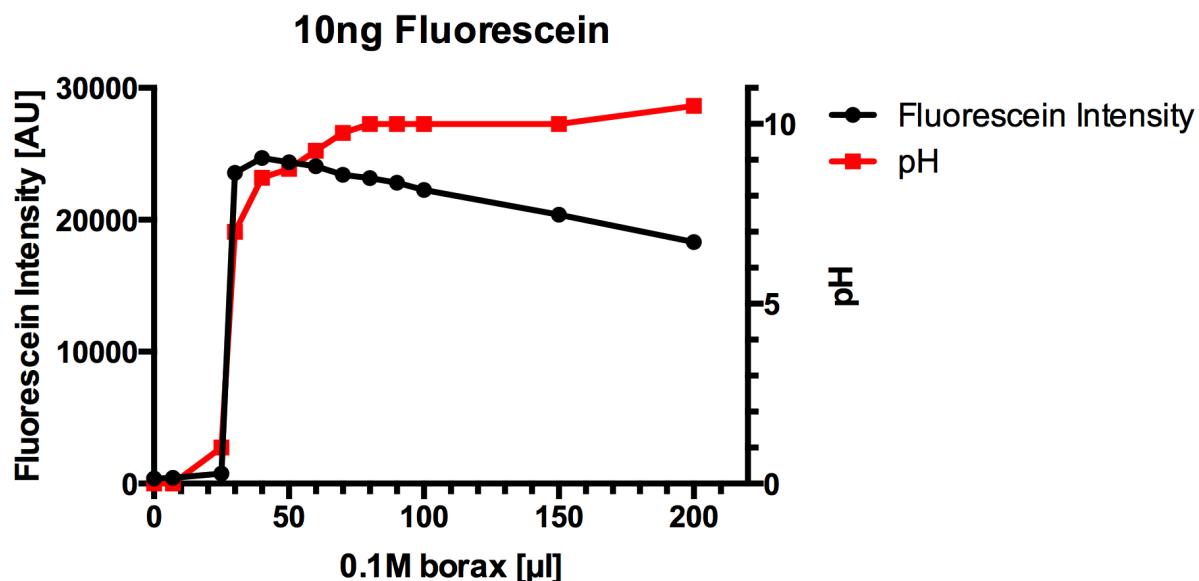


Figure 7: Fluorescence intensity of sodium fluorescein is pH dependent. Samples that contain trichloroacetic acid are mixed with borax buffer to ensure a pH of 10.

2.22. Cerebral Extraction Ratio (CER)

The Cerebral Extraction Ratio (CER) is the ratio of the absolute amount of sodium fluorescein in the brain sample to the absolute amount of sodium fluorescein in the serum sample of an animal. To compare CER results from different experiments the circulation time, injected amount of sodium fluorescein and perfusion regime should be the same. Furthermore the dilution steps during the sample processing have to be taken in to account.

$$\frac{[NaF]/[g\ brain]}{[NaF]/[ml\ blood]} * 100 = CER\ %$$

2.23. Statistics

Statistical analysis and graphs were done using the software GraphPad Prism 6. For comparison of two groups, independent sample t-tests were used. For comparison of more than 2 groups, one-way ANOVAs were employed and significant effects were further analyzed by multiple comparison with Tukey post-hoc test. Statistical significance was set to $p < 0.05$ unless indicated otherwise. Outliers were only excluded when a problem was noted during the experiment (for example poor perfusion results) or sample processing.

3. Results

3.1.1. *Apold1*

The hippocampus is a central brain structure targeted by the stress response, and it is one of the key brain regions involved in memory formation (Kim and Diamond 2002). In order to develop a better understanding of the genes involved in mediating the response to stress, previous work in the research group of Prof. Mansuy assessed gene expression in the hippocampus of female mice that were subjected to forced swim. Many genes showed altered expression levels following acute stress (see Figure 8, Bohacek et al., unpublished observations).

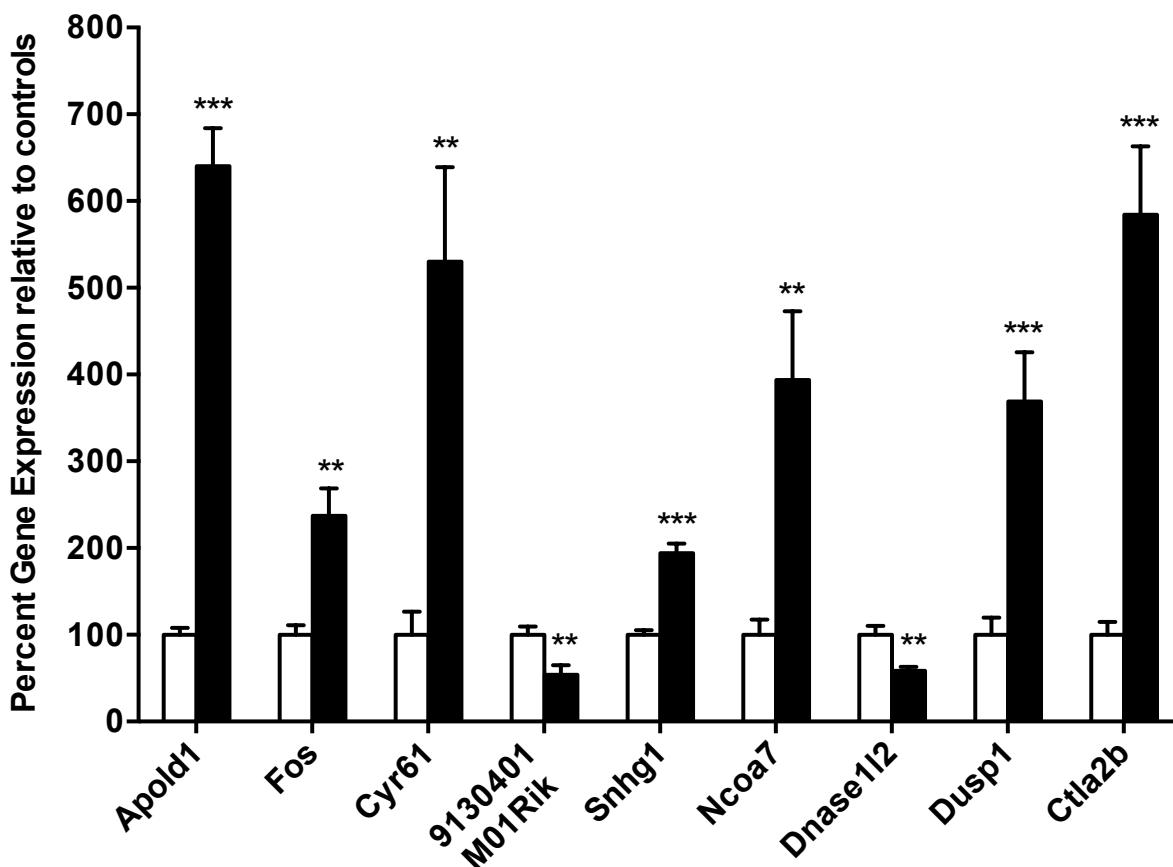


Figure 8: Altered gene expression in female mice hippocampus after 6 min forced swim in 18°C water.
***=p<0.001; **=p<0.01

The gene Apolipoprotein L domain containing 1 (*Apold1* or *Verge*) stood out in particular, as it showed the strongest increase in gene transcription. *Apold1* has previously been associated with angiogenesis in developing tissues (particularly

heart), ischemia and stroke (Liu et al. 2012; Mirza et al. 2013; Regard et al. 2004; Simonsen et al. 2010). It was also suggested that Apold1 functions as a dynamic regulator of endothelial cell signaling and vascular functions (Regard et al. 2004). We were interested in Apold1 for two reasons. First, we use it as a robust transcriptional readout to study the time course of gene expression following acute swim stress, as well as identifying the neurotransmitter systems involved in its regulation. Second, we hypothesize that Apold1 may play a critical role in orchestrating the vascular response to an acute stress challenge, possibly being involved in regulation of blood-brain barrier permeability or ensuring proper blood supply to the brain under stressful conditions.

3.1.2.Increased Apold1 Expression after Acute Stress

Forced swim for 6min in cold water causes a rapid and transient increase of Apold1 gene expression in the hippocampus ($F(4, 13)=12.55$, $p<0.001$), cerebellum ($F(4, 13)=9.062$, $p=0.001$) and prefrontal cortex ($F(4, 13)=9.608$, $p<0.001$) (see Figure 9 A-C). Apold1 gene expression increases rapidly within the first 15min after initiation of forced swim stress and peaks between 30 to 45 minutes, before decreasing back to baseline levels after 120min ($F(5, 24)=26.28$, $p<0.001$) (see Figure 10 A). Our results are in agreement with previous findings that Apold1 expression is rapidly and transiently induced and can be considered an immediate early gene (IEG) (Regard et al. 2004). Gene expression analysis on the same time course experiment shows that two other known IEGs such as Fos and Cyr61 ($F(5, 24)=27.54$, $p<0.001$) exhibit a similar temporal expression profile (see Figure 10 B and C), while other stress-related genes such as Sgk1 ($F(5, 24)=5.42$, $p=0.002$) and Per1 ($F(5, 24)=7.13$, $p<0.001$) show a delayed increase in transcript levels (see Figure 10 D and E).

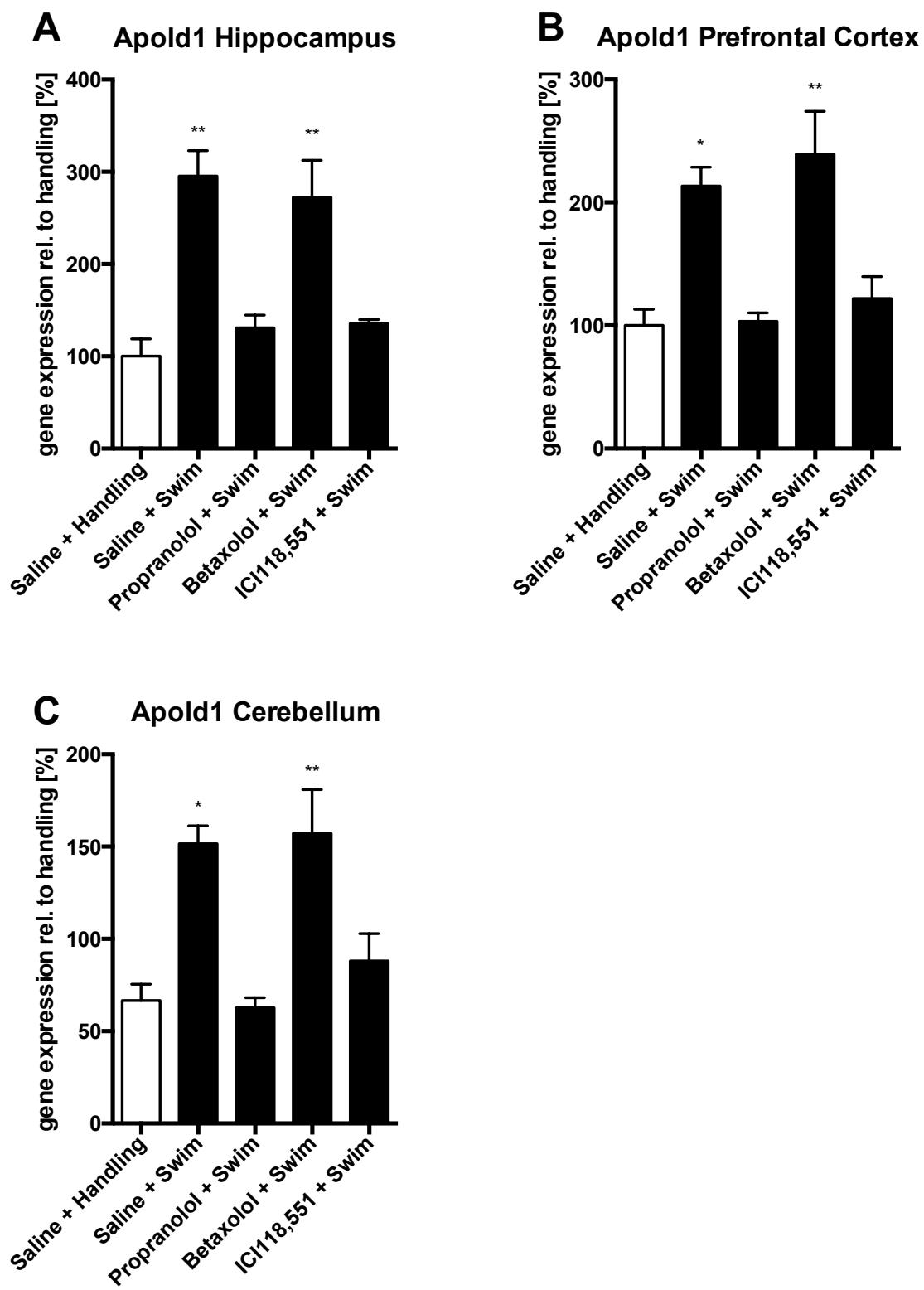


Figure 9: Gene expression increase of Apold1 in male mouse hippocampus (A), prefrontal cortex (B) and cerebellum (C) after forced swim is abolished in the presence of Propranolol and ICI-118,551 but not Betaxolol. Number of subjects per group: n(Saline+Handling)=3; n(Saline+Swim)=3; n(Propranolol+Swim)=4; n(Betaxolol+Swim)=4; n(ICI118,551+Swim)=4. **=p<0.01; *=p<0.05

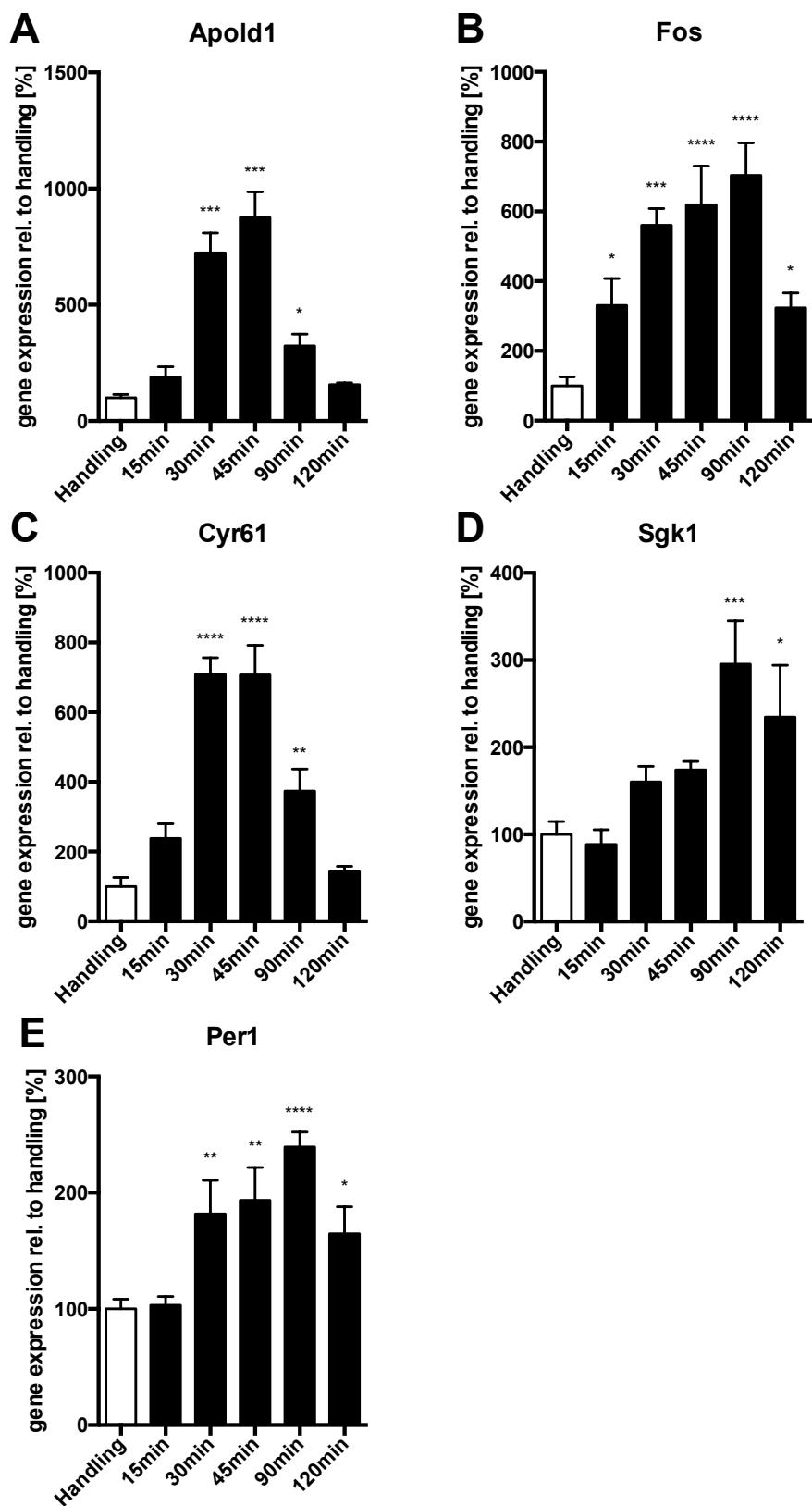


Figure 10: Increased gene expression of Apold1 (A), Fos (B), Cyr61 (C), Sgk1 (D) and Per1 (E) in female hippocampus after 6 min forced swim in 18°C water. Number of subjects per group: n=5. ****=p<0.0001; ***=p<0.001; **=p<0.01; * =p<0.05

3.1.3. Norepinephrine Mediates Apold1 Gene Expression

IEGs are key players in the genomic response to a wide range of different cellular and extracellular stimuli. Previous experiments conducted in the research group of Prof. Mansuy showed that the stress induced increase of Apold1 gene expression ($F(3, 17)=9.53$, $p<0.001$) in the hippocampus of female mice could be blocked by Propranolol (β -adrenoreceptor antagonist) but not Prazosin (α -adrenoreceptor antagonist) (see Figure 11).

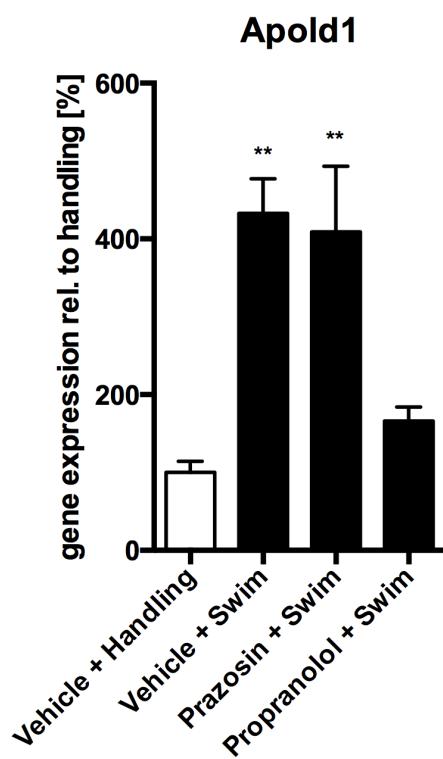


Figure 11: Changes in gene expression of Apold1 in the hippocampus of female mice after acute stress. Significant up regulation of Apold1 in Vehicle+Swim, Propranolol was the only drug that showed a significant effect. Number of subjects per group: n(Vehicle+Handling)=5; n(Vehicle+Swim)=5; n(Prazosin+Swim)=6; n(Propranolol+Swim)=5. **= $p<0.01$; *= $p<0.05$

In order to further characterize the regulatory mechanisms of Apold1 expression and validate the previous findings in female mice, we administered glutamate, serotonin and specific β -adrenergic receptor antagonists before acute stress. ICI118,551 (specific β_2 -adrenoreceptor antagonist) blocked the stress induced increase in Apold1 gene expression while Betaxolol (specific β_1 -adrenoreceptor antagonist) did not (see Figure 9 A). Furthermore, Volinanserin (5-HT₂ receptor antagonist) ($F(3, 20)=36.46$, $p<0.001$) also failed to block the stress induced increase in Apold1 gene expression and MK801 (NMDA receptor antagonist) seemed to increase gene

expression (see Figure 12). Similar results were obtained for Fos ($F(3, 20)=11.20$, $p<0.001$) and Cyr61 ($F(3, 20)=24.22$, $p<0.001$).

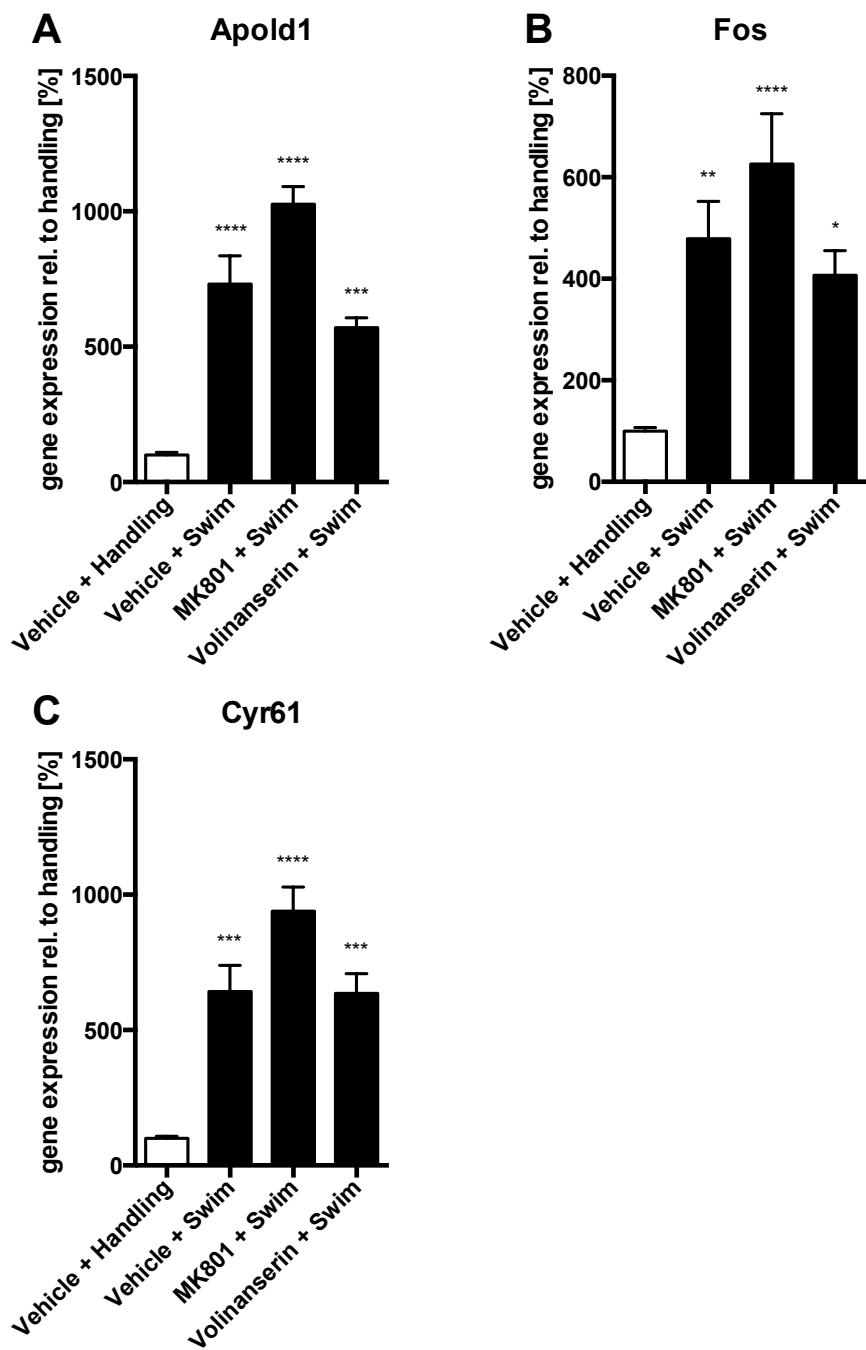


Figure 12: Stress-induced gene expression increase of Apold1 (A) in male mouse hippocampus is not blocked by MK801 or Volinanserin. Similar results can be observed for Fos (B) and Cyr61 (C). Number of subjects per group: $n=6$. ****= $p<0.0001$; ***= $p<0.001$; **= $p<0.01$; *= $p<0.05$

3.2. Protein Expression of Apold1

A previous report had shown a tight relationship between Apold1 gene expression and protein levels (Regard et al. 2004). Therefore, we wanted to assess Apold1 protein levels after acute stress by Western Blot. Unfortunately, none of the tested antibodies seemed to be specific for APOLD1 because they also showed a band in samples from Apold1 KO mice (see Figure 13 and Figure 14). The first tested antibodies had several bands which indicates that the antibody was binding non-specifically to other proteins (see Figure 13). Both antibodies were provided by abcam and predicted to work for mouse APOLD1.

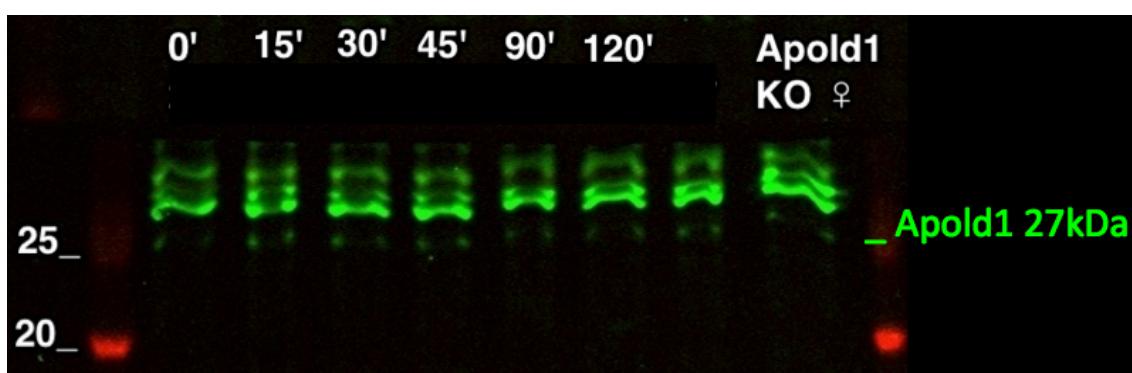


Figure 13: Anti-APOLD1 C-terminal antibody (abcam, ab179740) in WB showed multiple bands. A band of the right size was present (~27 kDa) in lanes 0', 15', 30', 45', 90' and 120' which were samples from the time course experiment. A band of similar size was also present in the knockout protein samples. Furthermore there were also other bands visible in the samples as well as knockout samples which indicates that the antibody was non-specific.

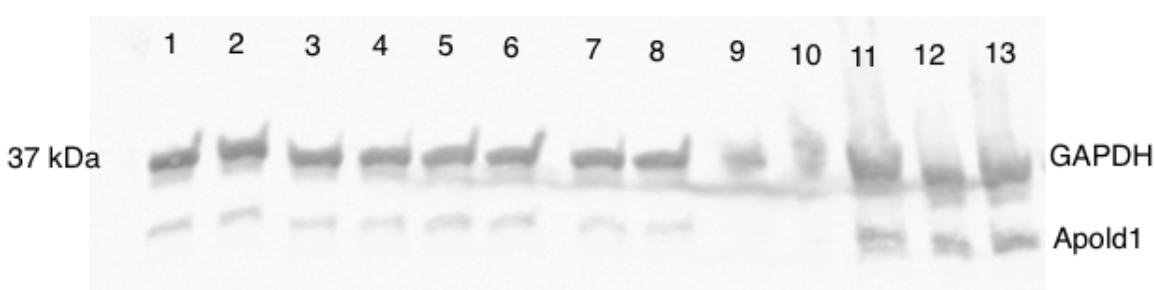


Figure 14: Anti-APOLD1 antibody (abcam, ab105079) in WB. A band of the right size was present (~27 kDa) in most samples (lanes 1-10) but it was also present in the knock-out protein samples (lanes 11-13).

3.3. Blood-Brain Barrier Permeability Assessment

To summarize our results thus far, Apold1 gene expression is strongly up-regulated after acute stress in a norepinephrine-dependent manner. Work from other groups suggests that Apold1 is localized in endothelial cells of blood vessels and is related to endothelial cell permeability in *in vitro* assays (Regard et al. 2004). Several previous reports have suggested that acute stress can increase BBB permeability (Esposito et al. 2001; Sarmento et al. 1991, 1994; Sharma et al. 1991) although this literature is quite inconsistent because other publications reported that BBB permeability was not increased (Ovadia et al. 2001; Park et al. 2008). We therefore hypothesized that blood-brain barrier permeability may be altered in our stress paradigm and that Apold1 could be involved in this process.

3.3.1. Method Validation by Transcranial Cold Lesion

In order to measure changes in BBBP we injected mice with sodium fluorescein. Sodium fluorescein is a small fluorescent dye that can pass through BBB and is detectable in the tissue afterwards. As this method was newly established in our lab, we first needed to determine that it could accurately detect changes in BBBP. Several methods exist to increase blood-brain barrier permeability. These methods usually lead to a global or localized breakdown of the blood-brain barrier and subsequent leakage of the indicator dye/molecule into the brain tissue. Transcranial cold lesion has been previously shown to be a reliable and robust method to induce BBB breakdown (Murakami et al. 1999). We therefore selected transcranial cold lesion as our method of choice to test whether we can detect an increase in BBBP.

Using transcranial cold lesion we were able to demonstrate that it causes the fluorescent dye to locally leak into the affected tissue. Cerebral Extraction Ratio (CER) was increased in the ipsilateral part of the cortex right below the part of the skull that received the cold treatment ($t(3)=2.64$, $p=0.039$), but not in the contralateral side where cold-lesion was not induced. Similarly, permeability changed only in the cortical area close to the cold-exposure, while deeper-lying structures such as the hippocampus were not affected ($t(3)=0.37$, $p=0.373$) (see Figure 15). This confirms the validity and sensitivity of our method to detect changes in BBBP *in vivo*.

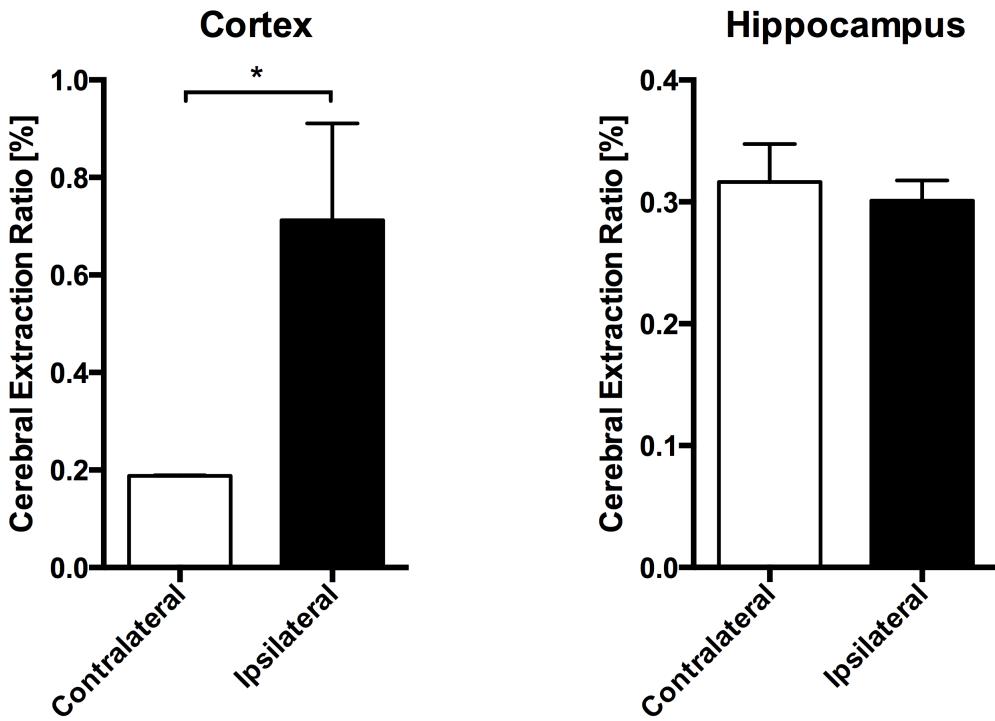


Figure 15: Increased Cerebral Extraction Ratio (CER) in the ipsilateral cortex hemisphere of female mice that were subjected to transcranial cold lesion. In the same animals CER was not changed in the hippocampus. The hippocampus was located below the cortex area. Number of subjects per group: n=4. *= $p<0.05$

3.3.2. Method validation: circulation times

A previous report has shown that a thorough perfusion after injection of fluorescent dyes is critical to avoid false positive results following stress-exposure (Ovadia et al. 2001). As sodium fluorescein – due to its small size - can steadily cross the BBB, more fluorescence should be detected in brain tissue the longer the dye is allowed to circulate in the system. An incomplete perfusion would cause a lot of sodium fluorescein to stay in the blood vessels and therefore similar amounts of the fluorescent dye would be detected between the two groups with different circulation times. Therefore, to ensure that perfusion in our setup was sufficient, we compared control animals of different experiments, where the fluorescent dye circulated for different periods of time. We observed a higher CER in cortex ($t(10)=3.26$, $p=0.009$) and hippocampus ($t(10)=2.65$, $p=0.024$) in animals that had sodium fluorescein for a longer time in the blood stream (see Figure 16), demonstrating that perfusion was sufficient in our experiments. As we also noted an increase in variability after longer circulation time, we selected a circulation time of 10min for all subsequent experiments in order to reduce variability between animals.

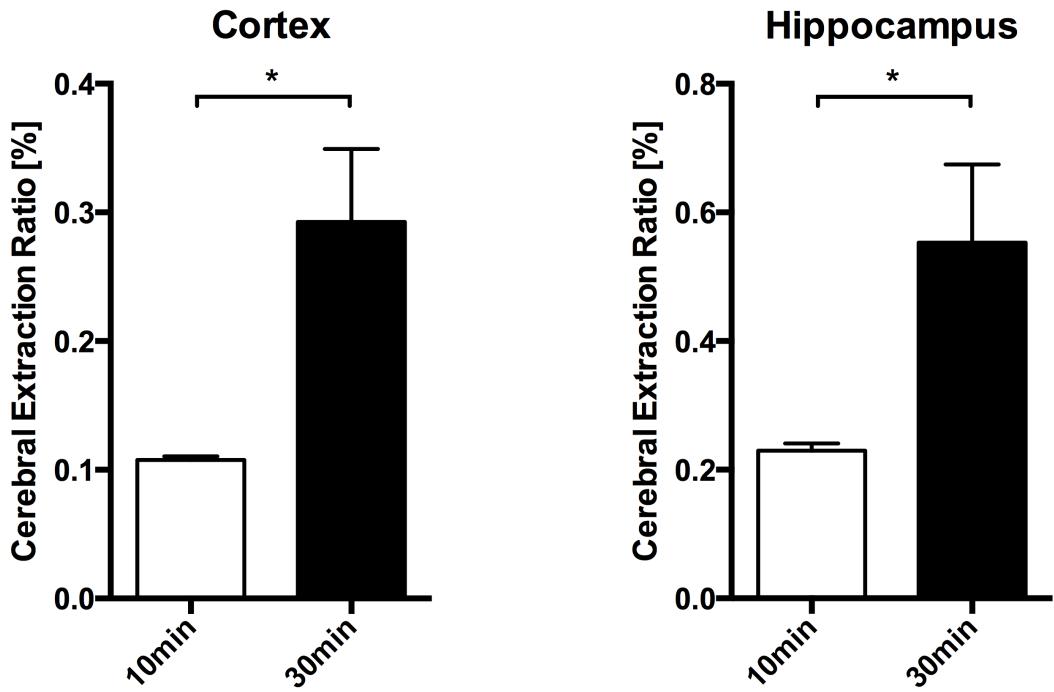


Figure 16: CER in cortex and hippocampus was higher when sodium fluorescein was circulating for 30min instead of 10min. Number of subjects per group: n=6. * $=p<0.05$

3.4. Blood-Brain Barrier Permeability after Acute Stress

We conducted several experiments to determine whether permeability for sodium fluorescein is altered after acute stress. Several stress paradigms were used to test the hypothesis that blood-brain barrier permeability is increased after acute stress.

3.4.1.Blood-Brain Barrier Permeability Experiment 1

In a first experiment we attempted to see if blood-brain barrier permeability is increased by acute stress in a similar time frame like the increase of Apold1 gene expression in previous experiments.

6 male mice were subjected to 6min forced swim in 18 °C cold water. 6 animals from the control group were briefly picked up by their tail and then put back in their cage. Immediately after forced swim animals were anesthetized and i.v. injected with 40 mg/kg sodium fluorescein in 0.9 % saline solution. 45min after the initial forced swim or handling animals were perfused and tissue and blood collected.

The cerebral extraction ratio (CER) was significantly increased in the forced swim animals in hippocampus by 62 % ($t(10)=2.73$, $p=0.021$), cortex 62 % ($t(10)=3.29$, $p=0.008$) and cerebellum by 64 % ($t(10)=3.26$, $p=0.009$). Surprisingly the serum levels of sodium fluorescein in the forced swim group were distinctively reduced by 62 % ($t(10)=2.22$, $p=0.050$) (see Figure 17).

Keeping in mind that the CER is calculated as the amount of sodium fluorescein in brain tissue relative to the amount of sodium fluorescein in serum, big differences in the serum levels between groups could give a positive result even if blood-brain barrier permeability is not changed. The cause for the decreased serum fluorescence in the forced swim group could not be determined with certainty. In the following experiments, sodium fluorescein applications were administered intraperitoneally to avoid anesthesia necessary for i.v. injections, a possible confounding factor.

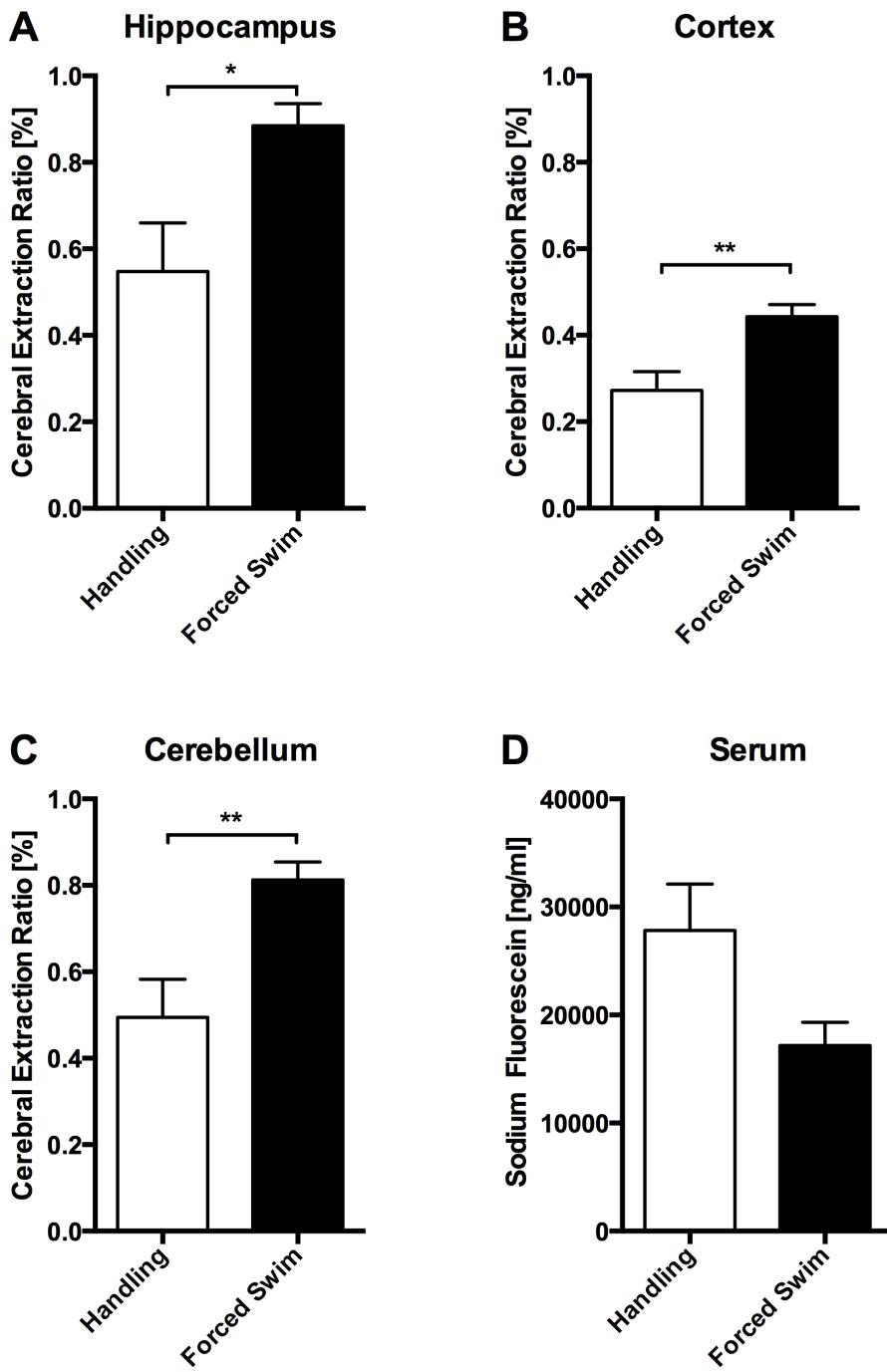


Figure 17: Male mice were subjected to 6min forced swim in 18 °C cold water. Immediately after forced swim they were anesthetized and i.v. injected with sodium fluorescein. CER was increased in hippocampus, cortex and cerebellum of mice that were forced to swim. Sodium fluorescein in the serum was decreased in the forced swim group. Number of subjects per group: n=6. **=p<0.01; *=p<0.05

3.4.2.Blood-Brain Barrier Permeability Experiment 2

We set out to repeat the first experiment because the results were inconclusive. Route of administration of sodium fluorescein was adapted to an intraperitoneal injection and a second time point was added.

Male mice were subjected to 6min forced swim in 18 °C cold water. Animals from the control group were briefly picked up by their tail and then put back in their cage. Immediately, 10min or 35min after the forced swim ended animals were i.p. injected with 40 mg/kg sodium fluorescein in 0.9 % saline solution. After a circulation time of 10min animals were perfused and tissue and blood. Furthermore the rectal temperature was measured right before blood sample collection.

In hippocampus ($F(2, 9)=7.13$, $p<0.001$) a decrease in CER can be observed 20min after forced swim that returns to baseline levels after 45min (see Figure 18 A). These changes in CER were also present in cortex ($F(2, 9)=2.55$, $p=0.133$) and cerebellum ($F(2, 9)=1.99$, $p=0.19$) although not significantly decreased (see Figure 18 B and C). Serum levels between groups were similar ($F(2, 9)=0.49$, $p=0.63$) (see Figure 18 D). Body temperature was 5.3 °C lower ($F(2, 9)=31.23$, $p<0.001$) in the 20min time point group when compared to handling and returned to baseline at the 45min time point group (see Figure 18 E).

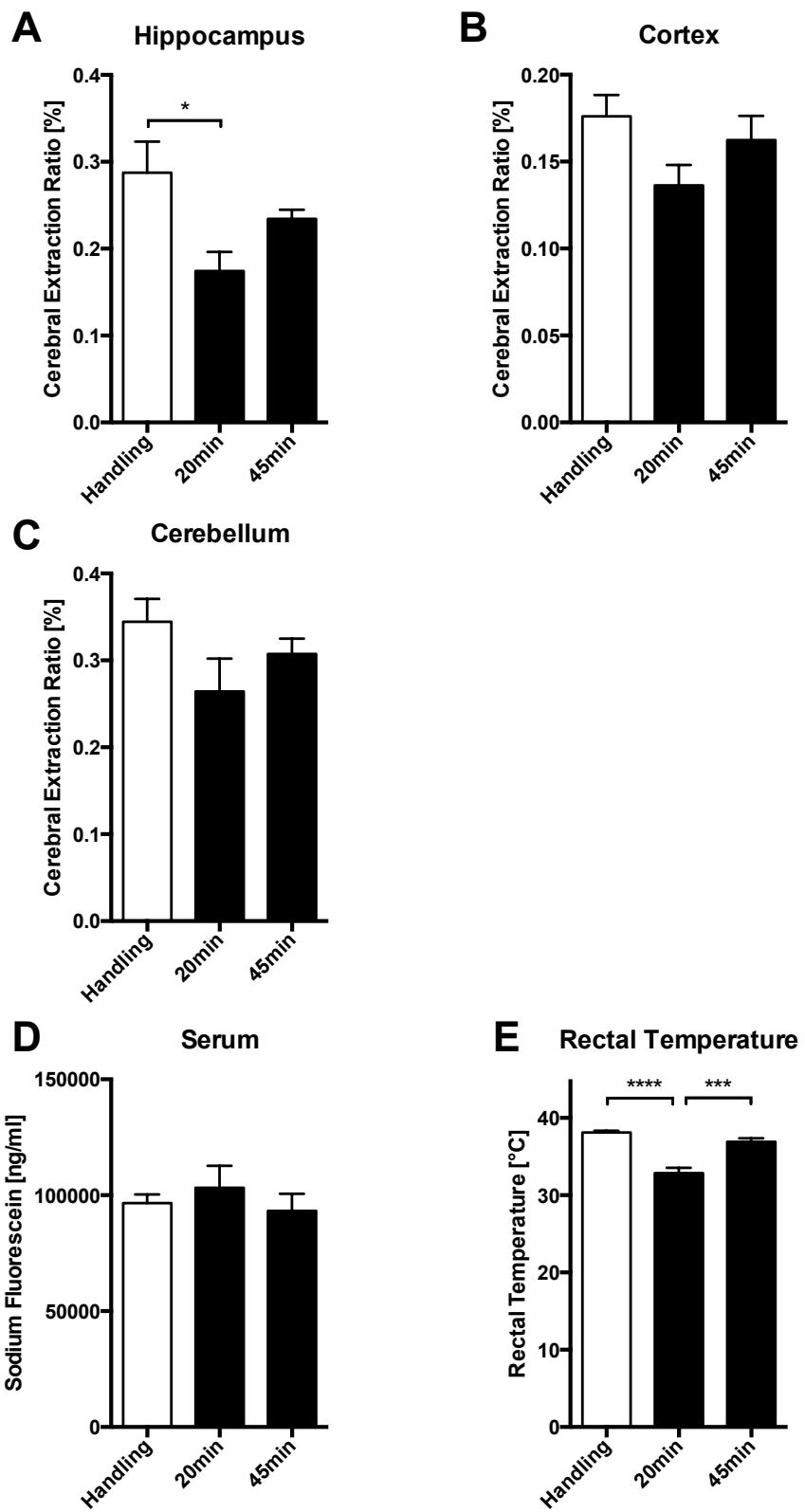


Figure 18: Male mice that were subjected to forced swim in 18 °C cold water had a lower CER in hippocampus 20min after forced swim but not 45min (A). A similar trend can be found in cortex (B) and cerebellum (C). Serum levels between groups were similar (D). Body temperature was significantly decreased in the 20min group (E). Number of subjects per group: n=4. ****=p<0.0001; ***=p<0.001; **=p<0.01; *=p<0.05

3.4.3.Blood-Brain Barrier Permeability Experiment 3

To replicate the findings from the previous experiment and to expand the time course we performed 3 additional experiments and pooled the results from all 4 experiments. Male mice from the same cohort as the previous experiment were subjected to 6min forced swim in 18 °C cold water. Animals from the control group were briefly picked up by their tail and then put back in their cage. Immediately or 10min after the forced swim ended animals were i.p. injected with 40 mg/kg sodium fluorescein in 0.9 % saline solution. After a circulation time of 10min animals were perfused and tissue and blood collected.

In hippocampus ($F(3, 26)=17.58, p<0.001$) and cortex ($F(3, 26)=13.34, p<0.001$) the CER was dramatically decreased at the 10min time point and returned to baseline levels in the 45min time point group (see Figure 19). The decrease was absent in the cerebellum ($F(3, 26)=1.12, p=0.358$). Serum levels ($F(3, 26)=0.75, p=0.53$) between groups were similar (see Figure 19).

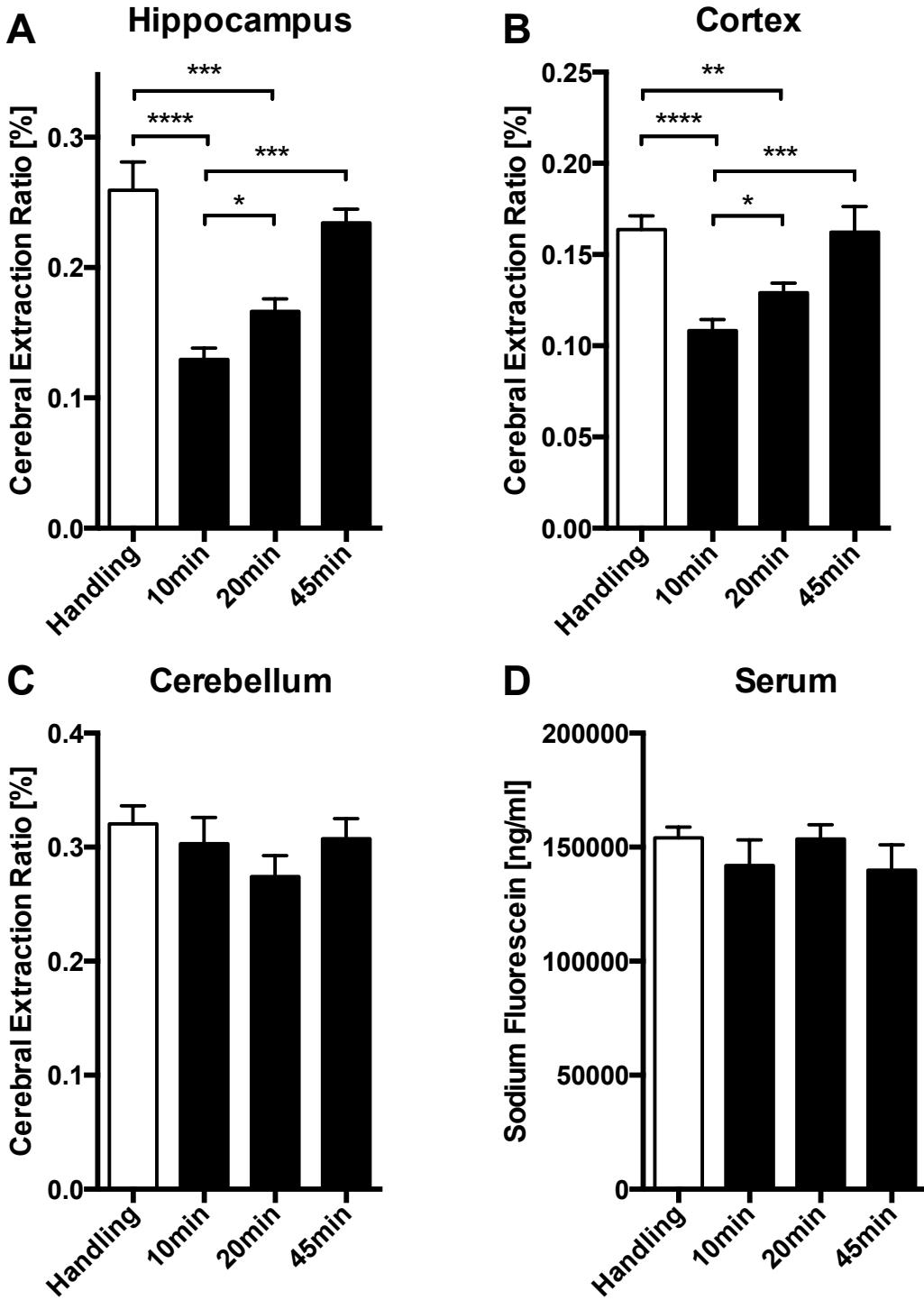


Figure 19: Male mice that were subjected to forced swim in cold water had a lower CER in hippocampus (A) and cortex (B) 10 and 20min after forced swim but not 45min. The CER of the cerebellum (C) was not significantly different. Serum levels (D) between groups were similar. Number of subjects per group: n(Handling)=8; n(10min)=8; n(20min)=10; n(45min)=4. ****=p<0.0001; ***=p<0.001; **=p<0.01; *=p<0.05

3.4.4.Blood-Brain Barrier Permeability Experiment 4

Next we tried to replicate previous findings that reported an increased blood-brain barrier permeability (BBBP) for the dye Evans Blue after acute stress (Friedman et al. 1996) as well as findings to the contrary that reported no such increase (Ovadia et al. 2001; Park et al. 2008).

6 male mice were subjected to a forced swim protocol consisting of two 4min long forced swimming in 22 °C cool water interrupted by one 4min break where they were put back in their cage. Animals from the control group were briefly picked up by their tail and then put back in their cage. 10min after the forced swim ended animals were i.p. injected with 120 mg/kg sodium fluorescein in 0.9% saline solution. After a circulation time of 10min animals were perfused and tissue and blood.

No significant increase in CER for sodium fluorescein could be detected in hippocampus ($t(9)=0.90$, $p=0.390$) or cerebellum ($t(9)=1.12$, $p=0.297$) and serum levels ($t(9)=0.29$, $p=0.778$) were similar between groups (see Figure 20). The CER for cortex was decreased by 15 % ($t(9)=4.363$, $p=0.002$).

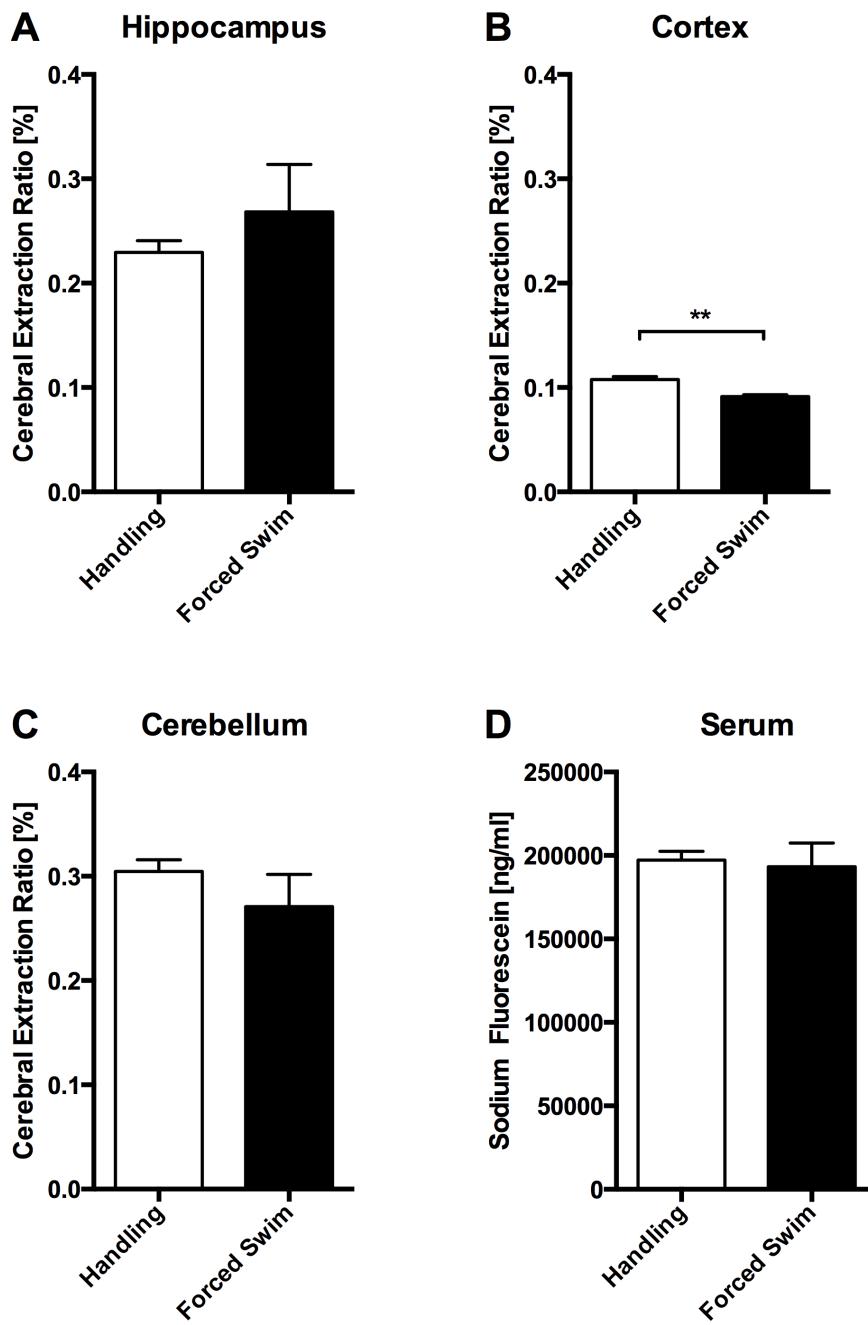


Figure 20: Male mice were subjected to two 4min forced swim with a break of 4min inbetween in 22 °C water. The CER in cortex (B) was decreased after forced swim and unchanged in hippocampus (A) and cerebellum (C). Serum levels (D) between groups were similar. Number of subjects per group: n(Handling)=6; n(Forced Swim)=5.
**=p<0.01

3.4.5.Blood-Brain Barrier Permeability Experiment 5

To further see whether acute stress can alter blood-brain barrier permeability (BBBP) we tested another stressor. Restraint has been previously reported to increase BBBP in rats (Esposito et al. 2001).

Male mice were subjected to 30min restraint in a 50 ml Falcon tube. Animals from the control group were briefly picked up by their tail and then put back in their cage. Immediately after the restraint ended animals were i.p. injected with 40 mg/kg sodium fluorescein in 0.9 % saline solution. After a circulation time of 10min animals were perfused and tissue and blood.

No changes in the CER could be observed for hippocampus ($t(5)=0.87$, $p=0.4232$), cortex ($t(5)=0.12$, $p=0.9094$), cerebellum ($t(5)=0.82$, $p=0.4506$), olfactory bulb ($t(5)=1.12$, $p=0.2885$) or prefrontal cortex ($t(5)=0.12$, $p=0.9093$) (See fig). Furthermore body temperature ($t(5)=2.17$, $p=0.0736$) and serum levels ($t(5)=1.23$, $p=0.275$) were similar between handling and restraint groups (see Figure 21).

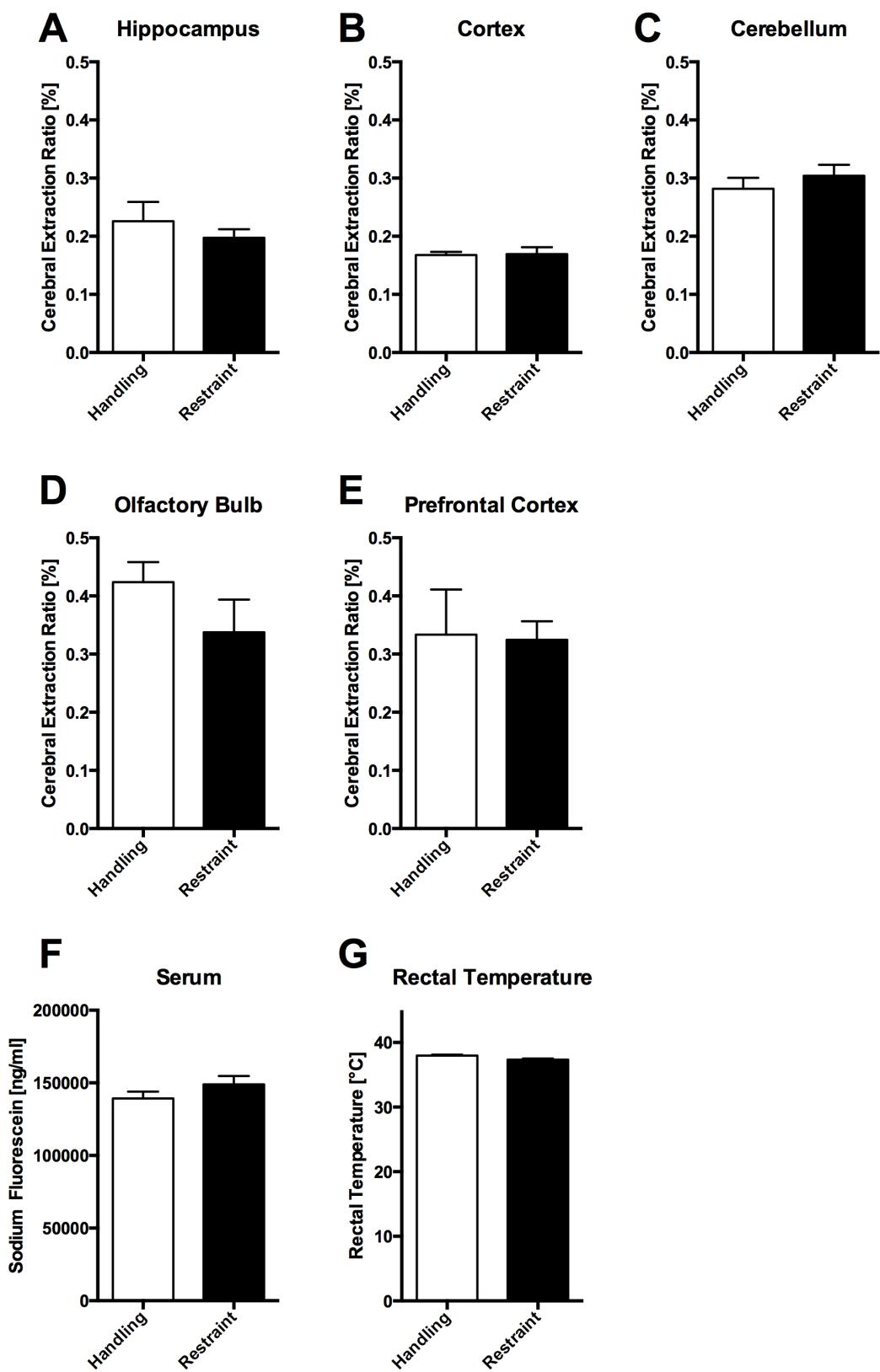


Figure 21: Male mice were restrained in a tube for 30min. CER was not changed for hippocampus (A), cortex (B), cerebellum (C), olfactory bulb (D) or prefrontal cortex (E). Serum levels (F) and rectal temperature (G) between groups were similar. Number of subjects per group: n(Handling)=3; n(Restraint)=4

3.4.6.Blood-Brain Barrier Permeability Experiment 6

In rats it had been reported that 30min of swimming in warm water led to an increase in BBBP (Sharma et al. 1991). Therefore, we tried to test the same protocol in mice. Male mice were subjected to 30min forced swim in 38 °C warm water. Animals from the control group were briefly picked up by their tail and then put back in their cage. Immediately after the forced swim ended animals were i.p. injected with 40 mg/kg sodium fluorescein in 0.9 % saline solution. After a circulation time of 10min animals were perfused and tissue and blood collected. In addition to hippocampus, cortex and cerebellum we also collected the olfactory bulbs and prefrontal cortex areas. A significant decrease in CER could be observed in cortex by 26 % ($t(5)=4.326$, $p=0.0075$), olfactory bulb by 27% ($t(5)=3.74$, $p=0.0134$) and prefrontal cortex by 25 % ($t(5)=6.06$, $p=0.0018$) but not in hippocampus ($t(5)=1.45$, $p=0.208$) or cerebellum ($t(5)=1.28$, $p=0.2564$) and serum levels between groups were similar ($t(5)=1.332$, $p=0.2405$) (see Figure 22). Even though we exchanged the water during the experiment to ensure a constant temperature, animals in the forced swim group had a significantly decreased body temperature by 1.4 °C ($t(5)=5.03$, $p=0.004$). The slight temperature decrease in the forced swim group is most likely due to lost body heat when the wet animals were put back in their cage at room temperature.

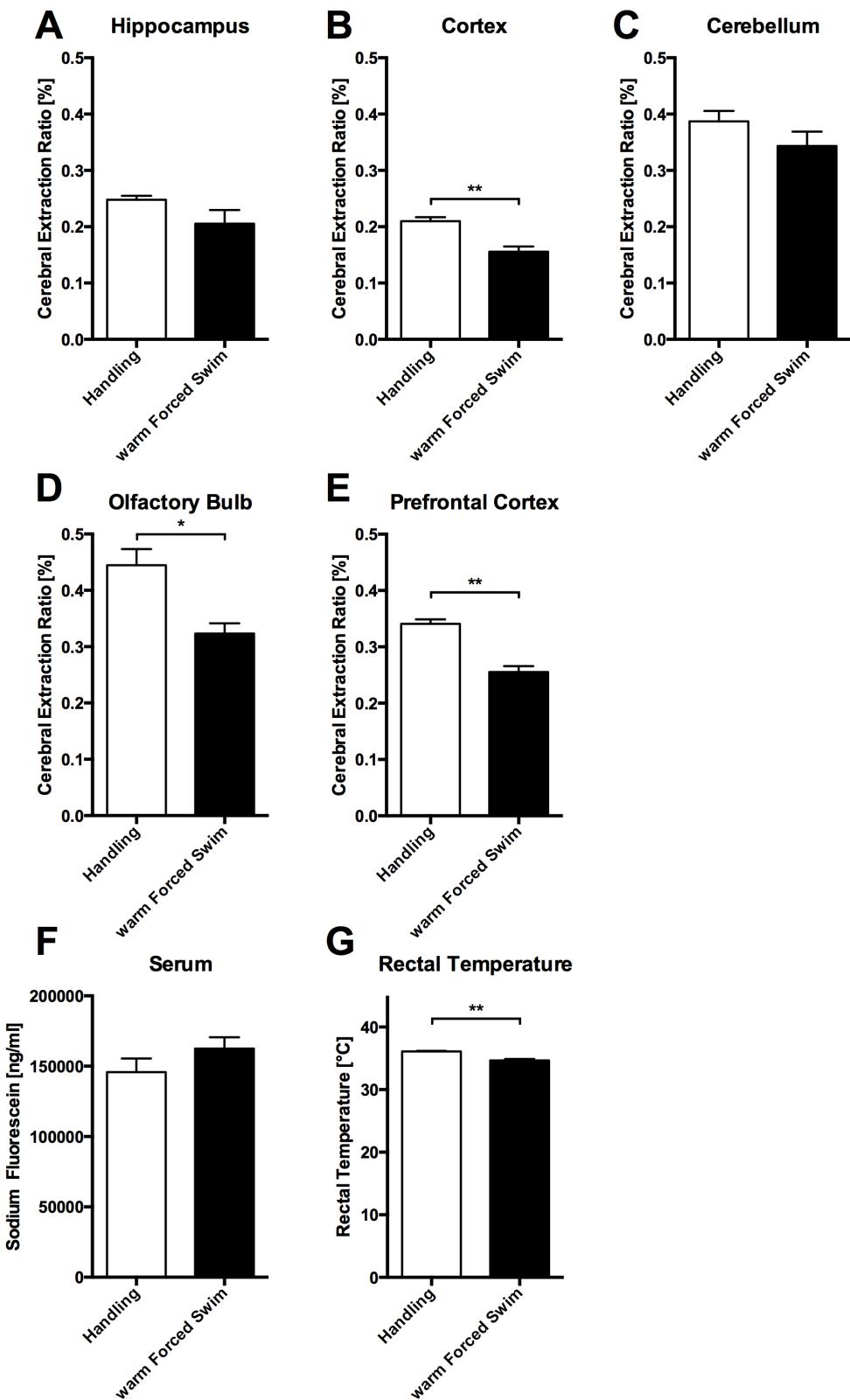


Figure 22: Male mice were subjected to 30min forced swim in 38 °C warm water. CER was decreased in cortex (B), olfactory bulb (D) and prefrontal cortex (E). A similar trend was apparent in hippocampus (A), cerebellum (C). Serum levels (F) between groups were similar. Rectal temperature (G) was decreased in the forced swim group. Number of subjects per group: n(Handling)=3; n(warm Forced Swim)=4. **=p<0.01; * =p<0.05

3.4.7.Blood-Brain Barrier Permeability Experiment 7

The fact that we did not observe changes in CER following several acute stress challenges, but a decrease in response to cold swim stress, we hypothesized that the cold temperature could explain the decrease in CER. To test this hypothesis, we subjected mice to a brief cold restraint to mimic the stress duration of the forced swim experiment in cold water. In contrast to forced swim stress, brief restraint stress is much less severe than swim stress and the physical activity of the animal is minimized.

Male mice were subjected to 6min restraint in a 50 ml Falcon tube. The tube was placed in a beaker with 18 °C cold water and partially immersed in the water. Animals from the control group were briefly picked up by their tail and then put back in their cage. Immediately after the restraint ended animals were i.p. injected with 40 mg/kg sodium fluorescein in 0.9% saline solution. After a circulation time of 10min animals were perfused and tissue and blood.

Similar to previous results in the forced swim in cold water experiments, decreased CER could be observed for hippocampus by 49% ($t(5)=3.35$, $p=0.020$), cortex by 40 % ($t(5)=2.63$, $p=0.047$), and olfactory bulb 47% ($t(5)=2.79$, $p=0.039$). Only a trend was observed for a decrease in the cerebellum ($t(5)=2.42$, $p=0.0598$), while no effect was observed in the prefrontal cortex ($t(5)=1.43$, $p=0.2121$). Body temperature was decreased by 6 °C ($t(5)=7.006$, $p<0.001$) and serum levels were similar between handling and restraint groups ($t(5)=1.49$, $p=0.197$) (see Figure 23).

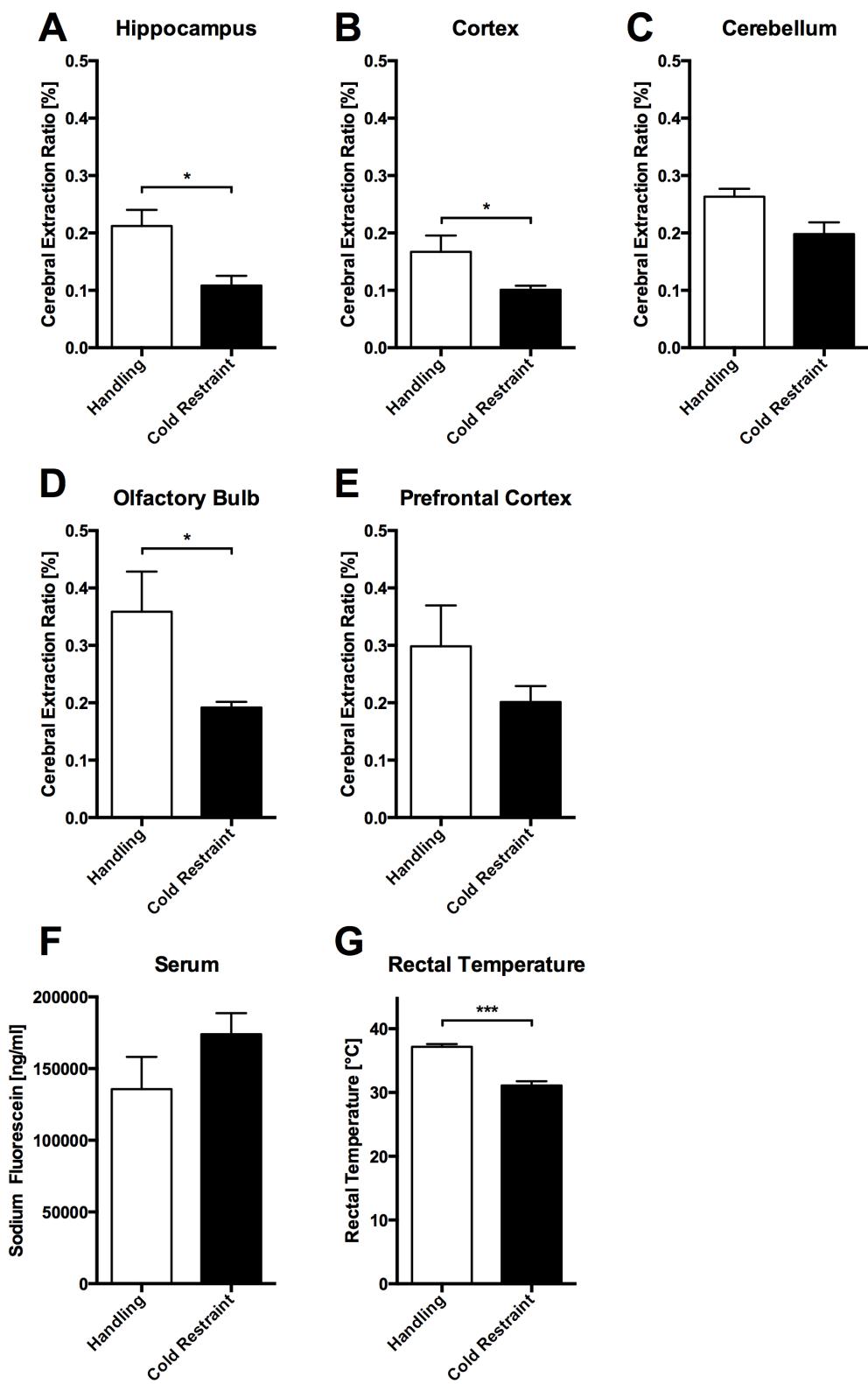


Figure 23: Male mice were restrained in a tube for 6min in 18 °C cold water. CER was decreased in hippocampus (A), cortex (B) and olfactory bulb (D). A similar trend was apparent in cerebellum (C) and prefrontal cortex (E). Serum levels (F) between groups were similar. Rectal temperature (G) was significantly decreased in the cold restrained group. Number of subjects per group: n(Handling)=3; n(Restraint)=4. ***=p<0.001; **=p<0.01; *p<0.05

4. Discussion

The work presented here characterizes for the first time the regulation of Apold1 in brain tissue following acute stress. We demonstrate that Apold1 gene expression is increased dramatically in several brain regions. Apold1 transcript levels are tightly regulated, increase shortly after stress exposure and peak 30-45min after initiation of stress, before returning to baseline between 90-120mins. We also demonstrate that the increase in Apold1 levels can be prevented by blocking β_2 -adrenergic receptors, suggesting an involvement of noradrenaline in the stress-induced regulation of Apold1.

Further, we present a series of studies that provide strong evidence against the hypothesis that acute stress can increase BBBP. We also demonstrate that cold-swim stress can lower the brain-uptake of sodium fluorescence and we identify cold temperature as the critical variable. Future research will need to focus on the relationship between this stress-induced decrease in fluorescence uptake following cold swim stress and the observed increase in Apold1 expression.

4.1. Apold1

Our findings, that Apold1 is rapidly increased after acute stress, shed new light on the possible role of this gene and its regulation. The peak in transcription at 45min and decrease to baseline levels within 120min supports previous findings that Apold1 is an immediate early gene (IEG) (Regard et al. 2004) with similar temporal expression pattern like other IEGs such as Fos or Cyr61.

We identified the β_2 -receptor antagonist ICI118,551 to be a potent inhibitor of stress-related Apold1 gene expression increase. This suggests that Apold1 gene expression is mediated by norepinephrine, specifically by the β_2 -adrenoreceptor pathway. While the systemic administration of an adrenergic receptor antagonist will certainly impact other physiological and cellular responses, it will be a useful tool to further study the functional role of Apold1. An important future experiment will be to inject ICI118,551 directly into the hippocampus to test if local administration can also block the stress-induced increase in Apold1 mRNA. Additionally, specific β_2 -adrenoreceptor agonists, such as salbutamol which can cross the BBB (Fischer, Gottschlich, and Seelig 1998), could be administered to test whether they lead to

increased gene expression of Apold1. A detailed quantification of norepinephrine levels in the hippocampus at different time points after stress exposure (see Figure 10 A) could give further insight in to the interaction between norepinephrine and Apold1 expression.

We detected increased transcript levels of Apold1 when MK801 was administered but a similar increase could be also observed in two other immediate early genes (see Figure 12 B and C) and therefore it was more likely a general cellular or systemic response to this drug and not specifically affecting Apold1 regulation. Further, it remains to be shown that at the selected doses MK-801 as well as the serotonin receptor 5-HT₂ antagonist volinanserin did successfully block their respective receptors in the hippocampus.

Our initial hypothesis that Apold1 might be involved in blood-brain barrier permeability is partly based on the assumption that Apold1 is predominantly expressed in endothelial cells of the vasculature (Regard et al. 2004). Thus far we could not confirm this observation because the anti-APOLD1 antibodies that we tested seemed to be unspecific against mouse APOLD1 (see Figure 13 and Figure 14). Obtaining a good antibody would allow us to test whether protein levels of Apold1 are indeed increased after stress, but also whether the protein is localized exclusively in endothelial cells or if it is also present in astrocytes or pericytes, which are part of the blood-brain barrier. A good antibody would further allow us to detect the subcellular localization using immunofluorescence imaging or if there are protein-protein interactions using a pull-down assay. Unfortunately, antibody design is expensive and time consuming. Mass spectrometry could be an alternative approach to address some of the questions stated above. While it would not enable us to determine with certainty where APOLD1 is localized, it could confirm whether the protein levels are increased after stress. Furthermore, it could provide information about possible post-transcriptional regulation of APOLD1 since phosphorylation sites are predicted (Regard et al. 2004). Another approach to further characterize the role of Apold1 would be to knock-down or knock-out Apold1 in cell culture and/or mice to screen for possible interaction partners by applying transcriptomics and proteomics.

4.2. Blood-Brain Barrier Permeability and Stress

The current scientific literature is split as to whether BBBP is affected by acute stress or not. While some studies report increased BBBP for dyes and drugs like pyridostigmine (Sarmento, Borges, and Azevedo 1991; Sarmento, Borges, and Lima 1994; Esposito et al. 2001; Sharma, Cervós-Navarro, and Dey 1991), subsequent studies failed to replicate the findings that stress promotes entry of pyridostigmine into the brain or that BBBP is increased due to stress (Ovadia et al. 2001; Park et al. 2008). Therefore, it remains controversial whether stress indeed alters BBB permeability, or whether other factors may account for the conflicting data.

Our results, that Apold1 expression is mediated by the adrenergic system (more specifically β_2 -adrenoreceptor pathway), and that Apold1 is involved in endothelial cell permeability (Regard et al. 2004), we hypothesized that Apold1 could play a role in the regulation of BBB, and may be involved in stress-induced alterations of BBBP. However, none of our experiments detected reliably any significant increase in cerebral extraction ratio (CER) in response to an acute stressors. In contrast, we detected a decrease in CER in stress paradigms during which the animals experienced a significant decrease in body temperature (see Figures 18, 22 and 23). One could argue that our method was simply not sensitive enough to detect changes in BBBP but our two method validations show conclusively that our experimental setup worked: As expected we saw increased CER after transcranial cold lesion in the targeted cortex area (see Figure 15). By comparing control animals after different time points of sodium fluorescein circulation, we detected a higher CER after longer circulation time (see Figure 16). These results fit the idea that a longer circulation time will lead to more sodium fluorescein passing through the blood-brain barrier in to the tissue and further supports the sensitivity of our method. Additionally, it also shows that our perfusion protocol was thorough, because if any sodium fluorescein leftover were present in the blood vessels, it would have contributed strongly to our detected fluorescence and resulted in similar CER independent of the circulation times.

In our first forced swim experiment (see Figure 17 A-C) we detected an increased cerebral extraction ratio but in this experiment the serum levels were lower in the forced swim group (see Figure 17 D). Keeping in mind that CER is calculated as the sodium fluorescein amount in the brain tissue relative to the sodium fluorescein amount in the serum, big differences in the serum levels between groups could give

a positive result even when the blood-brain barrier permeability is not changed. With the exception of one publication (Sharma et al. 1991), none of the previously mentioned publications that used dyes to detect changes in BBBP mentioned or showed dye levels in the blood or serum. This could potentially mean that previous publications failed to take into account that cerebral blood flow could be altered in their treatments and experimental groups. This would introduce a substantial bias in the reported cerebral extraction ratios.

We could not determine the cause for differences between groups in the serum levels of sodium fluorescein (see Figure 17 D). Initially we wanted to inject sodium fluorescein i.v. because we assumed that this would be the best way to deliver a precise amount of sodium fluorescein into the blood stream. We could not determine the cause for the difference in serum levels after isoflurane anesthesia. One possible explanation could be increased renal excretion or overall changes in liquid composition of the blood due to isoflurane anesthesia, but to our knowledge no studies have reported such effects. However, isoflurane is known to relax muscles and could therefore possibly also dilate blood vessels in the brain. Indeed studies conducted in mice, dogs and macaques have shown that isoflurane anesthesia increases CBF (Cucchiara, Theye, and Michenfelder 1974; Kehl et al. 2002; Li et al. 2014). The isoflurane anesthesia that we used for i.v. injections in combination with acute stress could therefore possibly have increased CBF which lead to increased CER (see Figure 17 A-C).

To avoid the difference in serum fluorescein levels, we chose to switch to an i.p. delivery of sodium fluorescein. One could again argue that the intraperitoneal absorption of sodium fluorescein is different in animals that were exposed to stress, but we did not detect differences in serum levels between groups. In subsequent blood-brain barrier experiments (2, 3, 6 and 7) we observed decreased CER despite comparable fluorescein levels in serum between groups. Even though we did not collect body temperature data in all those experiment it is apparent that mice exposed to cold water would have lower body temperature. It is possible that for some reason the pinocytosis, which is the main transport mechanism for sodium fluorescein through the BBB (Sarmento et al. 1991), gets slowed down due to decreased temperature but it would be very surprising if this effect was so big that it accounted for a 25% decrease (see Figure 19 A and B). A more reasonable explanation for our findings is the circumstance that heart rate and vasoconstriction

are highly dependent on the body temperature (Baker and Horvath 1964). We did not have the necessary equipment to measure these parameters in our experiments but previous findings in rats show that a loss of 5 °C in body temperature equaled a 25% decrease in heart rate, irrespective if animals were swimming or restrained (Baker and Horvath 1964). Such a decrease in heart rate would without a doubt be also reflected in a similar decrease in CBF. This could be a possible explanation why we did not see an increased CER in stressed animals that also lost body temperature. We propose to avoid the term BBBP, in the context of forced swim experiments where body temperature is often decreased, and instead use the broader term brain perfusion. Brain perfusion combines CBF and BBBP.

4.3. Possible Consequences of Decreased Brain Perfusion

In our experiments we show that brain perfusion appears to be decreased in stressful situations in combination with body temperature loss. In general, stressful situations lead to increased neuronal activity and therefore also an increased need for essential molecules such as water, gases, nutrients and other metabolites (Zheng et al. 2010). During a stressful situation in a cold environment this mismatch between reduced metabolite supply due to reduced CBF and increased demand will be more pronounced than under normal conditions. As a result, oxygen and energy supply in the brain could be impaired, although this is a hypothesis that remains to be tested. Out of all the affected metabolites, oxygen is probably the most critical one. In our forced swim experiments we could therefore inadvertently cause a temporal hypoxic condition in the brain.

Adequate oxygen supply in the brain is of utmost importance. Cells have to some degree the capability to switch to an anaerobic state when oxygen supply is impaired but prolonged oxygen deprivation usually causes cell damage and death. Local hypoxic conditions in brain tissue occur most often during a stroke and ischemia but global hypoxia can also be caused by impaired cerebral perfusion or decreased oxygen saturation in the blood.

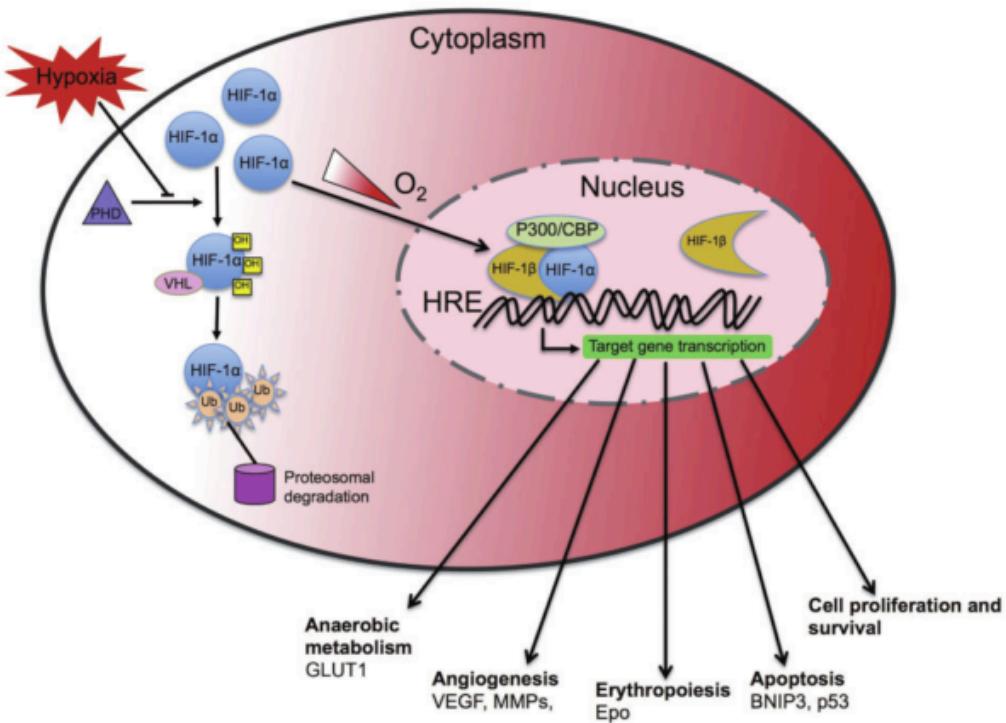


Figure 24: HIF-1 α is constitutively expressed and degraded through prolyl hydroxylase pathway. Decreased oxygen levels in the cell inhibit the degradation and HIF-1 α is stabilized. It translocates to the nucleus where it dimerizes with HIF-1 β . Binding to hypoxia-responsive elements (HRE) promotes transcription of specific genes. (Engelhardt, Patkar, and Ogunshola 2014)

Hypoxia inducible factors (HIF) have been identified to be master regulators of gene expression when a cell is deprived of oxygen. HIF-1 α is constantly expressed and degraded under normal conditions. When the oxygen concentration decreases this degradation is blocked and HIF-1 α is translocated to the nucleus. After it dimerizes with HIF-1 β it binds to hypoxia-responsive elements (HRE) (Adams et al. 2009). HREs are present in promoter regions of genes that are involved in the short and long term adaption to hypoxia. As a response to hypoxia vascular growth factors are secreted, cellular metabolism and overall gene expression are altered to minimize oxygen consumption (Engelhardt et al. 2014).

Not only could forced swim cause such a hypoxia response but there is also emerging evidence that stress and hypoxia response influence each other. Restraint induced an increase of norepinephrine in the periventricular nucleus of rats. This increase was further potentiated under hypoxic conditions (Chen, Du, and Wang 2004). In the other direction HIF-1 α increases the expression of PNMT in the adrenal medulla of rats (Tai et al. 2009). PNMT is involved in the biogenesis of epinephrine. These findings were further confirmed and HIF-1 α is implicated to be a possible

target to inhibit PNMT expression (Wong et al. 2010). The possible interaction between HIF-1 α and β_2 -adrenergic receptors pathway was also shown in mice exposed to chronic stress. They observed an up-regulation of several genes involved in tumor growth and angiogenesis which they could block by inhibiting HIF-1 α and β_2 -adrenergic receptor pathways (Shan et al. 2013). Norepinephrine-induced VEGF expression in cancer cells was also shown to involve HIF-1 α (Park et al. 2011). Experiments in cardiomyocytes show that specifically β_2 -adrenergic receptors activation is involved in the intracellular availability of oxygen (Li et al. 2010).

There are still a lot of missing pieces in understanding how these two systems are connected. Our antagonist experiments show that Apold1 gene expression increase is blocked when a specific β_2 -adrenergic receptor antagonist is injected. So far we did not check if the HIF pathway is triggered by our forced swim experiments but our BBBP experiments indicate that brain perfusion might be impaired, which in turn could also cause a temporary oxygen deficit.

4.4. Hypoxia and Apold1

As previously mentioned, activation of the HIF pathway leads to the up-regulation of a wide variety of genes involved in cellular energy metabolism, angiogenesis and erythropoiesis (red blood cell production) (Arrillaga-Romany, Reardon, and Wen 2014; Engelhardt et al. 2014).

Interestingly, previous studies have shown that Apold1 expression is rapidly increased by hypoxia and cerebral ischemia, which makes it a candidate for an immediate early gene (IEG) (Regard et al. 2004). So far only two other studies have taken a closer interest in Apold1. Both publications compared the outcome of stroke and ischemia between wild type and Apold1 knockout mice. In adult knockout mice less cerebral edema formation after stroke was reported (Liu et al. 2012). In neonatal Apold1 knockout mice, angiogenesis and neurogenesis in the ischemic hemisphere was reduced 30 days after stroke (Mirza et al. 2013). In ischemic tissue the oxygen supply is severely impaired which in turn will trigger the hypoxia-induced pathway. Hypoxia has been shown to promote the expression of Apold1 and the promoter region contains a possible target for hypoxia-inducible factor (Ortiz-Barahona et al. 2010). This prediction was confirmed and increased Apold1 transcription levels were detected in mouse hippocampus when exposed to an hypoxic environment (Xu, Lu, and Sharp 2011). Besides the hypoxia-induced gene expression increase in

hippocampus, Apold1 was also reported to be increased in retina tissue during hypoxia (Thiersch et al. 2008a), in brain and placenta of late-gestational mouse embryos (Trollmann et al. 2010), and in the brain of fetuses of mice that were treated with lipopolysaccharide (LPS) (Oskvig et al. 2012), which presumably results in fetal oxygen deprivation. Apold1 increase was also detected in a study about the hypoxia resistance in painted turtles (Bradley Shaffer et al. 2013). These turtles can hibernate and withstand long periods of low oxygen supply and Apold1 was massively up regulated in brain and heart tissue.

Although none of these data prove that Apold1 is up regulated through the hypoxia-induced pathway, it certainly raises the possibility that Apold1 plays a role in responding to hypoxic conditions and may be involved in the counter-regulatory cellular response to cope with reduced oxygen levels. Interestingly, some other genes that are prominently up-regulated by our cold swim stress paradigm such as Dusp1 (see Figure 8), Cyr61 and Sgk1 (see Figure 10 C and D), are also found to be up-regulated during hypoxia and possibly have a binding site for HIF in the promoter region (Lin et al. 2008; Liu et al. 2005; Ortiz-Barahona et al. 2010; Xu et al. 2011). Therefore, it could be of interest to take a closer look at the possibility that Apold1 and other genes that we identified to have altered gene expression after acute stress may be regulated by reduced oxygen supply and activation of HIFs. Such a relationship could have further implications for studies that use forced swim as a behavior readout and as a way to trigger stress response. Observed differences between control and treatment groups could be biased due to the proposed impact of cold and stress on CBF and oxygen supply.

Besides publications that used hypoxia as a model, Apold1 also shows up in energy metabolism and neuronal activity studies. In a human umbilical vein endothelial cell culture Apold1 increase, due to oxidative stress, could be inhibited with Exendin-4 treatment (Chien et al. 2014). Exendin-4 is a glucagon-like peptide-1 agonist involved in the regulation of glucose metabolism. In GRK2^{+/−} mice (GRK2 is a β-adrenergic receptor kinase) Apold1 was up regulated in the heart (Lucas et al. 2014). GRK2 is presumed to be involved in insulin signaling. Apold1 was shown to be up regulated in the barrel cortex of rats immediately as well as 4 hours after whisker stimulation in an enriched environment (Vallès et al. 2011). The same changes in transcription level could not be detected in other brain areas such as hippocampus, striatum, cortex or cerebellum. It appears that Apold1 might be up regulated in response to increased

neuronal activity and increased energy demand. Apolipoproteins are mainly known for their role in lipid transport in the blood but they are also involved in lipid transfer, uptake in tissues, receptor ligands and are cofactors of enzymes (Wang and Eckel 2014). Together with gene expression results from hypoxia (Ortiz-Barahona et al. 2010; Thiersch et al. 2008b; Xu et al. 2011), neuronal activity (Bowyer et al. 2013; Vallès et al. 2011) and glucose metabolism (Chien et al. 2014; Lucas et al. 2014) studies it is therefore conceivable that Apold1 may be involved in the intracellular energy metabolism.

5. Conclusion

The fact that Apold1 is the most strongly regulated gene identified by microarray following cold swim stress, as well as in the previously mentioned hypoxia-related publications, suggest that it plays a vital function in the immediate response to the environmental challenge. It is likely, that the observed reduction in brain perfusion observed after stress is intimately linked to the strong up regulation of Apold1. However, we still do not have enough information about Apold1 to deduct its precise function with certainty. Taking our findings and those from other publications together we propose a possible role of Apold1 in regulating energy metabolism during hypoxic conditions or conditions that challenge neuronal blood supply. These possibilities will need to be tested in future experiments.

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