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Induction of a neuroprotective phenotype of macrophages by the β-hydroxybutyrate receptor HCA_2

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Dedicated to my parents

Summary

Ketogenic diet is protective in models of ischemic stroke and neurodegenerative diseases. Currently, clinical trials are testing the efficacy of this diet in neurodegenerative diseases but its mode of action is still unclear. The ketone body β-hydroxybutyrate (BHB) is the endogenous agonist of the hydroxy-carboxylic acid receptor 2 (HCA_2, GPR109A) which is expressed in various immune cells; therefore, we tested the potential involvement of this receptor in a mouse model of ischemic stroke.

The protective effect of ketogenic diet and BHB was lost in *Hca2*$^{-/-}$ mice, demonstrating that HCA_2 receptors are responsible for neuroprotection. Similarly, nicotinic acid, a HCA_2 agonist, reduced the infarct size via a HCA_2-mediated mechanism. Immunohistochemical analysis of immune cells in *Hca2*^{mRFP} transgenic mice revealed HCA_2 expression in monocytes/macrophages. Bone marrow transplantation demonstrated that HCA_2 on monocytes/macrophages is required for the protective effect. Activation of HCA_2 receptors induced a neuroprotective phenotype of monocytes/macrophages that depended on PGD_2 production by COX-1 and the hematopoietic PGD_2 synthase. Our data reveal that HCA_2 activation by dietary or pharmacological means instructs monocytes/macrophages to carry a neuroprotective signal to the brain which can be used therapeutically.

Zusammenfassung

Eine ketogene Diät hat in Tiermodellen des ischämischen Schlagfalls und Modellen weiterer neurodegenerativer Erkrankungen einen protektiven Effekt. Aktuell wird auch in klinischen Studien die Effektivität einer ketogenen Diät bei neurodegenerativen Erkrankungen getestet. Der Wirkmechanismus ist jedoch bisher ungeklärt. Der Ketonkörper β-Hydroxybutyrat (BHB) ist der endogene Agonist des *hydroxy-carboxylic acid receptor 2 (*HCA_2 , GPR109A*)*, welcher in unterschiedlichen Zellen des Immunsystems exprimiert wird. In dieser Arbeit wird die Rolle des Rezeptors HCA_2 in einem Mausmodell des ischämischen Schlaganfalls untersucht.

Weder eine ketogene Diät, noch die Behandlung der Tiere mit BHB übten in *Hca*2-Knockout-Mäusen einen protektiven Effekt aus, was zeigt, dass die, in Kontrolltieren beobachtete, durch beide Behandlungen ausgelöste Neuroprotektion über den Rezeptor HCA_2 vermittelt wird. Analog dazu konnte auch der HCA_2-Agonist Nikotinsäure die Infarktgröße über einen HCA_2-abhängigen Mechanismus verringern. Eine immunhistochemische Analyse transgener *Hca2*^{mRFP}-Mäusen zeigte, dass *Hca*2 in Monozyten und Makrophagen exprimiert wird. Mittels Knochenmarktransplantation konnte demonstriert werden, dass *Hca2*-exprimierende Monozyten und Makrophagen für den protektiven Effekt verantwortlich sind. Die Aktivierung von HCA_2-Rezeptoren induzierte in Monozyten und Makrophagen einen neuroprotektiven Phänotyp, dessen Ausprägung abhängig von der Produktion von Prostaglandin D_2 (PGD_2) durch COX-1 und durch die hämatopoetische PGD $_2$-Synthase war. Zusammenfassend zeigt diese Studie, dass die Aktivierung von HCA_2 sowohl mittels einer Diät, als auch durch ein Pharmakon, Monozyten und Makrophagen dazu instruiert, ein neuroprotektives Signal in das

Gehirn zu übermitteln. Dieser Mechanismus stellt eine potentielle neue Therapiestrategie gegen neurodegenerative Erkrankungen dar.

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Introduction

1.1. Brain energy metabolism

The brain consumes more energy per mass unit than other tissues and has very limited energy stores; therefore it is highly dependent on a constant supply of nutrients. The brain receives approximately 15% of the cardiac output and consumes 25% of the body's energy although it encompasses only 2 % of the body's weight (Zauner et al, 2002). To generate ATP, a form of cellular energy, the brain uses more than 90% of its consumed oxygen (Zauner et al, 2002). Thus, uninterrupted blood supply is crucial for usual brain functions. Energy homeostasis is an important aspect of normal body function occurring as a consequence of a balance between energy intake and energy expenditure (Dupuis et al, 2011). The Brain uses energy that is produced mainly by oxidation of glucose to maintain ionic gradient across the cell membranes (Zauner et al, 2002). Maintenance of electrochemical gradients across the cell membrane is central for proper neuronal and glial function. Therefore, to maintain ionic gradients a substantial part of energy is used to fuel Na^+/K^+-ATPase activity even during the resting state (Shetty et al, 2012). Consequently, the cellular metabolic rate increases proportionately when activity increases (Zauner et al, 2002). Insufficient blood flow to the brain, such as occurs in stroke, leads to rapid loss of function and tissue demise. However, an altered energy balance seems to play a causal role in other neurodegenerative diseases as well, such as Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) (Dupuis et al, 2011; Exner et al, 2012). Under such circumstances, blood concentration of glucose and the ketone body β-hydroxybutyrate (BHB), the brain's main energy substrates, would be expected to affect a disease. However, the role of these two energy substrates in neurological

disorders is complex. Hyperglycemia aggravates rather than improves ischemic stroke and AD (Kruyt et al, 2010) but the basic mechanisms underlying this paradox are still unclear.

1.2. Cerebral ischemia and brain energy regulation

Cerebral ischemia

Reduction in blood flow to the brain results in altered cerebral function which eventually leads to cerebral ischemia. Stroke is of hemorrhagic origin which results from rapture of supplying blood vessels or ischemic where interrupted blood supply to the brain results from occlusion of a cerebral artery either by a thrombus or an embolus (Adibhatla et al, 2008). Global cerebral ischemia results when total blood flow to the brain is blocked due to events like cardiac arrest (Adibhatla et al, 2008).

Ischemic stroke is one of the leading causes of death and disability around the world. It negatively impacts not only the affected individuals but also the society as a whole (Flynn et al, 2008). The frequency of cerebral ischemia induced death is noteworthy in developing countries (Yepes et al, 2009).

Ischemic stroke is characterized by a ‘core’ where the central region of brain tissues rapidly undergoes infarction and ‘ischemic penumbra,’ the region surrounding the core where the blood flow reduces in a graded fashion due to the presence of collateral arteries (Smith, 2004). Ischemic penumbra gained more interest as a target to develop therapeutic intervention since the core is generally considered difficult to be rescued (Adibhatla et al, 2008).

Until now, thrombolysis with recombinant tissue plasminogen activator (tPA) and mechanical removal of the clot has been approaved by Food and Drug Administration (FDA,

www.fda.gov) for the treatment of patient with acute ischemic stroke (Yepes et al, 2009). However, treatment option with tPA is limited to a time window of 4.5 hours after the onset of stroke (Haile et al, 2011). Since presentation of stroke patients is often delayed in clinical practice and also because of comorbid diseases tPA therapy is effective in only about 5 to 10% of stroke patients (Lees et al, 2010). Therefore, further therapeutic intervention targeting wide patient groups is obligatory.

Cerebral ischemia and metabolic failure

Glucose is the major metabolic substrate in the brain. It is stored as glycogen. In brain, glycogen is found predominantly in astrocytes (Brown & Ransom, 2007). As soon as vascular occlusion ensues, brain tissues are deprived of glucose and oxygen and acidic metabolic byproducts start accumulating (Smith, 2004). As a result of substrate unavailability and decreased pH, mitochondrial electron transport chain activity diminishes leading to a sharp decline in ATP production (Smith, 2004). Na^+/K^+-ATPase activity fails due to lack of ATP resulting in increased intracellular Na^+ concentration. Continuous depolarization leads to an increase in intracellular Ca^{2+} and neurons release their transmitters nearby and at remote targets (Smith, 2004). Increased Ca^{2+} influx also damages mitochondria and thereby exaggerate energy failure even further (Smith, 2004). As glycolytic consumption increases significantly in this paradigm, tissues are in frantic need of glucose. Paradoxically, cellular damage increases distinctly when the tissue glucose level exceeds 16 to 20 mmol/L (Smith, 2004). In line with this glucose paradox, hyperglycemia aggravates human stroke (Bruno et al, 2002; Parsons et al, 2002).

1.3 Ketone bodies in brain energy regulation

β-hydroxybutyrate (BHB), acetoacetate (AcAc), and acetone are known as ketone bodies (Figure 1.1). The liver produces ketone bodies predominantly from β-oxidation of fatty acids and these ketone bodies are utilized by the brain and other organs as an alternative energy source in a situation when glucose utilization is compromised such as occurs in starvation and strenuous exercise (Laffel, 1999). Since the brain does not exploit fatty acids to generate energy when blood glucose levels drop, ketone bodies are the principal alternative fuel to meet the metabolic demand (Morris, 2005).

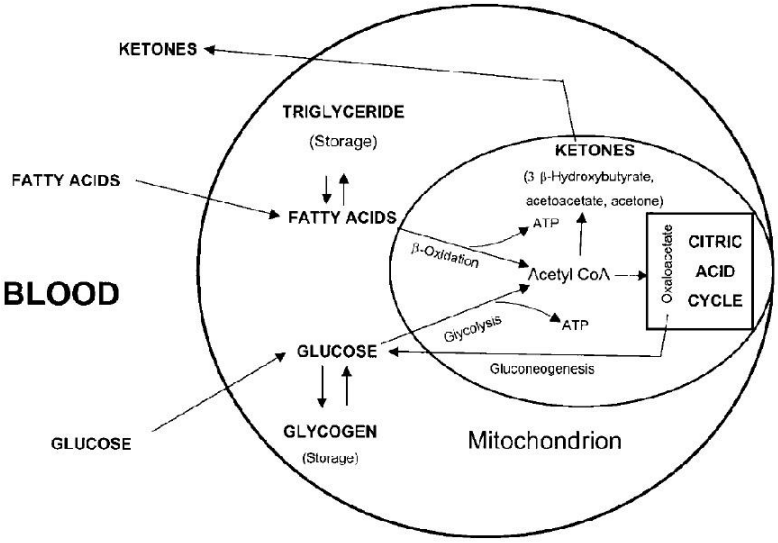


Figure 1.1. Formation of ketone bodies in hepatocytes. Adopted form Laffel et al. 1999. Descriptions are in the text.

Upon glycolysis and β-oxidation, acetyl CoA is formed in mitochondria of hepatocytes from glucose and fatty acid respectively which afterwards condensate with oxaloacetate and enter

into the citric acid cycle (Laffel, 1999). When glycolysis drops down to a very low level oxaloacetate is the privileged molecule being used in the process of gluconeogenesis and in this situation acetyl CoA is diverted from citric acid cycle and utilized to form ketone bodies since oxaloacetate is no more available to condense with acetyl CoA (Laffel, 1999).

1.4. Role of ketogenic diet and BHB in cerebral ischemia and other neurodegenerative diseases

Ketogenic diet

Ketogenic diet came in light during the 1920s as an option to treat epilepsy (Lutas & Yellen, 2013). It was launched to elevate the circulating concentration of ketone bodies. A classical form of ketogenic diet consists of fat to carbohydrates and proteins in a 4:1 ratio (Lutas & Yellen, 2013). With the advent of the first modern anticonvulsant diphenylhydantoin during the 1930s, ketogenic diet was promptly ignored in clinical practice despite of its reported efficacy in controlling epileptic seizures. However, during the 1990s, ketogenic diet regained its importance to treat pharmacoresistant childhood epilepsy since a substantial number of patients fail to get relief significantly from seizure with available anticonvulsants (Lutas & Yellen, 2013). Apart from epilepsy, protective functions of ketogenic diet have also been reported in animal models of stroke, Parkinson's disease, Alzheimer's disease and ALS (Lutas & Yellen, 2013; Prins, 2008; Puchowicz et al, 2008). Moreover, small clinical trials suggest that ketogenic diet is also effective in neurodegenerative diseases (Stafstrom & Rho, 2012). Currently, larger clinical trials are under way to test ketogenic diet in neurodegenerative diseases (clinicaltrials.gov, NCT01035710, NCT01016522, NCT01364545). Several mechanisms have been described that explain the antiepileptic

efficacy of a ketogenic diet but the mechanisms underlying its neuroprotective activity and the possible improvement in energy balance have not yet been elucidated (Gasior et al, 2006a; Lutas & Yellen, 2013; Prins, 2008).

β-hydroxybutyrate

BHB is a small water soluble carboxylic acid and an endogenous ligand to hydroxy-carboxylic acid receptor 2 (HCA_2, GPR109A) (Taggart et al, 2005). Being one of the major ketone bodies produced in the body during condition when glucose is unavailable as energy substrate, BHB has gained a lot of research interest in focal cerebral ischemia and in other CNS diseases (Puchowicz et al, 2008; Suzuki et al, 2002; Suzuki et al, 2001; Tieu et al, 2003). Suzuki et al. reported that BHB had protective effects on cerebral hypoxia, anoxia, and ischemia-induced metabolic changes. By using an animal model of cerebral ischemia they also demonstrated that BHB reduced the infarct volume as well as cerebral edema although the detailed mechanism underlying this effect was not clear (Suzuki et al, 2002). Long ago in 1968, Senior and Loridan found that under fasting conditions, BHB produced from free fatty acids in the liver reduces the release of free fatty acids from adipose tissue in a negative feedback loop (Senior & Loridan, 1968).This important homeostatic function is believed to be mediated by the HCA_2 receptor, that is activated by BHB on adipocytes (Taggart et al, 2005).

1.5. Hydroxy-carboxylic acid receptor 2 (HCA_2) and its ligand nicotinic acid

1.5.1. HCA_2 receptor

HCA_2 is a $G_{i\alpha}$ protein-coupled receptor and member of Class A rhodpsin-like GPCRs (Gille et al, 2008). After cloning in 1993 it was listed as an orphan receptor (Hanson et al, 2012). However, several groups in 2003 reported HCA_2 as a receptor for nicotinic acid (Soga et al,

2003; Tunaru et al, 2003; Wise et al, 2003) which was also referred to as GPR109A, HM74A in human, and PUMA-G in mice (Gille et al, 2008). HCA_2 is activated by BHB, nicotinic acid, and related drugs (Gille et al, 2008; Taggart et al, 2005).

Brown and white adipose tissue express HCA_2 (Gille et al, 2008). It is also expressed significantly in spleen and immune competent cells, specifically monocytes, macrophages, dendritic cells, and neutrophils (Gille et al, 2008). The expression pattern of HCA_2 in immune cells is regulated by various cytokines. GM-CSF up-regulate the expression of HCA_2 in neutrophils (Yousefi et al, 2000) where as in other immune cells, IFNγ increases its expression (Gille et al, 2008; Maciejewski-Lenoir et al, 2006; Schaub et al, 2001). In addition, expression of HCA_2 in the brain has been reported but the cellular localization was unknown (Miller & Dulay, 2008).

1.5.2. Metabolic effect of HCA_2 activation

Since HCA_2 is a $G_{i\alpha}$ protein-coupled receptor, its activation on adipocytes inhibits adenylyl cyclase activity which results in decreased intracellular cAMP levels (Figure 1.2) (Gille et al, 2008). Conversely, receptors that are G_s protein-coupled such as β-adrenergic receptors (β-AR) would increase cAMP level by increasing adenylyl cyclase activity. Therefore, HCA_2 activation by its ligand nicotinic acid counteracts the activity of β-adrenergic receptors as well as its protein kinase A (PKA) stimulatory effects (Gille et al, 2008). By phosphorylation of various proteins such as hormone sensitive lipase (HSL) and perilipin, PKA increases lipolysis. Since free fatty acids (FFA) are a substrate for hepatic triglyceride (TG) synthesis, substrate shortage occurs as a result of decreased FFA concentration in blood induced by HCA_2 activation (Gille et al, 2008). Consequently, the liver produces less triglycerides and

very low density lipoprotein (VLDL), which eventually leads to reduced plasma levels of triglycerides, VLDL, and low density lipoprotein (LDL) (Gille et al, 2008).

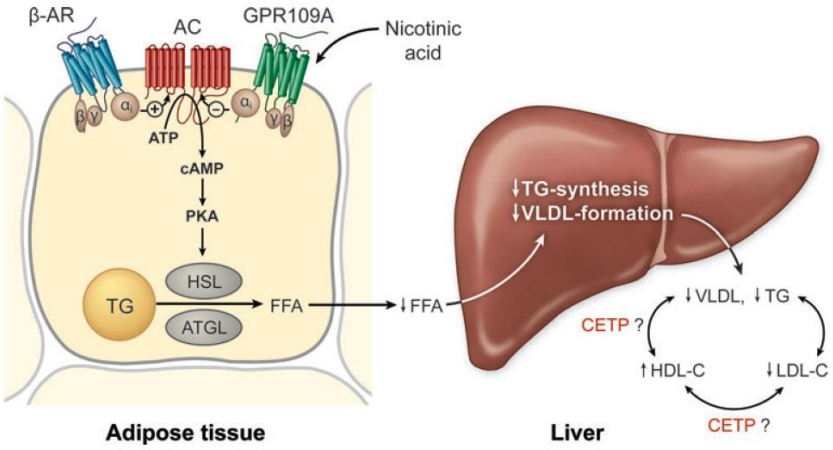


Figure 1.2. Metabolic role of HCA_2 activation. Adopted from Gille et al, 2008. Descriptions are in the text (CETP- Cholesteryl ester transfer protein, AC- Adenylyl cyclase, LDL-C- Low density lipoprotein cholesterol, HDL-C- High density lipoprotein cholesterol).

1.5.3. Nicotinic acid

Nicotinic acid is a water-soluble vitamin of the group B complex (B_3) and acts as a precursor of coenzymes NAD and NADP (Gille et al, 2008). In the beginning, nicotinic acid was used to treat pellagra, a disease caused by chronic deficiency of vitamin B_3 (Hanson et al, 2012). Rudolf Altschul et al. first described the lipid modifying properties of nicotinic acid when administered in gram quantities (Altschul et al, 1955). Since then extensive research has been carried out on nicotinic acid and its lipid lowering effects, which eventually introduced it into

clinical practice as a promising therapeutic intervention to treat dislipidemia and cardiovascular risk (Carlson, 2005; Hanson et al, 2012). The plasma lipid lowering effect of nicotinic acid is mediated by HCA_2 receptors as demonstrated by using mice lacking the gene that encodes HCA_2 (Tunaru et al, 2003). Nicotinamide is the vitamin counterpart of nicotinic acid. Nicotinic acid and nicotinamide together are called niacin (Gille et al, 2008). However, nicotinamide has no effect on plasma lipid levels and is inactive at HCA_2 even though both are chemically quite similar and serve as precursors for the coenzyme nicotinamide adenine dinucleotide (Carlson, 2005). This indicates that the carboxylic acid moiety of the ligand is critical in activating HCA_2 receptors (Gille et al, 2008). Being a potent 'broad-spectrum lipid lowering drug' (Carlson, 2005) nicotinic acid not only reduces total cholesterol, triglyceride, VLDL, and lipoprotein (a), but till now it is the most effective available lipid modifying drug that has been shown to raise high-density lipoprotein (HDL) as well (Kamanna & Kashyap, 2008). However, the mechanism by which nicotinic acid increases HDL cholesterol is still unknown. More recently, Lukasova et al. reported that activation of HCA_2 on immune cells by nicotinic acid is beneficial in reducing the progression of atherosclerosis and that this anti-inflammatory property mediated by HCA_2 is independent of its lipid modifying effect (Lukasova et al, 2011b). Nicotinic acid has been reported to be protective in cerebral ischemia although contributory mechanisms remained to be revealed (Shehadah et al, 2010b; Yan et al, 2012; Ye et al, 2011).

1.6 Inflammation in cerebral ischemia and the role of immune cells

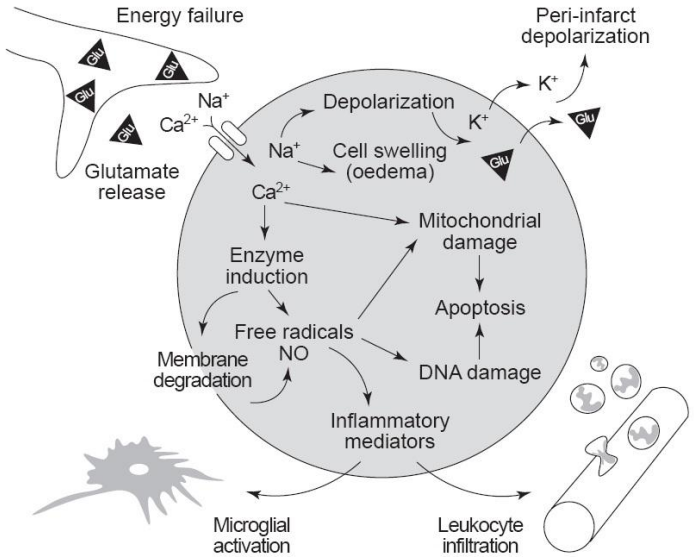
Inflammation is an integral part of the body's defense mechanisms and plays distinct functions upon injury and invasion of foreign particles and microbes. In response to injury,

vascularized tissues of the body initiate a characteristics cascade of responses manifested by changes in local blood vessels and exudation of plasma and circulating blood cell ultimately leading to inflammation (Zheng & Yenari, 2004). The pathophosiologic role of inflammation as a a key contributor in cerebral ischemia has been recognized more and more (Iadecola & Anrather, 2011). Elements of the immune systems are substantially involved in the ischemic cascade ranging from initial acute vascular events to ultimate brain damage and subsequent tissue repair that occurs at later time points (Iadecola & Anrather, 2011). Therefore, therapeutic interference targeting inflammation warrants careful consideration.

Pro-inflammatory signals can be generated within minutes after ischemic events (Iadecola & Anrather, 2011). Neurons are depolarized as a result of energy failure (Figure 1.3). Specific glutamate receptors become active and increase the intracellular concentration of Ca^{2+} and Na^{+} dramatically while at the same time release K^{+} into the extracellular space (Dirnagl et al, 1999). Diffusion of K^{+} and glutamate into the extracellular space results in peri-infarct depolarization. In parallel the second messenger Ca^{2+} increases the activation of proteolytic enzymes (Dirnagl et al, 1999). Free radicals are generated and damage DNA, mitochondria, and membranes, which in turn initiates apoptosis and induces the formation of inflammatory mediators (Figure 1.3). This activates microglia and induces peripheral immune cell infiltration into the ischemic brain (Dirnagl et al, 1999).

With the progress of the ischemic cascade cell death ensues leading to another stage of inflammation. Danger signals are released by the dying and dead cells leading to the activation of purinergic receptors on microglia and macrophages which in turn results in the production of pro-inflammatory cytokines (Iadecola & Anrather, 2011). Also damage-

associated molecular pattern molecules (DAMPs) are released by ischemic cell death which activate toll like receptors (TLRs) and subsequently up-regulate pro-inflammatory gene



expression (Iadecola & Anrather, 2011).

Figure 1.3. Pathophysiologic mechanisms leading to inflammation in focal cerebral ischemia. Adopted from Dirnagl et al. 1999 (Dirnagl et al, 1999). Explanations are in the text

Ischemic brain damage can be aggravated by inflammation in many ways. The degree of ischemia can be worsened by neutrophil-mediated microvascular obstraction (del Zoppo et al, 1991). In ischemic brain neutrophils produce iNOS, which is responsible for toxic amounts of NO having substantial pathogenic potential (Dirnagl et al, 1999). Cyclooxygenase 2 (COX-2) has been reported to be expressed by ischemic neurons. By producing superoxides and toxic prostanoids this enzyme contributes to ischemic injury (Dirnagl et al, 1999). The cytokine TNF is also able to intensify ischemic damage although a beneficial role in ischemic brain has

been reported as well (Barone et al, 1997; Bruce et al, 1996). The activated microglia has also been demonstrated to produce neurotoxins including NO, reactive oxygen species, and toxic prostanoids (Dirnagl et al, 1999).

1.7. Inflammation in post-ischemic repair mechanisms

Since post-ischemic inflammation is a dynamic as well as 'self-limiting'process, over time it ceases and participates in tissue remodeling and reconstruction following brain injury (Iadecola & Anrather, 2011; Zheng & Yenari, 2004). The exact mechanisms by which the resolution of inflammation and remodeling of the injured brain tissue happen are not completely understood. Clearing of dead cells and suppression of inflammation have been described as key events involved in this repair process (Iadecola & Anrather, 2011).

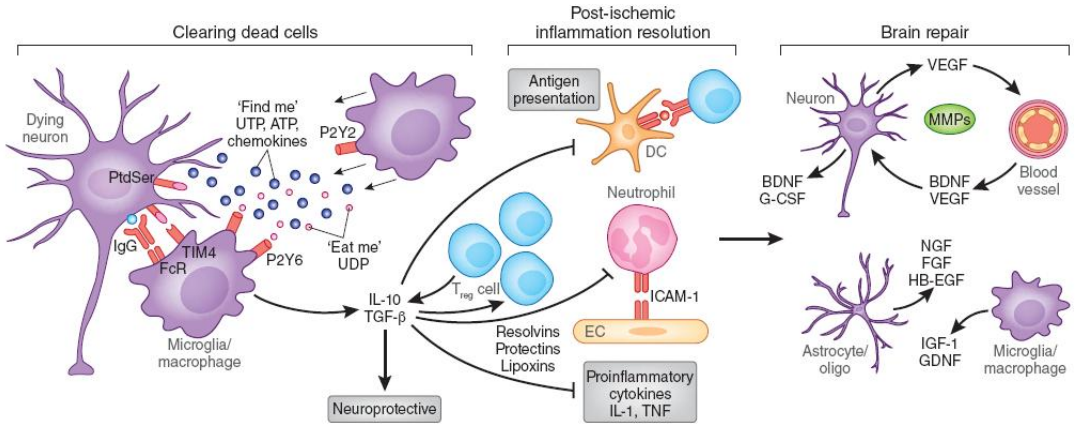


Figure 1.4. Repair process and resolution of inflammation after injury. Adopted and modified from Iadecola and Anrather et al. 2011(Iadecola & Anrather, 2011)

Microglia and infiltrating macrophages are the main cell types involved in removal of dead cells and tissue debris after stroke (Figure 1.4). After injury, microglia and macrophages are attracted to the site of injury by 'find-me' signals (UTP, ATP) and chemokines (Iadecola &

Anrather, 2011). Microglial phagocytosis is then stimulated by UDP 'eat-me' signals acting through P2Y6 receptors as well as phosphatidylserine (PtdSer) translocated to the outer membrane of apoptotic cells (Iadecola & Anrather, 2011). PtdSer-binding protein on microglia and T cell immunoglobulin and mucin domain-containing molecule 4 (TIM4) on macrophages are involved in clearing the dead cells (Figure 1.4). Phagocytosis induced TGF-β and IL-10 production play a central role in developing an anti-inflammatory environment coupled with tissue repair. Production of these cytokines results in suppression of antigen presentation, and pro-inflammatory cytokines while promoting T_{reg} formation (Iadecola & Anrather, 2011). However, TGF-β and IL-10 themselves are neuroprotective and may have the potential to facilitate the repair process. Metabolites of arachidonic acid may also dampen post-ischemic inflammation (Iadecola & Anrather, 2011). The post-ischemic repair process and tissue reorganization are achieved by the production of growth factors which have been reported to offer a favorable setting for neuronal sprouting, neurogenesis, angiogenesis, gliogenesis, and matrix reorganization (Iadecola & Anrather, 2011). For instances, neuronal sprouting after ischemia is attributed to insulin-like growth factor 1 (IGF-1), the full expression of which crucially depends on microglia (Figure 1.4) (Iadecola & Anrather, 2011).

1.8. The role of macrophages in cerebral ischemia

After neutrophils infiltrate the ischemic brain in the first place with a peak at 24-48 hours after ischemia, monocytes/macrophages are the decisive cell type at later stages of infarction (Iadecola, 1997).

1.8.1. The origin of macrophages

Macrophages are phagocytic cells and one of the important immune effectors that are involved in the body's defense mechanisms. They are involved in the process of removing cellular debris produced during tissue remodeling as well as efficient clearance of apoptotic cells (Mosser & Edwards, 2008). This clearing process could take place without the interference of immune cells since removal of apoptotic cells by unstimulated macrophages appears to result in little or no production of immune mediators (Mosser & Edwards, 2008). However, while removing necrotic debris, macrophages elicit inflammatory signals resulting in the production of cytokines and pro-inflammatory mediators (Mosser & Edwards, 2008).

Haematopoietic stem cells (M-CFU, myeloid colony-forming units) give rise to myeloid cells in the bone marrow of mature adults or in the yolk sac of the developing embryo (Lawrence & Natoli, 2011). First, the macrophage colony stimulating factor-1 (CSF-1) dependent macrophage and dendritic cell progenitor (MDP) is derived from M-CFU. This then gives rise to pro-monocytes (Lawrence & Natoli, 2011). In contrast to brain resident microglia and skin Langerhans cells, blood monocytes and most other tissue macrophages are developed from these pro-monocytes (Lawrence & Natoli, 2011). Derived from the yolk sac of developing embryos, microglial and Langerhans cells develop initially from M-CFUs. These radiation resistant cell populations are believed to be maintained by local proliferation (Lawrence & Natoli, 2011). In mice, two main subsets of monocytes have been described based on their expression of cell surface markers as well as the time they spend in the blood before migrating to the tissues (Auffray et al, 2009; Mosser & Edwards, 2008). $LY6C^-$ blood monocytes seem to be the source of most tissue macrophages and to be involved in patrolling blood vessels under homeostatic conditions (Auffray et al, 2009; Mosser & Edwards, 2008).

However, during inflammation, $LY6C^+$ blood monocytes are recruited and differentiate into inflammatory macrophages (Lawrence & Natoli, 2011). Upon the influence of environmental factors, inflammatory macrophages polarize into subtypes having specific phenotypes such as M1 or M2 macrophages. Nevertheless, resident tissue macrophages also have the potential to polarize into specific activation states during inflammation (Lawrence & Natoli, 2011).

1.8.2. Dual role of macrophages

Having a remarkable plasticity, macrophages can attain distinctive functional phenotypes in order to respond competently to micro-environmental signals. Two classes of macrophages have been described based on polarized phenotypes. Classically activated macrophages are designated as M1 and M2 represents the macrophages that are alternatively activated (Lawrence & Natoli, 2011).

Upon stimulation with interferone –γ (INF-γ) and TNF, cell-mediated immune responses generate classically activated M1 macrophages having increased microbicidial or tumoricidal capacity (Mosser & Edwards, 2008). Hence, classically activated macrophages are crucial for host defense. Since M1 macrophages secret high levels of pro-inflammatory cytokines and mediators, accurate titration of their activation must be maintained to prevent host-tissue damage (Mosser & Edwards, 2008).

Interleukin-4 (IL-4) and IL-13 induce M2 macrophage activation (Gordon, 2003), which is associated with anti-inflammatory and homeostatic functions in wound healing, fibrosis, and tissue repair (Lawrence & Natoli, 2011). In mice, these macrophage subtypes significantly express arginase1, macrophage mannose receptor 1(Mrc1/CD206), resistin-like-α (Fizz1/Retnla), chitinase 3-like 3 (Ym1), and IL-10 (Lawrence & Natoli, 2011).

In the acute stage, infiltration of immune cells to the ischemic brain leading to tissue damage is well known (Iadecola & Anrather, 2011). Conversely , they also contribute to tissue remodeling and reconstruction processes following brain injury and are critical for the integrity of the neurovascular unit (Gliem et al, 2012a) (Iadecola & Anrather, 2011). M2 macrophages have been described by several authors as beneficial after cerebral ischemia (Frieler et al, 2011; Fumagalli et al, 2013; Hu et al, 2012; Xu et al, 2012). Therefore, treatment strategies targeting infiltrating macrophages should critically consider the M1/M2 dynamics.

1.9. Cyclooxygenase 1 (COX-1) and prostaglandin D_2 (PGD_2) in neuroinflammation

Inflammation has been recognized as one of the key contributor to various neurological and neurodegenerative diseases (Choi et al, 2009). Activation of microglia/macrophages has been described clinically and experimentally following ischemia (Liu et al, 2009). Microglia and infiltrating macrophages are involved in secondary infarct expansion since they produce several chemokines and pro-inflammatory mediators (Liu et al, 2009). Bone marrow-derived macrophages and activated microglia also produce arachidonic acid (AA) and its lipid metabolites, which have been reported to play a critical and differential role in neuroinflammation (Choi et al, 2009; Zhao et al, 2013b).

Prostaglandins (PGs) are 20-carbon polyunsaturated fatty acids derived from AA (Joo & Sadikot, 2012). When various hormones interact with their cell surface receptors, prostaglandin synthesis is initiated through the activation of one or more cellular lipases, specially phospholipae A_2 (PLA_2) (Smith, 2002). Activation of PLA_2 leads to the release of arachidonic acid from membrane phospholipids. COX-1 and COX-2 convert arachidonic acid

into PGG_2 and PGH_2 (Harris et al, 2002). Subsequently, PGH_2 is converted into a series of prostaglandins such as PGD_2, PGE_2, PGI_2, and $PGF_{2\alpha}$ by the action of cell specific enzymes, the prostaglandin synthases (Harris et al, 2002). For instances, PGD_2 is synthesized from PGH_2 by the enzyme prostaglandin D synthase.

PGD_2 is the most abundant prostaglandin in brain and appears to be involved in maintaining homeostatic functions (Joo & Sadikot, 2012). Although mast cells in peripheral tissues mainly produce PGD_2, it can be produced by other leukocytes as well (Ricciotti & FitzGerald, 2011). The rate limiting enzyme in the process of PGs generation is COX (Choi et al, 2009). Two distinct isoforms of COX enzymes have been described, namely COX-1 and COX-2. In the central nervous system, tissue distribution and privileged pairing with upstream and downstream enzymes differ between these two isoforms (Choi et al, 2009). COX-1 is expressed constitutively in most tissues and believed to be responsible for synthesizing PGs in homeostatic conditions, whereas COX-2 is an inducible enzyme involved mostly in regulation of inflammation (Harris et al, 2002).

In the brain, COX-1 is expressed constitutively in microglia and endothelial cells (Choi et al, 2009; Depboylu et al, 2011). Depboylu et al. reported its expression on macrophages and multinucleated giant cells upon SIV infection. The role of COX-1 in cerebral ischemia is controversial. Iadecola et al. reported increased susceptibility to focal cerebral ischemia upon genetic deletion of *Cox-1* (Iadecola et al, 2001b), whereas pharmacologic inhibition of COX-1 resulted in reduced neuronal injury and oxidative stress during transient global cerebral ischemia (Candelario-Jalil, 2003). Therefore, manipulation of COX-1 signaling to combat brain inflammation requires further investigations.

Downstream of COX-1, prostaglandin D synthase catalyzes the production of prostaglandin D_2 from its precursor PGH_2. Lipocalin-type prostaglandin D synthase (L-PGDS) and hematopoietic prostaglandin D synthase (HPGDS) are the two prostaglandin D synthase enzyme that have been identified (Taniguchi et al, 2007b). In the brain, oligodendrocytes express L-PGDS, whereas HPGDS is expressed by microglia (Taniguchi et al, 2007b). HPGDS has also been reported to be expressed in dendritic cells, Langerhans cells, mast cells, Th2 cells, and megakaryoblasts (Joo & Sadikot, 2012). A neuroprotective role of HPGDS in focal cerebral ischemia as well as in hypoxic ischemia has been demonstrated (Liu et al, 2009; Taniguchi et al, 2007b)

Under physiological conditions, PGD_2 is involved in regulation of sleep and body temperature, olfactory function, hormone release, and nociception in the central nervous system (Joo & Sadikot, 2012). On the contrary, several studies provide evidence that it is an important mediator of inflammation (Taniguchi et al, 2007b). PGD_2 exerts its effect by interacting with its two receptors DP1 and DP2. DP1 is a Gs protein-coupled receptor and has been reported to be expressed in neurons and endothelial cells (Taniguchi et al, 2007b) as well as in dendritic cells, platelets, and bronchial and vascular smooth muscle cells (Pettipher et al, 2007). Alternatively, DP2 is a G_i protein-coupled receptor expressed in Th2 lymphocytes, eosinophil, basophil, and neural cells (Pettipher et al, 2007). Both receptors are involved in mediating diverse biological response. Taniguchi et al. reported a neuroprotective role of PGD_2 which was mediated by the DP1 receptor in hypoxic-ischemic injury (Taniguchi et al, 2007b). Deletion of the DP1 receptor increased the susceptiblity of mice to brain damage induced by middle cerebral artery occlusion (MCAO), while pharmacological activation of DP1 receptor exerted beneficial effects in transient cerebral ischemia (Ahmad et al, 2010).

Aim of the study

The G protein coupled receptor HCA_2 is expressed on immune cells in addition to adipocytes. BHB is an endogenous ligand to this receptor and regulates the release of free fatty acid under fasting conditions. This important homeostatic function is thought to be mediated by HCA_2. Although ketogenic diet and BHB have been reported to be protective in stroke, underlying mechanisms are not completely understood. HCA_2 receptors are also stimulated by nicotinic acid and related drugs that are in clinical use to lower plasma lipids and protect against atherosclerotic disorders. Therefore, in the current study we aimed to investigate the effect of HCA_2 activation in cerebral ischemia by addressing the following aspects:

1. Which cells express HCA_2 in the brain?
2. Does HCA_2 mediate the neuroprotective effect of ketogenic diet and BHB?
3. Does HCA_2 mediate the neuroprotective effect of nicotinic acid in cerebral ischemia?
4. Do hemotapoietic cells mediate the neuroprotective effect of nicotinic acid?
5. Which macrophagic cell population infiltrates the brain?
6. Does nicotinic acid affect the polarization of macrophages after cerebral ischemia?
7. Does COX-1 mediate the neuroprotective effect of nicotinic acid in cerebral ischemia?
8. Does HPGDS mediate the neuroprotective effect of nicotinic acid in cerebral ischemia?

2. Materials and methods

2.1 Materials

2.1.1. Reagents

Reagents	Company
2,2,2-Tribromoethanol	Sigma-Aldrich, Steinheim
2 Methyl 2-butanol	Sigma-Aldrich, Germany
Acetone	Merck, Darmstadt, Germany
Alzet pumps (2001D)	Cupertino, CA
Ammonium chloride	Merk, Germany
Ammonia solution 25%	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	Roth , Karlsruhe, Germany
Cell strainer, 40 µm	BD Biosciences, Germany
Cell lysis solution	Applied bio system, UK
Collagenase A	Roche, Mannheim, Germany
DAPI	Sigma-Aldrich, Deisenhofen, Germany
DMEM	Invitrogen, Germany
DNAse	Roche, Mannheim, Germany
Dulbecco's phosphate-buffered saline (dPBS)	Invitrogen, Germany
EDTA, disodium ethylenediamintetra- acetate	Merck, Darmstadt, Germany
Formaldehyde	Merck, Darmstadt, Germany
Heparin, 5000 U/ml	Braun, Melsungen
HQL-79	Tocris bioscience , R & D system

Hydrochloric acid, HCl 37%	Merck, Darmstadt, Germany
Hydroquinone	Fluka, Buchs
ssniff EF R/M ketogenic diet with 80% fat, long-chain fatty acid	Ssniff Spezialdiäten GmbH, Germany
Lithium carbonate	Riedel-de Haen, Seelze
Methyl cellulose	Fluka, Germany
Mowiol	Merck, Darmstadt, Germany
Nucleic acid purification lysis solution	Applied biosystem
Nicotinic acid	Sigma-Aldrich, Germany
O.C.T freezing medium	Leica Microsystems, Nussloch
Paraformaldehyde	Merck, Darmstadt
Platinum SYBER Green qPCR supermix	Invitrogen
Polysine slides	Thermoscientific
Potassium bicarbonate	Merck ,Germany
Ringer's solution	Braun, Melsungen
Safety-multifly-set	Sarstedt, Germany
Saline , physiological	Diaco, Triest
Silver nitrate	Riedel-de Haen, Seelze
Sodium citrate	J.T. Baker, Deventer
Sodium hydroxide	Carl Roth, Karlsruhe
Titration complex	Roth, Germany
Triton X 100	Promega, USA
β-hydroxybutyrate	Sigma-Aldrich, Germany

2.1.2. Equipments

Device	Company
6100 Nucleic Acid Prepstation	Applied biosystem
ABI prism 7000 sequence detection system	Applied Biosystem
Centrifuge 2.ORS	Heraeus , Sepatech
Coagulator ERBE ICC50	Erbe, Tübingen, Germany
Cryostat CM 3050	Leica, Nussloch, Germany
Driller Proxxon micromot 50/F	Proxxon, Luxemberg
Fluostar Optima	BMG Labtech
MoFlo Legacy, 100 µm nozzle, 20 psi	Beckman Coulter, Krefeld, Germany
Nanodrop 2000 spectrophotometer	Thermo scientific
Olympus AU 400 analyzer	Beckman Coulter, Krefeld, Germany
Scanner CanoScan 9000F	Cannon, Krefeld
Surgery microscope, Hund SM33	Wetzlar, Germany
Temperature control module TKM-0902	FMI, Seeheim-Ober Beerbaach
Ultra-turrax T8	Werner Hassa
Water Bath	B. Braun, Melsungen AG

2.1.3. Antibodies

Primary antibodies

Antibody	Host/Type	Dilution	Company
CD11b	Rat/monoclonal	1:100	AbD Serotec
GFAP	Rabbit/polyclonal	1:500	DAKO
Iba1	Rabbit/polyclonal	1:100	Wako
NeuN	Mouse/monoclonal	1:500	Chemicon

Secondary antibodies

Antibody	Host	Conjugate	Dilution	Company
Anti-Rabbit	Donkey	Alexa 488	1:400	Invitrogen
Anti-Rat	Donkey	Alexa 488	1:400	Invitrogen
Anti-Mouse	Donkey	Alexa 488	1:400	Invitrogen

Antibodies used for flow cytometry and cell sorting

Antibody	Host	Conjugate	Company
Anti-mouse CD11b	Rat	APC	BD Pharmingen
Anti-mouse CD16/32, F_c block	Rat	-	BD Pharmingen
Anti-mouse CD45	Rat	PE	eBioscience
Anti-mouse CD45	Rat	PerCP	BD Pharmingen
Anti-mouse Ly-6C	Rat	PE-Cy7	BD Pharmingen

APC Rat IgG2b, kappa isotype control	Rat	APC	BD Pharmingen
PE Rat IgG2b, kappa isotype control	Rat	PE	BD Pharmingen
PerCP Rat IgG2b, kappa Isotype Control	Rat	PerCP	BD Pharmingen
PE-Cy7 Rat IgGM, kappa isotype control	Rat	PE-Cy7	BD Pharmingen

2.1.4. Kits

Kit	**Company**
Cloned AMV First Stand Synthesis Kit	Invitrogen, Germany
Prostaglandin D_2-MOX EIA Kit	Cayman Chemicals. USA
Platinum SYBER Green qPCR Supermix-UDG with ROX	Invitrogen, Germany

2.1.5 Buffers and solutions

Buffer	**Ingredients**	**Quantity**	**Remarks**
10x PBS (1 L)	NaCl KCl Na_2HPO_4 x7H_2O KH_2PO_4 Water	80.0 g 2.0 g 26.8 g 2.4 g ad 1000 ml	pH 7.4
1x PBS (1 L)	10x PBS H_2O	100 ml Ad 1000 ml	pH 7.4
30 % Percoll B (60 ml)	90% percoll DMEM	20 ml 40 ml	Stored at 4^0 C
78 % Percoll A (60 ml)	90 % percoll 1x PBS	47 ml 13 ml	Stored at 4^0 C
90 % Percoll (100 ml)	Percoll plus (100 %)	90 ml	Stored at 4^0 C

	10x PBS	10 ml	
Avertin (100 ml)	2,2,2-Tribromoethanol 2-Methyl-2-butanol Isotonic NaCl	2.5 g 2.5 ml 97.5 ml	Protected from light
Blocking solution for Immunohistochemistry	BSA Triton-X 100 1x PBS	5 g 0.3 ml Ad 100 ml	Stored at 4^0 C
Digestion solution (100 ml)	DMEM Collagenase A DNase	100 ml 100 mg 10 mg	Aliquoted at 5 ml and stored at -20^0 C
Erylysis-buffer (1 L)	0.15 M Ammonium chloride 10 mM Potassium hydrogen carbonate 0.1 mM Titration complex III (Na_2 EDTA)	8.02 g 1.00 g 0.037 g	pH 7.2-7.4
FACS Buffer	1x PBS 1 M NaN_3 (0.02 %) 0.5 % BSA (Albumin, Fraction V)	500 ml 1.54 ml 2.5 g	Stored at 4^0 C
Mowiol^R	Mowiol® 4-88 Glycerol Tris-HCl	10 % W/V 25 % W/V 0.1 M	 pH 8.5

2.1.6. Primers used in real-time RT-PCR

Gene	Sequence	Amplicon length (bp)
Ang	F, 5´- CCCCACCCCGTCACATGAGC-3´ R, 5´- TCCAACAGAGATTCCAAAGCTGGC-3´	144
Arg1	F, 5´-TGGTGTGGTGGCAGAGGTCCA-3´ R, 5´-ACTGCCAGACTGTGGTCTCCACC-3´	72
Arg2	F, 5´-CCTTGCGTCCTGACGAGATCC-3´ R, 5´-GGTGGCATCCCAACCTGGAGAG-3´	148
Ccl17	F, 5´-CCAGGGATGCCATCGTGTTTCTG-3´ R, 5´-TCAGCGGGAAGGTCATGGCCT-3´	122
Ccl2	F, 5´-GCTCAGCCAGATGCAGTTAACGC-3´ R, 5´-GCTTCTTTGGGACACCTGCTGCT-3´	122
Ccl5	F, 5´-GCCTCACCATATGGCTCGGACA-3´ R, 5´-ACTCCTTGACGTGGGCACGA-3´	85
Chi3l3	F, 5´-AGCCAGCAGAAGCTCTCCAGAAGC-3´ R, 5´-TGCCAGACCTGTGACAAGAATGAGC-3´	72
Clec10a	F, 5´-ACCCAAGAGCCTGGTAAAGCAGC -3´ R, 5´-TGGGAATTTTGGGATCCAATCACGG-3´	140
Dab2	F, 5´- AGCCAGCCCCGAGACAAGGT-3´ R, 5´- GGCTGAGAAACCACAGAGGGGT-3´	112
Fcrls	F, 5´-GCTGAAAACGCCTGGGGTACCA-3´ R, 5´-ACTTTGGGTGGGGGCTCTGTGA-3´	71
Gas6	F, 5‘- GGGGACGCGCGATGCAAGAA-3´ R, 5‘- TGGCACTCGTCCACATCTTGGC-3´	107
Hpgds	F, 5´- AACACAGATTTGGCTGGGAAGACAG-3´ R, 5´- CATCCAGCGTGTCCACCACTGC-3´	70
Igf1	F, 5´- GCAGCCCGCTCTATCCGTGC-3´ R, 5´- TGTCGATAGGGACGGGGACTTCT-3´	72
Il1b	F, 5‘-CGAGGCCTAATAGGCTCATCT G-3´ R, 5‘-CACTGTCAAAAGGTGGCATTTC-3´	117
Il1rn	F, 5´-TTGCCTTGCTGTGGCCTCGG-3´ R, 5´-ATTCTGAAGGCTTGCATCTTGCAGG-3´	143
Itgax	F, 5´- AGCCTTTCTTCTGCTGTTGGGGTT-3´ R, 5´- TGTCCGAACTCAGCACCGTCCA-3´	99
Mgl2	F, 5´-TGGAGCGGGAAGAGAAAAACCAGG-3´ R, 5´-TGGGAATTTTGGGATCCAATCACGG-3´	196
Mrc1	F, 5´-GGGACGTTTCGGTGGACTGTGG-3´	76

	R, 5´-CCGCCTTTCGTCCTGGCATGT-3´	
Nos2	F, 5´-GCCCGGCAAACCCAAGGTCT-3´ R, 5´-ACATCCCGAGCCATGCGCAC-3´	129
Ppia	F, 5´-AGGTCCTGGCATCTTGTCCAT-3´ R, 5´-GAACCGTTTGTGTTTGGTCCA-3´	51
Retnla	F, 5´-TCCTGCCCTGCTGGGATGACTGCTA-3´ R, 5´-CAGCGGGCAGTGGTCCAGTCAA-3´	125
Rnase4	F, 5´- GCAACGCCGACCTCACCCAT-3´ R, 5´- ACCTAGAAAGTGCCTGGACCCGGA-3´	135
Stard8	F, 5´- CCTCGTGGTGGGTGCCTCCT-3´ R, 5´- GGAGAACGGCCCCTGAGGTC-3´	170
Tnf	F, 5´-TGTAGCCCACGTCGTAGCAAA-3´ R, 5´-GCTGGCACCACTAGTTGGTTGT-3´	120
Wwp1	F, 5´- TCCCTCTGCCAGTGCGGAAGT-3´ R, 5´- TGTTCCCACCCTGATGGCAAAGC-3´	174

2.2. Animals

The mice were housed and bred under appropriate conditions at the Central Animal Facilities of the University of Heidelberg and University of Lübeck.

Mice

Mice	**Reference/source**
C57BL/6	Charles River, Germany
Cox1$^{-/-}$	(Langenbach et al, 1995)
Hca2$^{-/-}$	(Tunaru et al, 2003)
Hca2^{mRFP} *(Gpr109a*^{mRFP}*)*	(Hanson et al, 2010)

2.3. Methods

2.3.1 Mouse model of stroke

In the model, 8- to 12-week-old male mice were subjected to left MCAO as described previously (Bargiotas et al, 2012). Briefly, the mice were anesthetized with 15 µl 2.5% tribromoethanol per gram body weight. Panthenol eye ointment was used to prevent eye dryness. A 4-cm long skin incision was made between the ear and the orbit on the left side. The temporal muscle was removed and a burr hole was drilled to expose the stem of the middle cerebral artery (MCA). The MCA was then occluded by microbipolar electrocoagulation (Modell ICC 50, Erbe, Tübingen, Germany). The surgery was done under a microscope (Hund, Wetzlar, Germany) and rectal temperature was maintained at 37^0 C during surgery by a heating pad. The skin incision was then closed by suture and the mice were placed under a heating lamp until they fully recovered. After 24 or 48 h of MCAO, mice were deeply reanesthetized with tribromoethanol and perfused intracardially with 15 to 20 ml of Ringer's solution. Brains were carefully removed and coronally cryosectioned (20-µm thick) every 400 µm. Coronal sections were then stained with a silver technique (Lubjuhn et al, 2009) and the infarct volume was determined using ImageJ and corrected for brain edema as described previously (Herrmann et al, 2005a; Lubjuhn et al, 2009). All experiments were performed according to the German animal protection law and approved by the local animal welfare authorities (Regierungspräsidium Karlsruhe; Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume, Kiel, Germany). I was blinded to the treatment or genotype of mice or to both in all experiments. Mice were randomized to the treatment groups. If not indicated otherwise, nicotinic acid or vehicle was administered 10 min before MCAO and 4 h, 8 h, 24 h, 28 h, and 32 h after MCAO. Due to its short half-life, we

administered BHB through subcutaneous Alzet pumps (2001D, releasing 8 µl/h BHB, 1mg/ml, dissolved in normal saline) that were implanted subcutaneously 10 h before MCAO under isoflurane anesthesia. HQL-79 was administered by oral gavage 1 h before each nicotinic acid dose.

The mice were reluctant to eat ketogenic diet. Therefore, to habituate mice to ketogenic diet (sniff EF R/M ketogenic diet with 80% fat, long-chain fatty acids) we mixed it with normal chow (ssniff M-Z, containing 48% carbohydrates, 15% fat, and 37% protein) and increased the fraction of ketogenic diet in a stepwise manner (50% for 3 days, 70% for 5 days, 90% for 6 days, 100% for 4 days).

2.3.2. Silver staining

For silver staining, silver impregnation and developing solutions were applied to determine the infarct area on brain cryosections (Lubjuhn et al, 2009).

2.3.2.1. Impregnation solution

To prepare impregnation solution for 60 slides, 0.81 g lithium carbonate was mixed with 67.5 ml of water to generate a saturated solution of lithium carbonate. This solution was then mixed with 33.75 ml of 10% silver nitrate which formed precipitate. The precipitate was then carefully titrated by dropwise adding 25% ammonia. Precautions were taken not to add an excess of ammonia since this could make the staining faint. Finally, 506.25 ml of distilled water was added and the solution was protected from light by wrapping the container with aluminum foil.

2.3.2.2. Developing solution

To prepare developing solution, 6.6 g sodium citrate was dissolved in 420 ml of distilled water. After adding filtered formaldehyde (37%, 120 ml) the solution was well mixed at room temperature. Finally, 1.8 g hydroquinone and 90 ml acetone were added. This solution was mixed well at room temperature for about 30 minutes.

2.3.2.3. Staining procedure

Both the silver impregnation and the developing solutions were used only once for each 60 slides and staining was performed as described previously (Vogel et al, 1999). First the slides were arranged in a slide rack and incubated in silver impregnation solution for 2 minutes. Then, the slides were washed 6 times (1 minute each) in distilled water. Finally, slides were dipped in developing solution for 3 minutes followed by 3 washing steps (1 minute each) in distilled water. The stained sections were air dried over night.

2.3.3. Scanning, measurement and calculation of infarct sizes

Air-dried stained sections were scanned at 300 dpi along with a ruler to set the scale. Digitized images were analyzed with 'ImageJ' software. The area of the silver deficit (infarcted area) and the left (ischemic) and right hemisphere (non-ischemic) were measured. Edema correction was achieved by the following formula (Swanson et al, 1990).

$Vol_{infarct} = RH - (LH - SD)$

$Vol_{infarct}$ = Corrected Infarct volume (mm^3),

RH = Right hemisphere (non-ischemic)

LH = Left hemisphere (ischemic)

SD = Silver deficit

2.3.4. Bone marrow transplantation*

Bone marrow transplantation was performed as described previously (Muhammad et al, 2008) with modifications.

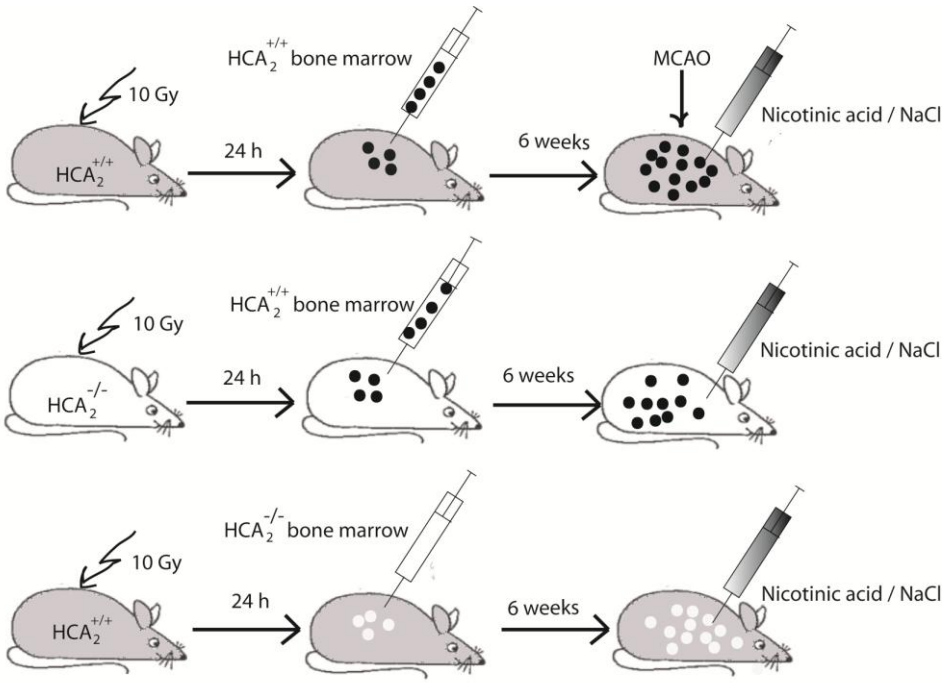


Figure 2.1. Schematic diagram of bone marrow transplantation. Descriptions are in the text.

2.3.4.1. Preparation of bone marrow cells from donor mice

Mice were euthanized by cervical dislocation and bone marrow was collected aseptically from femurs and tibias by flushing with 1x PBS. Collected bone marrow cells were passed through 23-gauge needles to obtain single cell suspensions. These unfractionated cells were resuspended in sterile PBS ($5x10^6$ cells/0.25 ml PBS).

*This experiment was performed together with Sajjad Muhammad (SM). Preparation of the recipient mice was done by myself. Bone marrow cells were prepared by SM and myself. MCAO surgery of the recipient mice were done by SM. Cryosection, silver staining, calculation and analysis of infarct volume were done by myself.

2.3.4.2. Irradiation of recipient mice and bone marrow transplantation

To generate chimeric mice by bone marrow transplantation we employed $HCA_2^{+/+}$ and $HCA_2^{-/-}$ mice. The following 3 groups of chimeric mice were generated: $HCA_2^{+/+}$ to $HCA_2^{+/+}$, $HCA_2^{+/+}$ to $HCA_2^{-/-}$, and $HCA_2^{-/-}$ to $HCA_2^{+/+}$. The recipient $HCA_2^{+/+}$ or $HCA_2^{-/-}$ mice (10 to 13 weeks old) were lethally irradiated with 10 Gy γ-radiation (in 2 divided sessions, 5 Gy each time with 4 hours interval) at the Deutsches Krebs Forschungs Zentrum (DKFZ), Heidelberg. One day after irradiation, 5 million bone marrow cells suspended in 0.25 ml PBS are reconstituted by injection into the retroorbital venous plexus (Hall et al, 2007). Six weeks after bone marrow reconstitution, mice were subjected to MCAO and the infarct volume was measured 48 hours later.

2.3.5. Behavioral analysis

To evaluate sensory motor function after cerebral ischemia, we used the following three established tests.

2.3.5.1. Corner test

The corner test has been described previously (Lubjuhn et al, 2009). In this test, mice were allowed to enter a 30x20-cm corner with an angle of 30^0. A food pellet between the two boards stimulated the mice to enter the corner. Mice were placed half way between the boards facing the corner and had the choice to explore the environment. When entering the corner, due to bilateral stimulation of vibrissae mice tended to turn around either left or right on rearing. Number of right and left turns on rearing out of 12 trials were counted before and 48 hours after MCAO.

2.3.5.2. Latency to move

Lubjuhn et al. previously described the latency-to-move test where a plain board was used to perform the test (Lubjuhn et al, 2009). Mice were placed at the centre of a plain board and the time to cross one full body length (7 cm) was measured before and 48 hours after MCAO. The test was performed three times for each mice and the mean value was calculated.

2.3.5.3. Sticky-tape-removal-test

In the sticky-tape-removal test, a small circular adhesive tape (HERMA No 2212, 8 mm) was placed on forepaws before and 48 hours after MCAO. The time until mice first tried to remove the adhesive tape as well as the total time needed to remove adhesive tapes were determined. In this study mice were trained before MCAO 2 times on two days.

2.3.6. Flow cytometry and cell sorting*

To perform flow cytometry and cell sorting, mice were deeply anesthetized with tribromoethanol 48 h after MCAO and perfused intracardially with Ringer's solution. Brains were dissected and olfactory bulbs, right hemispheres, and cerebella were removed. Two left hemispheres were pooled and digested in DMEM (Invitrogen) containing collagenase A (1 mg/ml, Roche) and DNAse (0.1 mg/ml, Roche) for 30 min at 37°C. Then, tissues were mixed thoroughly with a 10 ml pipette and filtered through a 40-µm nylon cell strainer (BD Biosciences) followed by washing the strainer with 40 ml cold PBS. After centrifugation at 310 g for 10 minutes at 4^0 C, cells were resuspended in 5 ml standard erythrocyte lysis buffer and incubated for 7 min on ice to lyse the red blood cells. After centrifugation, cells were resuspended in 2.8 ml percoll B (30 %). The cell suspension was placed on top of 2.8 ml percoll A (78%) carefully and centrifuged again at 1350 g for 30 minutes without break at

*Preperation of the cells were done by myself. Flow cytometry and cell sorting were performed together with Dr.Tillman Vollbrandt at FACS core facility, University of Lübeck.

4^0C. Myelin and debris were separated and cells were collected carefully from the interface of the gradient and washed with 10 ml PBS containing 0.5% BSA. After treatment with purified rat anti-mouse CD16/32 (Fc Block, BD Pharmingen, 2 µl for 100 µl cell suspension) for 10 minutes on ice, cells were incubated with the antibodies and respective isotype controls for 30 minutes on ice. The $CD45^{hi}CD11b^{+}$ and $CD45^{int}CD11b^{+}$ cells were sorted on a MoFlo Legacy (Beckman Coulter, 100 µm nozzle, 20 psi) with the laser line 488 nm at 100 mW and 635 nm at 25mW. Then, cells were lysed with Nucleic Acid Purification Lysis solution (Applied Biosystem) and used for quantitative RT-PCR. To analyze monocytes and macrophages, $CD45^{hi}CD11b^{+}$ cells were gated and subpopulations of monocytes ($Ly\text{-}6C^{hi}$ and $Ly\text{-}6C^{lo}$) were identified and quantified.

2.3.7. RNA extraction, reverse transcription and quantitative RT-PCR

2.3.7.1. RNA extraction from cerebral cortex and sorted cells

Total RNA was extracted using a 6100 Nucleic Acid PrepStation. Twenty four or 48 hours after MCAO, mice were perfused with Ringer's solution. Left and right cerebral cortices were dissected. Separated cortices were lysed in 1X Nucleic Acid Purification Lysis solution (1 ml/cortex) with a Ultra Turrax homogenizer. Two µl of proteinase K (20mg/ml) was added to 100 µl of homogenized solution and incubated for about 1 hour at room temperature. This solution was ready for extracting RNA with the ABI 6100 Nucleic Acid PrepStation.

To extract RNA from sorted cells, 1X Nucleic Acid Purification Lysis Solution (500 µl/$10.3x10^4$ cells) was used to resuspend the cell pellets after sorting. The sample solutions obtained from brain and sorted cells (500 µl) were loaded in pre-wetted wells (RNA Purification Wash Solution 1) of the Total RNA Purification tray. RNA extraction was

performed as described by the manufacturer's protocol. Briefly, loaded samples were incubated in the wells for 2 minutes followed by washing the wells with RNA Purification Wash Solution 1 and 2 each for 2 minutes. To collect RNA, 70 µl Nucleic Acid Purification Elution Solution was added to the wells. The concentration of the RNA was measured with the Nanodrop 2000 spectrophotometer (Thermo scientific). RNA samples were stored at -80^0 C.

2.3.7.2. cDNA Synthesis

cDNA was synthesized from extracted RNA using Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. In brief, 9 µl RNA, 1 µl Oligo (dT) (0.5µg/µl) and 2 µl dNTP mix (10 mM) were combined and denaturated by incubating at 65^{0}C for 5 minutes. Then, this solution was placed on ice. After mixing for 5 second, 5x cDNA Synthesis Buffer was used to prepared the following master reaction mix.

Component	**For 1 Reaction**
5x cDNA Synthesis Buffer	4 µl
0.1 M DTT	1 µl
RNaseOUT (40 units / µl)	1 µl
DEPC-treated water	1 µl
Cloned AMV RT (15 units /µl)	1 µl
Total Volume	**8 µl**

Eight µl of the master mix was then pipeted into each reaction tube on ice and transferred to a preheated thermal cycler where reaction tubes were incubated for 60 minutes at 50^{0}C. Finally, the reaction was terminated by incubating at 85^{0}C for 5 minutes. cDNA was then stored at -20^{0}C for quantative RT-PCR.

2.3.7.3. Quantitative RT-PCR

Quantitative RT-PCR was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystem) using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). The experiment was performed according to manufactuere's instructions. First, the real-time instrument was programmed as follows.

50^0C for 2 minutes hold (UDG incubation) 95^0C for 2 minutes hold 40 cycles of: 95^0C, 15 seconds 60^0C, 30 seconds

The following components were then added to the each well of the qPCR plate

Component	**Quantity for single reaction**
cDNA	2 µl
Platinum SYBR Green qPCR superMix-UDG with ROX	12.5 µl
Forward primer	0.75 µl
Reverse primer	0.75 µl
DEPC-treated water	9 µl
Total	25 µl

The qPCR plate was sealed and mixed gently. In order to make sure that all components were at the bottom of the tube, a brief centrifugation was performed. The reaction plate was then placed in a preheated real-time instrument programmed as described above. Data were collected when the reaction was finished. Quantified results of each cDNA samples were normalized to cyclophilin using the ΔΔCt method. The purity of the amplified products was checked by the dissociation curve. The primers used to detect specific cDNA are listed in Table 2.1.6.

2.3.8. Measurement of ketone bodies*

Total ketone bodies (acetoacetate + BHB), BHB, and free fatty acids in plasma were measured photometrically on an Olympus AU 400 analyzer (Beckman Coulter, Krefeld, Germany) using Autokit Total Ketone Bodies, Autokit 3-HB and NEFA C kit from Wako Chemicals GmbH (Neuss, Germany). The acetoacetate concentration was calculated by subtracting the BHB levels from the total ketone bodies levels.

2.3.9. Measurement of PGD_2 with Prostaglandin D_2-MOX EIA Kit

Plasma concentrations of PGD_2 were measured using an ELISA kit (Cayman Chemicals) and following the manufacturer's protocol.

2.3.9.1. Preparation of the buffers and samples

The EIA buffer was prepared by adding 90 ml of Ultra Pure water to the contents of one vial of EIA Buffer Concentrate (10X). To prepare the wash buffer, 1 ml Wash Buffer Concentrate (400X) and 200 µl of polysorbate 20 were added to 400 ml of Ultra Pure water. Plasma samples were obtained from mice at 2 minutes and 10 minutes after nicotinic acid injections. Samples were diluted 10 times with Ultra Pure water to determine the PGD_2 concentration.

2.3.9.3. Derivatization of prostaglandin D_2 to prostaglandin D_2-MOX

Derivatization of the prostaglandin D_2 EIA standard

Methoxylamine HCl (0.04 g) was dissolved with 10:90 ethanol:water (4 ml). After adding sodium acetate (0.328 g), the solution was mixed to prepare the methyloximating reagent. Prostaglandin D_2 EIA Standard (20 µl) was transferred to a clean tube and diluted with 180 µl of Ultra Pure water and mixed well. After adding the methyl oximating reagent (200 µl), the

*The measurement was performed at the Children's Hospital, University of Heidelberg in collaboration with Dr. Okun.

mixture was heated at 60^0C for 30 minutes. The concentration of this bulk standard solution was 20 ng/ml. This bulk solution was used to generate standard curves.

Derivatization of the prostaglandin D_2 samples

The Methyl Oximating reagent (30 µl) was added to 30 µl of plasma samples. The mixture was heated at 60^0C for 30 minutes. Subsequently, samples were centrifuged at 7000 rpm and the supernantant was diluted 1:10 and used in the assay.

2.3.9.4. Assay specific reagents preparation

The PGD_2 EIA methoximated bulk standard (100 µl) was diluted with 300 µl Ultra Pure water. Serial dilution was performed to obtain PGD_2-MOX EIA standard. PGD_2-MOX Ach tracer and PGD_2-MOX EIA antiserum were reconstituted by adding 6 ml of EIA buffer to each vial.

2.3.9.5. Assay procedure

First, the template sheet was labeled. Then, 100µl of EIA buffer was added to Non-specific Binding (NSB) wells and 50 µl to maximum binding (B_0) wells of the 96-well plate. Standards (50 µl) and samples (50 µl) were transferred to respective wells. PGD_2-MOX AChE (50 µl) Tracer was added to every well except Total Activity (TA) and blank (Blk) wells. PGD_2-MOX EIA Antiserum (50 µl) was added to every well except TA, NSB, and Blk wells. The plate was then covered and incubated overnight at 4^0C. After 5 washing steps, 200 µl Ellman's reagent (reconstituted by adding 20 ml Ultra Pure water to the vial) was added to each well. The PGD_2-MOX AChE Tracer (5µl) was added to TA wells and the plate was covered. The plate was placed on a shaker in the dark at room temperature for approx. 90 minutes. The absorbence was read at 405 nm with the Fluostar Optima.

2.3.10. Immunohistochemistry*

Forty-eight hours after MCAO, *Hca2*^{mRFP} mice were deeply anesthetized with tribromoethanol and perfused with Ringer's solution and 4% PFA. Then, 20-µm-thick coronal cryosections were permeabilized with 0.3% Triton X-100 in PBS for 30 min and blocked with 5% BSA. The sections were incubated with rabbit anti-mouse Iba1 (Wako, 1:100), rat anti-mouse CD11b (AbD Serotec, 1:100), mouse anti-NeuN (Chemicon, 1:500), and rabbit anti-GFAP (DAKO, 1.500) overnight at 4°C. After washing with PBS we used the following secondary antibodies to visualize the staining using a confocal microscope (LSM, Lyca): Alexa 488-labeled donkey anti-rabbit (Invitrogen, 1:400) and Alexa 488-labeled donkey anti-rat (Invitrogen). The sections were then washed with PBS containing DAPI (Sigma, 1: 5000) and mounted with Mowiol.

2.4. Statistical analysis

GraphPad Prism 5 software was used for statistical analysis of the acquired data. The data are presented as mean ± SEM. To compare multiple groups, either two-way repeated-measures ANOVA followed by Bonferroni posthoc test or one-way ANOVA followed by Newman-Keuls posthoc were used as indicated. Student's t test was used to compare 2 groups.

*The staining was performed together with Dr. Dirk A Ridder.

3. Results

With the aid of pharmacological as well as genetic tools the current study aimed to investigate the role of HCA_2 activation in cerebral ischemia. We also explored potential cellular and molecular mediators mediating the protective function of HCA_2 activation.

3.1. Expression of HCA_2 receptors in brain

After the discovery of the HCA_2 receptor, extensive research has been carried out to uncover its impact in physiological as well as pathological aspects (Lukasova et al, 2011b; Tunaru et al, 2003). In addition to adipocytes where HCA_2 plays an important homeostatic function in mobilizing free fatty acids, significant expression has been observed in immune competent cells (Taggart et al, 2005). Expression of HCA_2 receptors has been reported in the brain but its cellular localization was unknown (Miller & Dulay, 2008). Therefore, to analyze the expression of HCA_2 in brain, we used the BAC-transgenic mouse line *Hca2*^{mRFP} (*Gpr109a*^{mRFP}), in which the *Hca2* locus directs the expression of monomeric red fluorescent protein (mRFP) (Hanson et al, 2010). To identify brain cell types that could potentially express HCA_2 receptors, we used immunohistochemistry in conjunction with cell specific marker antibodies. Under normal conditions, mRFP was expressed exclusively by CD11b$^+$ microglia (Figure 3.1A). To investigate the expression of HCA_2 in other brain cells, we used anti-GFAP and anti-NeuN antibodies as markers for astrocytes and neurons, respectively. We found that astrocyte and neuron do not express mRFP (Figure 3.1B, C). Since infiltration of immune cells in the brain is common after cerebral ischemia and immune cells express HCA_2, we characterize HCA_2 expressing immune cells after cerebral ischemia induced by occlusion of the middle cerebral artery. This procedure induces mainly cortical infarcts. Forty eight

hours after MCAO, confocal microscopy revealed that $mRFP^+$ cells accumulated in the periphery of the ischemic area (Figure 3.1D). Most of them expressed CD11b (87.5±14.6% of $mRFP^+$ cells) and Iba1 (98.7±18.0% of $mRFP^+$ cells), indicating that HCA_2 is present in microglia or monocyte/macrophages that infiltrated the ischemic brain.

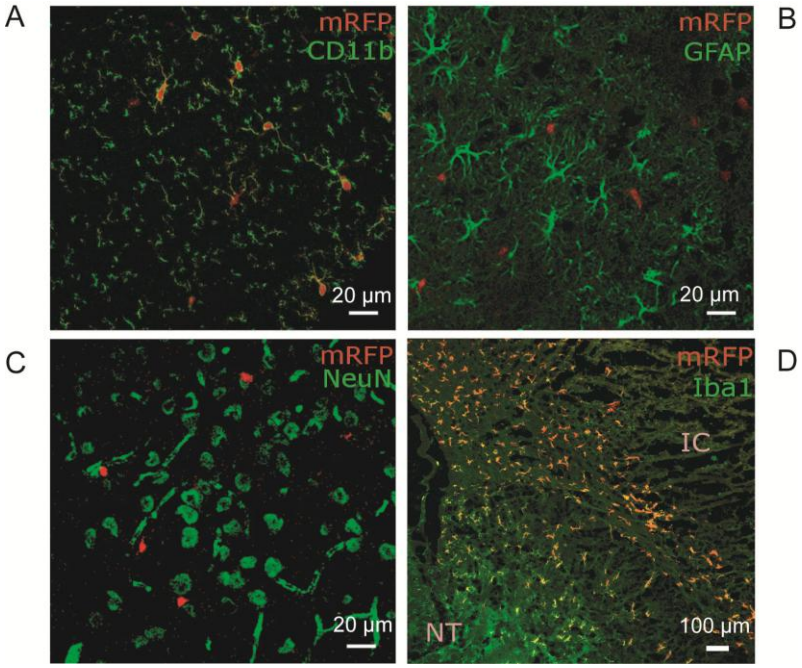


Figure 3.1. HCA_2 receptors are expressed in $CD11b^+$ cells. *(A-C) Evidence for the expression of HCA_2 in $CD11b^+$ microglia under normal conditions. In $Hca2^{mRFP}$ mice, mRFP (red) reflects HCA_2 expression. CD11b, the astrocyte marker GFAP, and the neuronal marker NeuN were detected by immunohistochemistry. (D) $mRFP^+$ cells markedly increased in the periphery of the ischemic area 48 h after MCAO. Most $mRFP^+$ cells expressed Iba1, a marker of microglia and infiltrating monocytes/macrophages. IC, ischemic core. NT, normal tissue.*

3.2. HCA_2 receptors mediate the neuroprotective effect of ketogenic diet

Ketogenic diet has already been established as a treatment option for pharmacoresistant childhood epilepsies (Lutas & Yellen, 2013). To explore HCA_2 function in ketogenic diet-induced neuroprotection, we employed *Hca2*$^{-/-}$ mice. When we fed wild-type (*Hca2*$^{+/+}$) or *Hca2*$^{-/-}$ mice with a ketogenic diet, body weight did not change but plasma concentrations of BHB increased markedly (Figure 3.2A). Compliant with the concept that HCA_2 provides a negative feedback on ketone body production by inhibiting fatty acid release, the BHB levels were even higher in *Hca2*$^{-/-}$ than in wild-type mice (Figure 3.2A). Plasma levels of free fatty acids and acetoacetate increased similarly (Table 3.1). Forty-eight hours after MCAO, infarcts were significantly smaller in wild-type mice on a ketogenic diet than in animals on a normal diet (Figure 3.2B). Interestingly, the protective effect of the diet was lost in *Hca2*$^{-/-}$ mice, although they had higher plasma levels of ketone bodies (Figure 3.2A-B, Table 3.1). However, there was no significant difference in the infarct size between the genotypes when mice were fed a normal diet.

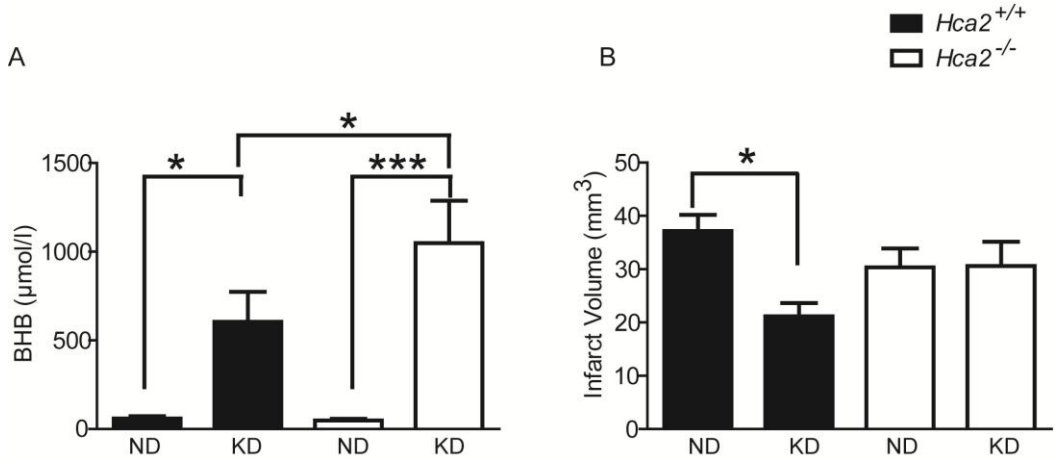


Figure 3.2. HCA_2 receptors mediate the neuroprotective effect of ketogenic diet (KD) in cerebral ischemia*. (A) BHB plasma concentrations increased when mice were fed a ketogenic diet (KD). Controls received normal chow (ND). ANOVA, F(3/36)=10.85, p<0.0001. *p<0.05, ***p<0.0001 (Newman-Keuls posthoc test). Values are means ± SEM (n=10). (B) Ketogenic diet reduced the infarct volume in $Hca2^{+/+}$ but not in $Hca2^{-/-}$ animals. ANOVA, F(3/35)=3.692, p<0.05. *p<0.05 (Newman-Keuls posthoc test). Values are means ± SEM (n=9-10).*

Table 3.1. Plasma concentrations of free fatty acids and acetoacetate in mice on a ketogenic diet (KD) in comparison to animals on a normal diet (ND). *One-way ANOVA showed a statistically significant difference only for free fatty acids, F(3/28)=10.61, p<0.0001. **p<0.001 in comparison to $Hca2^{+/+}$ mice on ND, ***p<0.0001 in comparison to $Hca2^{-/-}$ on ND (Newman-Keuls posthoc test). Values are means ± SEM (n=6-10).*

	***Hca2*$^{+/+}$ / ND**	***Hca2*$^{+/+}$ / KD**	***Hca2*$^{-/-}$ / ND**	***Hca2*$^{-/-}$ / KD**
Free fatty acids (µmol/l)	82.7±25.0	371.4±60.5**	181.9±35.2	503.6±68.9***
Acetoacetate (µmol/l)	2.1±0.9	12.2±3.5	9.2±4.3	19.8±7.0

3.3. β-hydroxybutyrate (BHB) is protective in cerebral ischemia acting through HCA_2 receptors

The liver produces ketone bodies, which serve as alternative energy source when the normal supply of glucose is compromised such as occurs during fasting, strenuous exercise, and hypoxic conditions. BHB is one of the two dominant ketone bodies produced in mitochondria by reduction of acetoacetate (Laffel, 1999). BHB is an endogenous ligand of HCA_2 receptors

(Taggart et al, 2005). To test whether BHB is involved in reducing the infarct size via HCA_2 receptors, we administered BHB by implanting subcutaneous pumps because of its short half-life. This treatment elevated plasma levels of BHB 48 hours after MCAO (Figure 3.3A). In parallel, BHB decreased the infarct volume in wild-type but not in *Hca2*$^{-/-}$ animals, demonstrating a neuroprotective effect of BHB through HCA_2 receptors (Figure 3.3B).

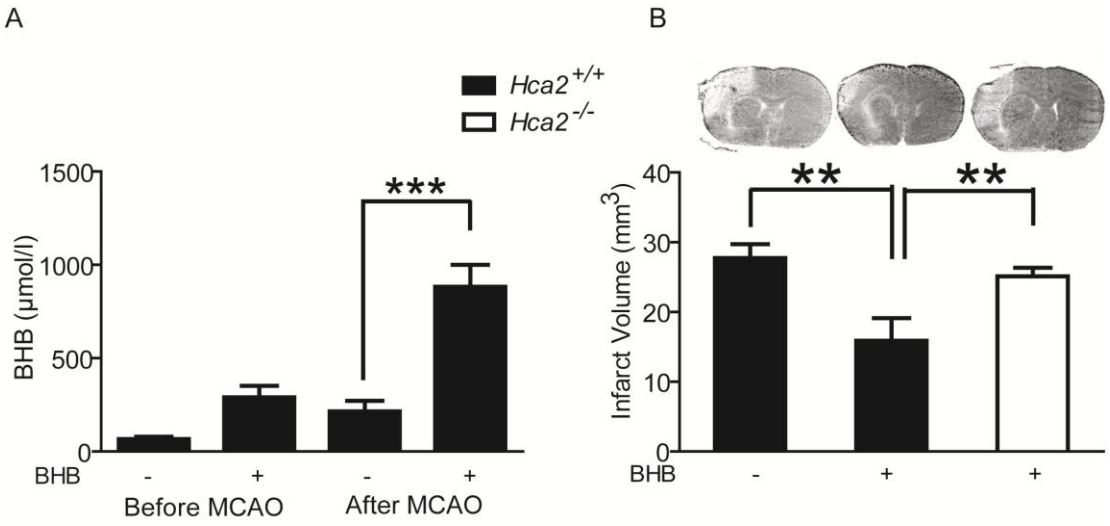


Figure 3.3. Protective effect of BHB in cerebral ischemia. *(A) BHB plasma concentrations increased when mice were treated with BHB to similar levels as on ketogenic diet. Measurements were performed immediately before MCAO or 48 h after MCAO. ANOVA, F(3/8)=24.28, p=0.0002. ***p<0.0001 (Newman-Keuls posthoc test). Values are means ± SEM (n=3). (B) BHB treatment reduced the infarct volume in Hca2*$^{+/+}$ *but not in Hca2*$^{-/-}$ *mice. ANOVA, F(2/27)=7.29, p=0.0029. **p<0.001 (Newman-Keuls posthoc test). Values are means ± SEM (n=10).*

3.4. The HCA_2 agonist nicotinic acid ameliorates the consequence of ischemic stroke.

The ability of nicotinic acid to lower plasma cholesterol was discovered by Rudolf Altschul in the middle of 20th century followed by a landmark study confirming that nicotinic acid administration in gram quantities lowered total plasma cholesterol in healthy human subjects and in patients with high level of cholesterol (Altschul & Herman, 1954; Altschul et al, 1955; Carlson, 2005). Eventually, nicotinic acid had been tested in controlled clinical trials establishing it for the treatment of coronary heart diseases (Carlson, 2005; Gille et al, 2008). After the discovery of the HCA_2 (GPR109A) receptor, it had been shown that nicotinic acid could also stimulate HCA_2 and protect against atherosclerotic disorders (Lukasova et al, 2011b; Tunaru et al, 2003). Based on these data and also because both BHB and nicotinic acid are small carboxylic acids we aimed to investigate the role of nicotinic acid and its corresponding HCA_2 receptor in the context of cerebral ischemia. We subjected male C57BL/6 mice to MCAO. Immediately before the occlusion of the artery, nicotinic acid was injected intraperitonially. The treatment was continued three times daily for two consecutive days. Forty eight hours after the MCAO, mice were sacrificed and the infarct volumes were analyzed on silver stained serial sections.

In clinically relevant doses, nicotinic acid reduced the infarct size significantly in comparison to the vehicle control (Figure 3.4.1A). Based on this dose-effect relationship of nicotinic acid on cerebral ischemia we chose a dose of 100 mg/kg body weight for the subsequent experiments. When we repeated the experiment in wild-type and *Hca2*$^{-/-}$ mice, nicotinic acid was only effective in wild-type but not in *Hca2*$^{-/-}$ mice, proving that by activating HCA_2 receptors a neuroprotective effect is produced (Figure 3.4.1B).

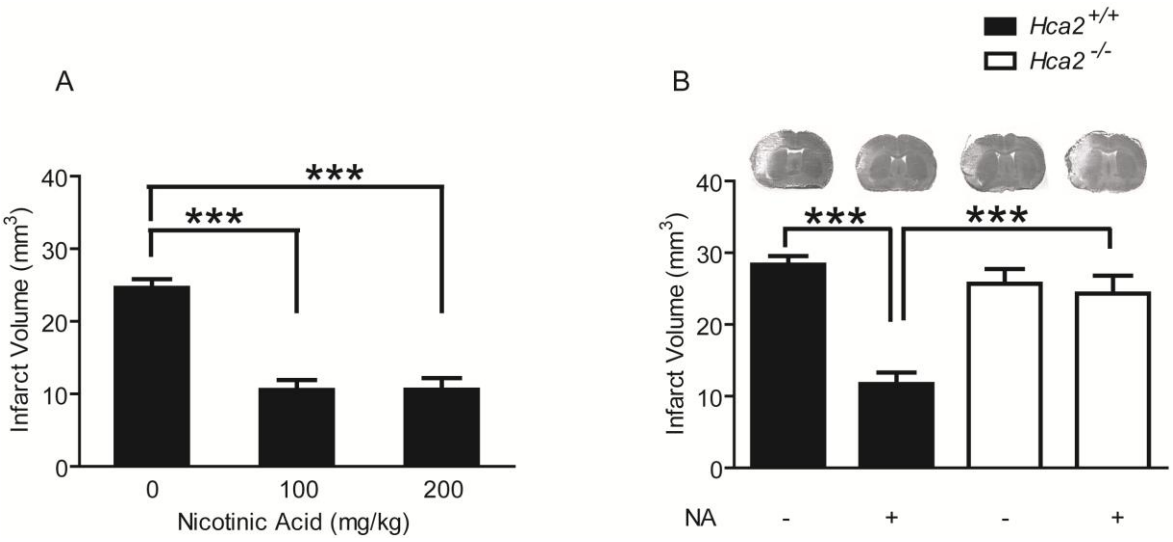


Figure 3.4.1. The HCA_2 agonist nicotinic acid ameliorates the consequences of ischemic stroke

**(A) Nicotinic acid at doses similar to those used in the clinic reduced the infarct volume. The infarct volume was determined 48 h after MCAO. ANOVA, $F(2/59)=37.05$, $p<0.0001$. ***$p<0.0001$ (Newman-Keuls posthoc test). (B) The protective effect of nicotinic acid (100 mg/kg) was lost in $Hca2^{-/-}$ mice. The infarct volume was determined 48 h after MCAO. ANOVA, $F(3/48)=15.81$, $p<0.0001$. ***, $p<0.0001$ (Newman-Keuls posthoc test). Values are means ± SEM (n=12-14).*

*Data from individual experiments were pooled.

Thrombolysis with tissue plasminogen activator (tPA) is one of the approaches that has been investigated for the treatment of acute ischemic stroke. In addition to mechanical removal of the clot, thrombolysis with tPA has been approved by the Food and Drug Administration (FDA)(Yepes et al, 2009). However, this treatment option is limited to a time window of 4.5 hours after the onset of ischemic events. On the other hand, deleterious effects of tPA in the ischemic brain have been reported (Yepes et al, 2009). In clinical practice, treatment of stroke is often delayed excluding effective therapy. Therefore, we administered nicotinic acid after onset of MCAO. Although the delayed dosage reduced the efficacy, nicotinic acid still decreased the infarct size significantly when administered up to 4.5 hours after MCAO (Figure 3.4.2).

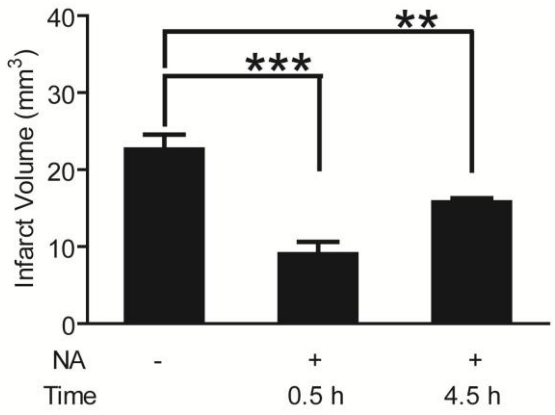


Figure 3.4.2. Delayed treatment with nicotinic acid is protective in cerebral ischemia. *Nicotinic acid (100 mg/kg) reduced the infarct volume when administered 30 min or 4.5 h after MCAO. The infarct volume was determined 48 h after MCAO. ANOVA, F(2/21)=21.05, p<0.0001. **p<0.001, ***p<0.0001 (Newman-Keuls posthoc test). Values are means ± SEM (n=8).*

3.5. Nicotinic acid improves the stroke-induced neurological deficit

Cerebral ischemia is a leading cause of death and disability around the world. Millions of stroke survivors every year have to adapt to a restricted lifestyle (Flynn et al, 2008; Yepes et al, 2009). Therefore, treatment options should address cognitive impairment and functional outcome after ischemic events. To investigate functional outcome of nicotinic acid treatment after cerebral ischemia, we performed three established tests of sensorimotor function (Lubjuhn et al, 2009). In the corner test, mice were allowed to enter into a 30^0 angled corner and their turning behavior on rearing was evaluated before and 48 hours after MCAO. Mice did not show a significant bias before MCAO. However, after MCAO, mice tended to turn more often to the contralateral (i.e. right) than to the ipsilateral side which was normalized by nicotinic acid treatment (Figure 3.5A, D). When we placed the mice on an open board during the latency-to-move test after MCAO, mice took more time to cross one body length (7 cm).Treatment with nicotinic acid normalized this parameter to its basal level (Figure 3.5B, D). Finally, in the sticky-tape-removal test, adhesive tapes were placed onto both forepaws of mice and the time until mice first sensed the tape and started to remove it was measured. After MCAO, mice needed more time to sense the tape on the contralateral forepaw. Nicotinic acid treatment also reduced this time (Figure 3.5C).

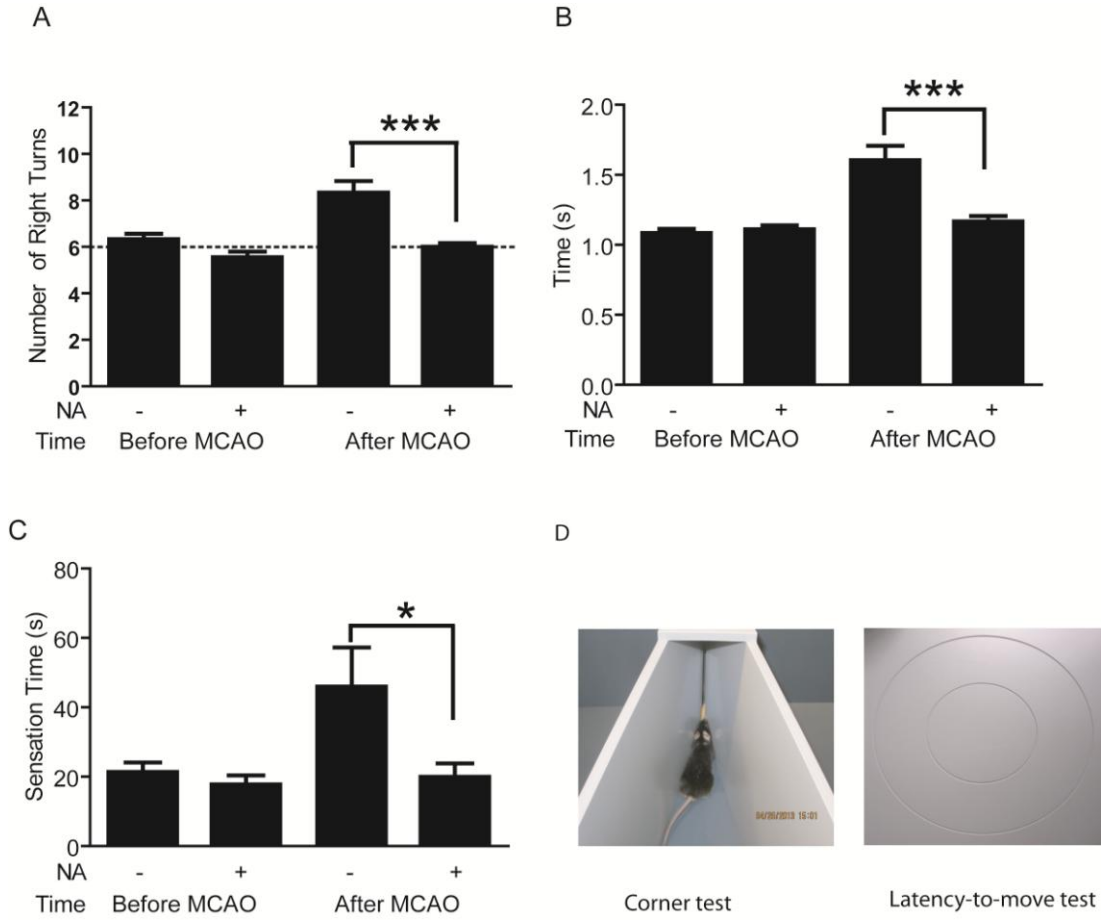


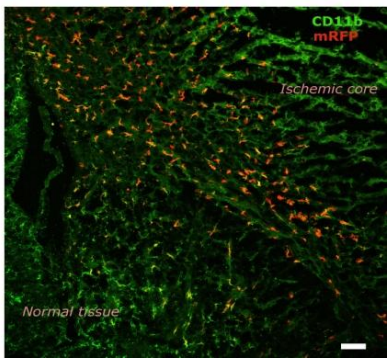
Figure 3.5. Functional outcome in stroke after nicotinic acid treatment. *(A) In the corner test, nicotinic acid treatment (100 mg/kg) improved the preference that was observed in vehicle-treated animals to turn to the right side 48 h after MCAO. The dashed line indicates the expected behavior without a side preference in 12 trials of the corner test. Two-way repeated-measures ANOVA, F(1/16)=20.36, p=0.0004. ***p<0.001 (Bonferroni posthoc test). Values are means ± SEM (n=9). (B) Nicotinic acid treatment (100 mg/kg) improved the latency to move that was increased 48 h after MCAO. Two-way repeated-measures ANOVA, F(1/15)=13.30, p=0.0024. ***p<0.001 (Bonferroni posthoc test). Values are means ± SEM (n=8-9). (C) After nicotinic acid treatment (100 mg/kg) mice started to remove the sticky tape*

*fixed to the right frontpaw earlier. Nicotinic acid treatment only started 30 min after MCAO with the dosing intervals specified in the Methods section. The behavioral test was performed 48 h after MCAO. Two-way repeated-measures ANOVA, F(1/18)=11.28, p<0.05. *p<0.05 (Bonferroni posthoc test). Values are means ± SEM (n=10). (D) The equipment used for the corner and latency-to-move tests.*

3.6. Impact of nicotinic acid on HCA_2 expressing cells.

Most of the mRFP expressing cells express CD11b (Figure 3.6A) after MCAO. We wanted to investigate the effect of nicotinic acid on this cell population. We subjected the BAC-based transgenic *Hca2*^{mRFP} mice to MCAO and treated them with nicotinic acid three times a day for two consecutive days. While analyzing coronal cryosections 48 hours after MCAO, confocal microscopy revealed a characteristic expression of mRFP$^+$ cells in the periphery of the infarct (Figure 3.6.B) as shown above (Figure 3.1D). Treatment with nicotinic acid significantly reduced the number and the expression of HCA_2 as revealed by a reduced integrated density of the mRFP signal (Figure 3.6.B, C). This suggests that the neuroprotective effect of nicotinic acid could depend on HCA_2 expressing CD11b$^+$ monocytes/macrophages.

A



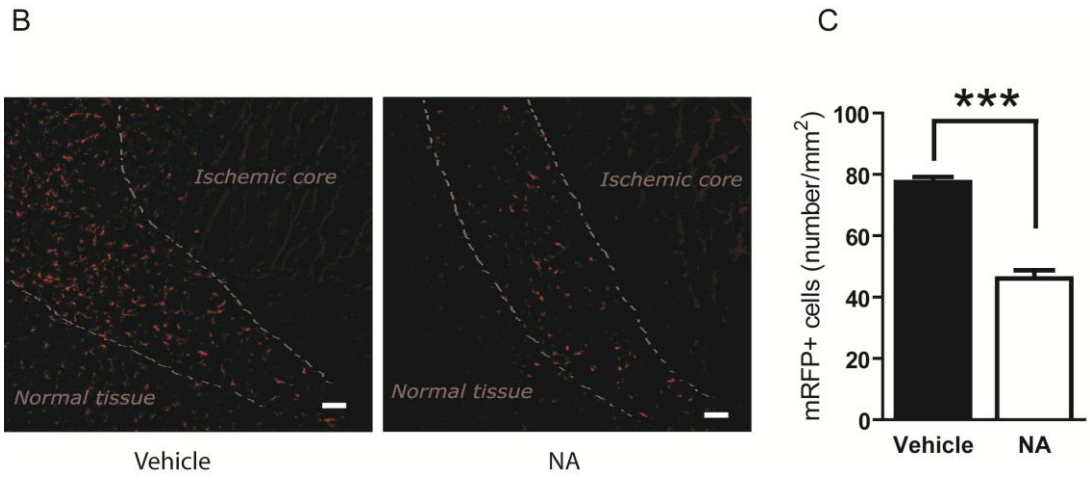


Figure 3.6. Effect of nicotinic acid on mRFP expressing cells. *(A) Most of the mRFP expressing cells also express CD11b, a marker for monocyte/macrophages. (B, C) Accumulation of mRFP$^+$ cells in the periphery of the infarct was reduced by nicotinic acid treatment (NA, 100 mg/kg). Hca2^{mRFP} mice were subjected to MCAO. Scale bar, 100 µm. Unpaired t test, ***p<0.0001, values are mean ± SEM (n = 4/group).*

3.7. Activation of HCA_2 receptors in bone marrow-derived monocytes/macrophages is neuroprotective.

$CD11b^+$ cell in the ischemic brain represent a composite population consisting of bone marrow-derived peripheral macrophages and resident microglia. Both microglia and macrophages have been reported to exert beneficial and/or deleterious effects not only on ischemic brain damage but also in other neurodegenerative diseases such as Alzheimer´s disease, ALS, and PD (Butovsky et al, 2006; Gliem et al, 2012a; Lo et al, 2003; Prinz & Mildner, 2011; Reichmann et al, 2002). Therefore, to determine whether bone marrow–derived monocytes/macrophages or resident microglial cells mediate the protective effect of HCA_2 in stroke, we generated chimeric mice by bone marrow transplantation. Six weeks after

successful reconstitution mice were subjected to MCAO and infarct volumes were measured. In line with our expectation, *Hca2*$^{+/+}$ mice that received *Hca2*$^{+/+}$ bone marrow were protected by nicotinic acid treatment as revealed by a significant reduction in the infarct volume (Figure 3.7). Transplantation of *Hca2*$^{+/+}$ bone marrow rescued the response of *Hca2*$^{-/-}$ animals, whereas nicotinic acid lost its activity when *Hca2*$^{-/-}$ bone marrow was transplanted to *Hca2*$^{+/+}$ mice, which demonstrates that HCA_2 present in bone marrow-derived macrophages mediates the protective effect of nicotinic acid (Figure 3.7)

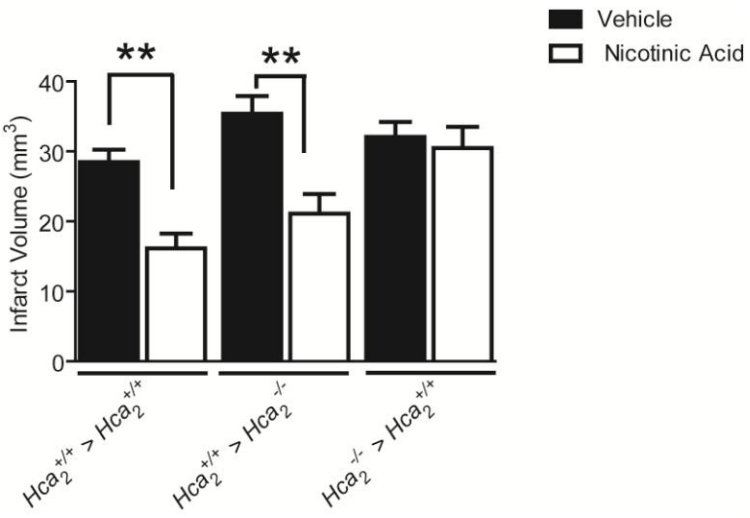


Figure 3.7. Activation of HCA_2 in bone marrow-derived monocytes/macrophages is neuroprotective. *After transplanting Hca2*$^{+/+}$ *bone marrow to either Hca2*$^{+/+}$ *mice (Hca2*$^{+/+}$*>Hca2*$^{+/+}$*) or to Hca2*$^{-/-}$ *mice (Hca2*$^{+/+}$*>Hca2*$^{-/-}$*) nicotinic acid (100 mg/kg) reduced the infarct size. However, when Hca2*$^{-/-}$ *bone marrow was transplanted to Hca2*$^{+/+}$ *mice (Hca2*$^{-/-}$*>Hca2*$^{+/+}$*) nicotinic acid was no longer effective. The infarct volume was measured 48 h after MCAO. ANOVA, F(5/50)=8.454, p<0.0001. **p<0.001 (Newman-Keuls posthoc test). Values are means ± SEM (n=9-10).*

3.8. Characterization of infiltrating monocytes/macrophages

Macrophages and its precursor monocytes encompass functionally distinct subpopulations (Gliem et al, 2012a). To characterize monocytes/macrophages in the ischemic brain we performed flow cytometry (Figure 3.8). After 48 hours of MCAO, substantial number of $CD45^{hi}CD11b^{+}$ monocytes/macrophages infiltrated the brain (Figure 3.8, top left panel). Most of the cells in this subpopulation also expressed Ly-6C, a marker for monocytes (Figure 3.8, upper right panel). The amount of $CD11b^{+}Ly\text{-}6C^{hi}$ cells was substantially increased in the ischemic hemisphere when compared to non-ischemic hemisphere (Figure 3.8, upper right and lower right panel). However, $CD11b^{+}Ly\text{-}6C^{lo}$ cells were not affected significantly by ischemia.

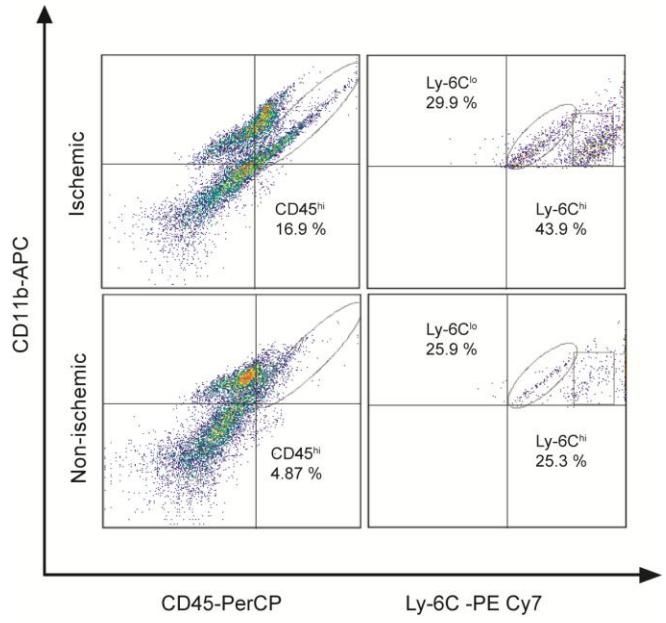


Figure 3.8. $CD11b^{+}Ly\text{-}6C^{+}$ monocytes migrated to the brain after MCAO *Increased infiltration of $CD45^{hi}CD11b^{+}$ cells in the ischemic hemisphere (upper left panel) after 48 hours of MCAO. Gated $CD45^{hi}CD11b^{+}$ cells were plotted against Ly-6C (right panel).*

3.9. Nicotinic acid treatment induces M2 polarization of macrophages in vivo.

Macrophages originate from the differentiation of circulating peripheral-blood mononuclear cells (PBMCs). In response to inflammation or under normal conditions they migrate into the tissues (Mosser & Edwards, 2008). Plasticity is one of the noteworthy characteristics of macrophages. This plastic nature permits them to modulate their phenotype in order to respond efficiently to environmental signals (Liao et al, 2011). Therefore, macrophages have been categorized in a simplified manner where the classically activated macrophages are designated as M1 representing a pro-inflammatory state and the alternatively polarized macrophages as M2 representing an anti-inflammatory state. Thus, in titrating inflammatory responses, M1 and M2 macrophages play divergent roles (Gordon, 2003; Mosser & Edwards, 2008).

To approach the question of how monocytes/macrophages mediate the neuroprotective effect of HCA_2 we investigated the polarization of monoccytes/macrophages by quantifying mRNA levels of marker genes for pro-inflammatory M1-polarized and anti-inflammatory M2 polarized monocytes/macrophages (Frieler et al, 2011; Raes, 2004). M1-related genes were upregulated by cerebral ischemia but treatment with nicotinic acid did not affect expression levels (Table 3.2). Out of 8 M2-related genes only *Retnla* and *Mrc1* were increased by nicotinic acid treatment (3.9.A, B), which suggests only a partial M2 polarization, if any. A recent meta-analysis of gene expression signatures in mouse leukocytes reported that *Mrc1* is part of a specific cluster of genes coordinately expressed mainly in macrophages and bone marrow-derived dendritic cells (cluster 60) (Mabbott et al, 2010). Therefore, we investigated the expression level of other *Mrc1* cluster members. Out of 9 *Mrc1* cluster genes, 6 were regulated by nicotinic acid treatment 24 or 48 h after MCAO (Figure 3.9.C, D and Table 3.2).

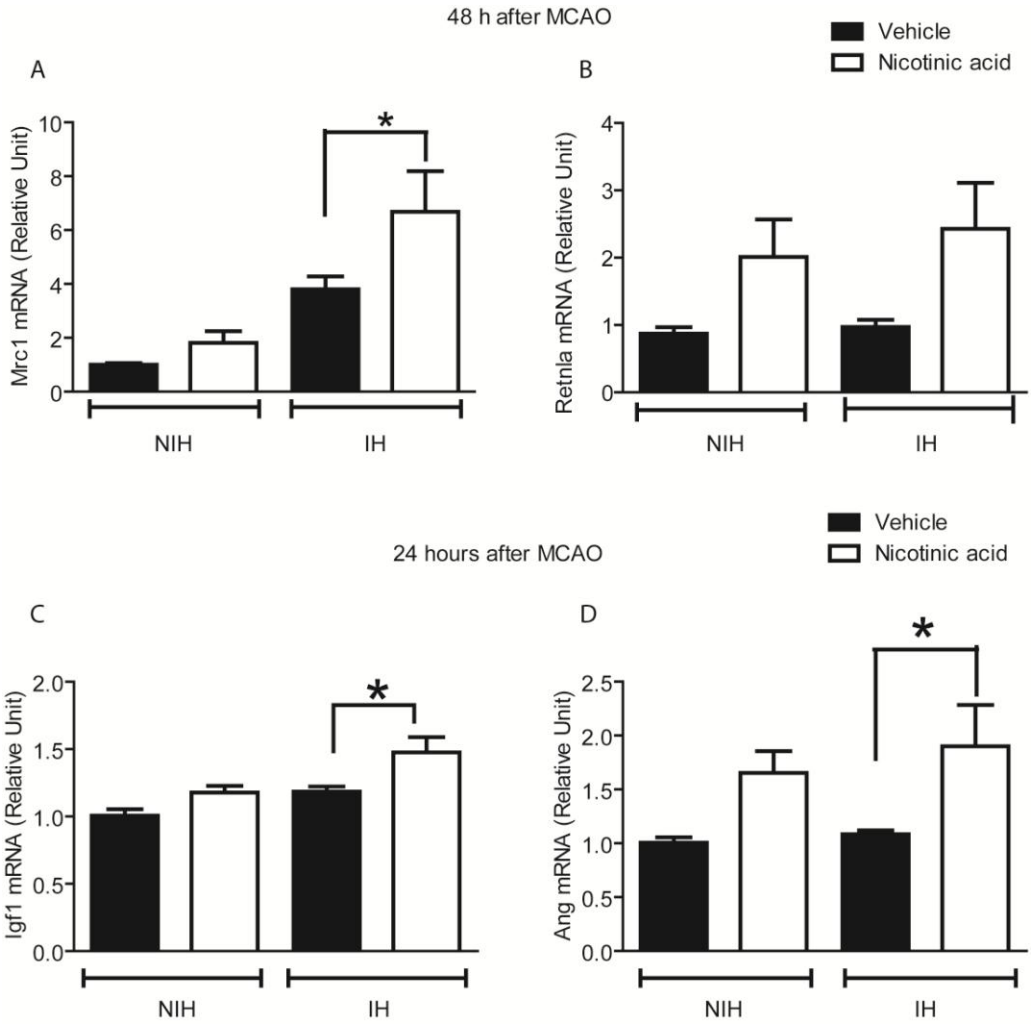


***Figure 3.9. Nicotinic acid partially induced alternative activation of macrophages in* vivo.** *(A) Relative mRNA expression of Mrc1, a marker gene that is involved in the alternative polarization of macrophages. Nicotinic acid treatment increases the expression of Mrc1 48 h after MCAO in brain. Two-way repeated-measures ANOVA, F(1/8)=5.48, p=0.047. *p<0.05 (Bonferroni posthoc test). Values are means ± SEM (n=5). (B) Relative mRNA expression of Retnla increases upon nicotinic acid treatment in brain. Two-way repeated-measures ANOVA, F (1/9)=6.73, p=0.029. Values are means ± SEM (n=5-6). (C, D) Brain mRNA*

*expression of Mrc1 gene cluster members. Brain mRNA expression of Igf1(C) and Ang (D) 24 h after MCAO was increased significantly upon nicotinic acid treatment. Two-way repeated-measures ANOVA, Igf1, F (1/10)= 7.391, p= 0.0216, *p<0.05 (Bonferroni posthoc test). Values are means ± SEM (n=6). Ang,.F (1/10)=7.314, p=0.022, *p<0.05 (Bonferroni posthoc test). Values are means ± SEM (n=6). NIH, Non-ischemic hemisphere. IH, Ischemic hemisphere.*

Table 3.2. mRNA levels of macrophagic genes in the non-ischemic (NIH) and ischemic hemispheres (IH) of mice that were treated with nicotinic acid (NA, 100 mg/kg) or vehicle. Only statistically significant differences of the two-way repeated-measures ANOVA are given. For a description of 'cluster 60' see the publication of Mabbot et al. (2010).

	24 h after MCAO							48 h after MCAO						
	Vehicle		NA		2-way ANOVA			Vehicle		NA		2-way ANOVA		
Gene	**NIH**	**IH**	**NIH**	**IH**	**NA (P, F)**	**Ischemia**	**Interaction**	**NIH**	**IH**	**NIH**	**IH**	**NA (P, F)**	**Ischemia**	**Interaction**
M2-related genes														
Arg1	1.1±0.3	3.3±0.1	0.6±0.1	1.7±0.5		P=0.0018, F=14.86		1.4±0.5	4.0±0.9	2.5±1.0	6.4±2.0		P=0.0006, F=19.40	
Retnla	1.2±0.2	1.6±0.5	0.6±0.2	0.9±0.3				0.9±0.1	1.0±0.1	2.0±1.6	2.4±0.6	P=0.029,F=6.73		
Chi3l3	1.3±0.4	11.3±7.3	0.7±0.2	3.9±2.0				1.0±0.2	1.9±0.2	1.6±0.4	5.0±1.6		P=0.02, F=7.67	
Mrc1	1.1±0.3	1.9±0.4	0.9±0.4	1.8±0.4		P=0.007, F=12.51		1.0±0.1	3.8±0.5	1.8±0.4	6.7±1.5	P=0.04, F=5.487	P=0.002, F=20.15	
Fcrls	1.3±0.3	1.8±0.5	0.8±0.2	1.7±0.4		P=0.005, F=11.05		2.8±1.8	4.9±2.0	1.4±0.5	3.1±0.9			
Clec10a	1.2±0.3	0.7±0.2	1.2±0.6	1.2±0.2				1.1±0.1	2.9±0.8	2.6±0.7	4.1±1.1		P=0.017, F=7.664	
Mgl2	0.9±0.2	0.9±0.2	0.9±0.2	0.9±0.1				1.1±0.1	1.5±0.3	2.0±0.6	2.4±0.6			
Il1rn	1.8±0.9	17.6±10.0	1.0±0.5	7.1±3.2		P=0.04, F=5.06		1.6±0.6	14.1±2.3	3.5±0.7	23.1±6.3		P=0.0002,F=26.42	
M1-related genes														
Tnf	1.2±0.2	6.2±10.0	1.4±0.8	6.6±1.7		P=0.0002, F=26.04		1.3±0.4	2.8±0.9	1.4±0.4	2.5±0.5		P=0.016, F=7.739	
Il1b	1.2±0.2	11.2±3.8	3.0±2.0	15.1±7.0		P=0.004, F=11.75		1.0±0.1	9.8±3.0	2.0 ±0.5	16.9±8.0		P=0.018, F=7.454	
Ccl2	1.3±0.5	20.7±6.5	0.6±0.2	18.8±4.8		P=0.0002, F=24.05		1.7±1.0	21.3±4.7	1.8±0.5	24.7±5.6		P=0.0001, F=38.41	
Ccl5	1.7±0.8	3.2±1.5	0.4±0.1	2.5±1.4				1.3±0.4	3.6±1.0	3.2±1.6	4.3±1.5			
Ccl17	1.2±0.2	1.1±0.3	0.8±0.2	0.7±0.1				2.6±1.9	3.1±2.0	1.8±0.4	1.4±0.4			P=0.039, F=5.394
Nos2	2.4±1.7	17.9±13.5	0.3±0.1	0.4±0.1				1.2±0.4	1.6±0.1	1.9±0.5	2.5±0.7			
Arg2	1.3±0.3	0.9±0.2	0.6±0.1	0.7±0.1	P=0.041, F=5.106			1.0±0.3	1.1±0.3	1.3±0.2	1.6±0.3			
Mrc1 cluster genes														
Ang	1.0±0.1	1.1±0.2	1.7±0.2	1.9±0.3	P=0.022, F=7.314			1.1±0.1	1.5±0.2	1.7±0.4	1.9±0.1		P=0.054, F= 4.735	
Dab2	1.0±0.1	2.5±0.2	1.3±0.1	3.1±0.1	P=0.013, F=9.079	P<0.0001, F=141.1		1.0±0.1	3.9±0.3	1.7±0.4	3.6±0.6		P =<0.0001, F =56.46	
Gas6	1.0±0.1	1.1±0.1	0.8±0.1	0.9±0.1	P=0.017, F=8.009	P=0.044, F=5.254		1.0±0.1	1.3±0.2	1.0±0.1	1.0±0.1			
Igf1	1.0±0.1	1.2±0.1	1.2±0.1	1.5±0.1	P=0.021, F=7.391	P=0.0005, F=26.01		1.0±0.1	1.6±0.2	1.1±0.1	1.4±0.2		P = 0.002, F=15.95	
Hpgds	1.0±0.1	1.5±0.1	0.9±0.1	1.3±0.1		P<0.0001, F=95.07		1.0±0.1	2.5±0.3	1.2±0.3	2.1±0.2		P = <0.001, F =55.84	
Rnase4	1.0±0.1	1.1±0.1	1.0±0.1	1.0±0.2				1.0±0.1	1.7±0.2	1.3±0.1	1.4±0.1		P= 0.004, F=13.43	P= 0.045, F=5.210
Stard8	1.0±0.1	0.9±0.1	1.0±0.1	0.8±0.1				1.0±0.1	1.0±0.1	1.3±0.1	1.0±0.1			
Wwp1	1.0±0.1	1.1±0.1	0.8±0.1	0.8±0.1	P=0.004, F =12.93			1.0±0.1	1.1±0.1	1.0±0.1	0.9±0.1			
Itgax	1.0±0.1	1.1±0.1	1.6±0.2	1.8±0.3	P=0.020, F=7.548			1.0±0.1	0.9±0.1	1.3±0.2	1.5±0.1	P=0.0171, F=8.153		P=.022, F=7.254

3.10. HCA_2 activation by nicotinic acid induces neuroprotective factors

Since we noticed a regulated expression of the *Mrc1* cluster genes in vivo, we wanted to analyze the regulation of these genes in isolated monocytes/macrophages after cerebral ischemia. We sorted $CD45^{int}CD11b^{+}$ cells, corresponding to resident microglia and $CD45^{hi}CD11b^{+}$ infiltrating monocytes/macrophages from the ischemic hemisphere (Gliem et al, 2012b; Sedgwick et al, 1991). Quantitative real-time RT-PCR of these sorted cells revealed that treatment with nicotinic acid increased the expression of the two neuroprotective genes *Igf1* and *Ang* (Figure 3.10A, B).

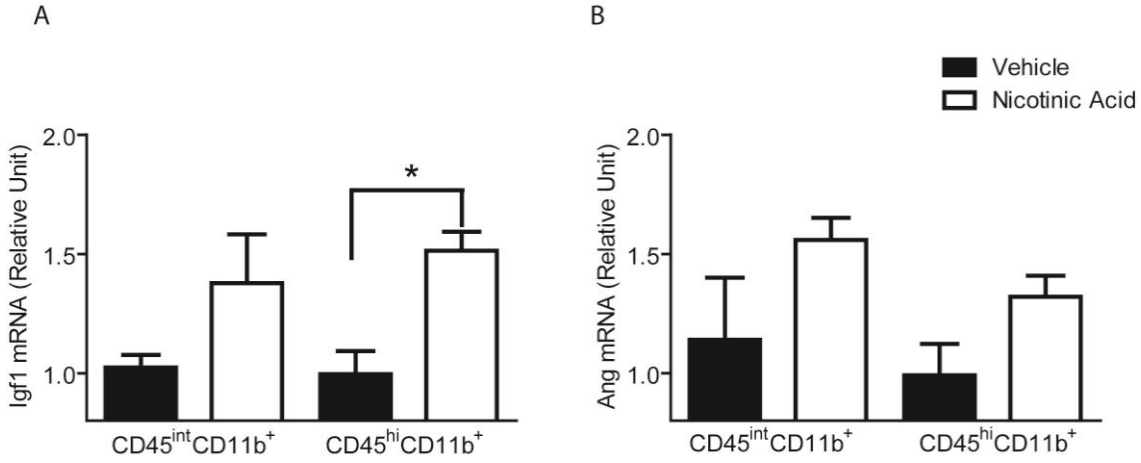


Figure 3.10. HCA_2 activation by nicotinic acid induces neuroprotective factors. *(A, B) Nicotinic acid treatment (100 mg/kg) increased the expression of Igf1 and Ang in $CD45^{int}CD11b^{+}$ and $CD45^{hi}CD11b^{+}$ cells that were sorted from the ischemic hemisphere of mice 48 h after MCAO. Expression levels were determined by quantitative RT-PCR. Two-way repeated measures ANOVA of Igf mRNA for nicotinic treatment, $F(1/6)=11.01$, $p=0.016$. $*p<0.05$ (Bonferroni posthoc test). Ang mRNA, $F(1/12)=9.533$, $p<0.01$. Values are means ± SEM (n=4-7).*

3.11. Protective effect of HCA_2 activation depends on COX-1 and prostaglandin D_2

Hematopoietic PGD_2 synthase (*Hpgds)* is another member of the *Mrc1* gene cluster (Mabbott et al, 2010) that was upregulated by cerebral ischemia but not by nicotinic acid treatment (Table 2). HPGDS and COX-1 are responsible for synthesizing PGD_2 and its derivatives in macrophages (Knowles et al, 2006; Zhao et al, 2013a). In accordance with the finding that HCA_2 activation stimulates PGD_2 release from macrophages (Meyers et al, 2007b), plasma concentrations of PGD_2 increased upon nicotinic acid treatment (Figure 3.11A). To test the role of PGD_2 synthesis in HCA_2-mediated neuroprotection we used *Cox1*$^{-/-}$ mice and an inhibitor of HPGDS. In our stroke model, the infarct volume in vehicle-treated *Cox1*$^{-/-}$ mice was similar as in *Cox1*$^{+/+}$ animals in line with some but not all previous studies (Cheung et al, 2002; Iadecola et al, 2001a; Zou et al, 2006). Interestingly, nicotinic acid had no effect on the infarct volume in *Cox1*$^{-/-}$ mice whereas *Cox1*$^{+/+}$ littermates were protected (Figure 3.11B). Furthermore, when we inhibited HPGDS in mice with the small molecule compound HQL-79, the protective effect of nicotinic acid was partially reversed (Figure 3.11C), suggesting that COX1 and HPGDS mediate the effect of nicotinic acid.

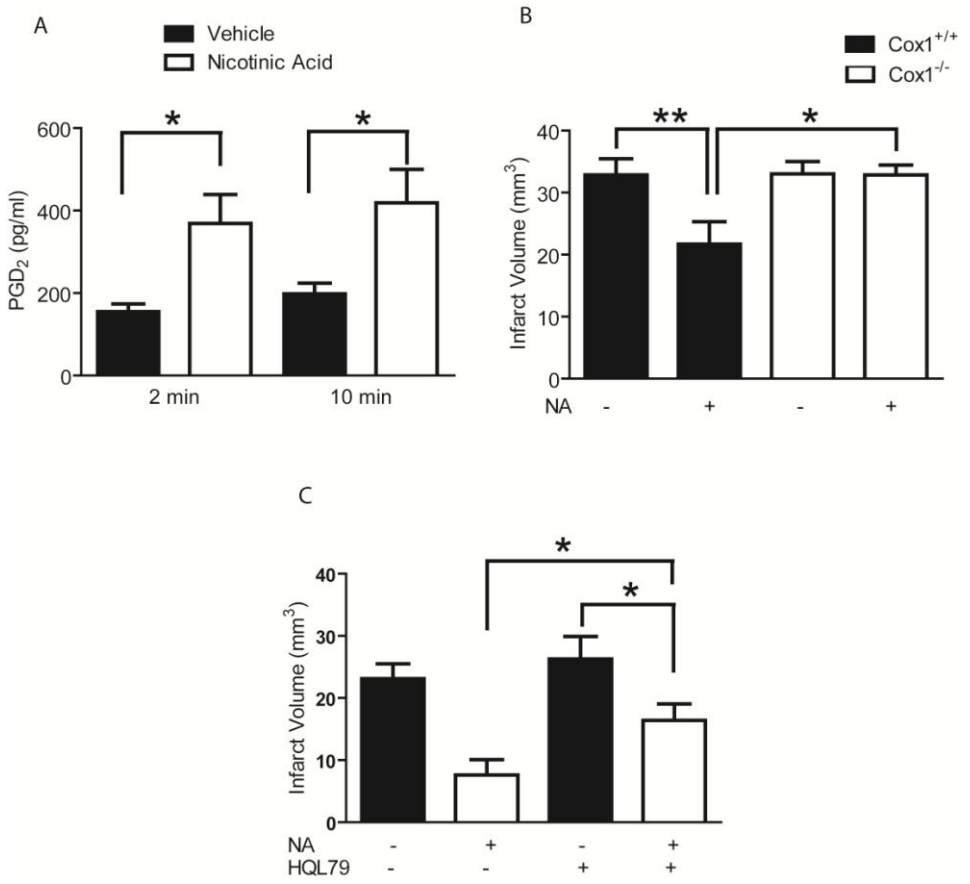


Figure 3.11. COX-1 and PGD_2 dependent protective effect of HCA_2 activation. *(A) PGD_2 plasma concentrations increased after nicotinic acid treatment. The time between nicotinic acid (100 mg/kg) treatment and blood sampling is indicated. Two-way repeated-measures ANOVA, F(1/15)=16.45, p=0.001. *p<0.05 (Bonferroni posthoc test). Values are means ± SEM (n=8-9). (B) The protective effect of nicotinic acid (100 mg/kg) was lost in $Cox1^{-/-}$ mice. The infarct volume was determined 48 h after MCAO. ANOVA, F(3/30)=4.617, p<0.01. *p<0.05, **p<0.001 (Newman-Keuls posthoc test). Values are means ± SEM (n=7-8). (C) Inhibition of HPGDS by HQL-79 (30 mg/kg) partially reversed the neuroprotective effect of nicotinic acid (100 mg/kg). Infarct volumes were determined 48 h after MCAO. One-way ANOVA, F (3/36)=8.471. p=0.0002. *p<0.05 (Bonferroni posthoc test). Values are means ± SEM (n=10).*

4. Discussion

Extensive research has been carried out to understand the biology of HCA_2 receptors and their potential implications in disease models. Nevertheless, the impact of HCA_2 activation in the context of cerebral ischemia has not been elucidated so far. Apart from their well recognized metabolic role in lipid mobilization, little was known about the activation of HCA_2 receptors in brain and the associated immune modulation especially in the context of cerebral ischemia. In this study we demonstrated that activation of HCA_2 by ketogenic diet, BHB or nicotinic acid is neuroprotective. The current study demonstrated not only a protective role of HCA_2 activation in cerebral ischemia but also provide insight into how it can modulate immune cells to carry a neuroprotective signal to the brain after cerebral ischemia.

4.1. HCA_2-dependent neuroprotective effects of ketogenic diet and BHB

Over 90 years of experience with ketogenic diet have provided evidence for its antiepileptic and neuroprotective efficacy. It is now an established treatment for pharmacoresistant childhood epilepsies and constitutes an experimental therapy for various neurodegenerative diseases. However, compliance is often low because the diet is unpalatable. Elucidating its neuroprotective mechanisms may guide drug development and may ultimately lead to a 'ketogenic diet in a pill' (Gasior et al, 2006b).

Here we provide evidence that the neuroprotective action of ketogenic diet and of BHB is mediated by HCA_2 receptors (Figure 3.2, 3.3). So far it has been established that HCA_2 receptors on adipocytes are activated by the endogenous ketone body BHB and by the anti-dyslipidemic drug nicotinic acid (Lukasova et al, 2011a; Taggart et al, 2005). However, CNS effects mediated by this G protein-coupled receptor were unknown. We showed here now that

chronic treatment with ketogenic diet prophylacticaly could generate therapeutic concentrations of the ketone body BHB (Figure 3.2A) that were able to activate HCA_2 receptor and thereby to protect the ischemic brain (Figure 3.2B). Pharmacological infusion of BHB through osmotic pumps induced a similar outcome as ketogenic diet which confirms the neuroprotective role of BHB. Until now, clinical trials of ketogenic diet in patients with stroke have not been performed although several animal studies reported a neuroprotective potential of ketogenic diet (Puchowicz et al, 2008; Stafstrom & Rho, 2012).

Ketone metabolism has some distinctive features that places it as favorable energy substrate for the brain in various pathological conditions. In contrast to 11 biochemical steps to process glucose only 3 enzymatic steps are involved to produce acetyl-CoA from β-hydroxybutyrate metabolism (Prins, 2008). It improves mitochondrial metabolism and decreases oxygen consumption. Production of free radicals is also decreased by ketone metabolism since it decreases the reduced form of coenzyme Q and thereby decreases its reaction with O_2 to form superoxide $O_2^{-\bullet}$ (Prins, 2008). Cerebral blood flow has also been reported to be substantially increased upon ketone metabolism (Prins, 2008). Considering all these findings, it is speculative that multiple mechanisms are involved in mediating the neuroprotective effect of ketogenic diet.

Puchowicz et al. have reported a link between the neuroprotective role of ketogenic diet and hypoxia inducible factor (HIF-1α) (Puchowicz et al, 2008). In their study, pretreatment with BHB leads to an increased content of brain succinate as well as HIF-1α and Bcl-2 after MCAO. In an earlier study they also found an increased brain capillary density after 3 weeks of treatment with ketogenic diet. HIF-1α is important in angiogenesis and anti-apoptotic

activity (Stafstrom & Rho, 2012). On the other hand, angiogenesis is known to play an important role in improving stroke outcome. In an animal model of stroke, we demonstrated that activation of HCA_2 by nicotinic acid leads to significantly higher expression of *Ang* and and *Igf1* in brain after nicotinic acid treatment (Figure.3.9C, D). These macrophagic genes have been involved in neuroprotection and angiogenesis (Butovsky et al, 2006; Butovsky et al, 2007; Kieran et al, 2008; Sebastia et al, 2009; van Es et al, 2011).

4.2. The HCA_2 agonist nicotinic acid in cerebral ischemia

HCA_2 receptors are activated by nicotinic acid, a drug that is used clinically to lower serum lipid concentrations (Tunaru et al, 2003). Although previous studies reported that nicotinic acid ameliorates ischemic brain damage (Chen et al, 2007; Shehadah et al, 2010a), its mode of action was unclear. Our data now show that the neuroprotective activity of nicotinic acid depends on HCA_2, very much as that of BHB and ketogenic diet. Nicotinic acid is able to penetrate the blood-brain barrier (Hankes et al, 1991). Under normal conditions HCA_2 receptors are expressed by microglia. In ischemic stroke and in chronic neurodegenerative diseases, such as AD, PD, and ALS, monocytes/macrophages infiltrate the brain (Biju et al, 2010; Butovsky et al, 2012a; Prinz et al, 2011a). In accordance with a recent study infiltrating monocytes/macrophages alone seem to have little effect on the infarct size in ischemic stroke (Gliem et al, 2012b). However, they express HCA_2 (Figure 3.1.D) and mediate its neuroprotective action as demonstrated by our experiments in which bone marrow was transplanted. These data exclude an indirect effect secondary to altered lipid levels or increased release of the neuroprotective factor adiponectin from adipocytes in response to HCA_2 activation (Digby et al, 2010; Nishimura et al, 2008). Furthermore, the experiments

argue against a major role of resident microglia in HCA_2-induced neuroprotection because this cell population is not exchanged by bone marrow transplantation (Mildner et al, 2007).

Delay in treating stroke patient is a common situation faced in clinical practice. The only available pharmacological approach till now to treat stroke patients is tPA. In this study we demonstrated now that nicotinic acid-induced activation of HCA_2 receptors retained its efficacy even 4.5 hours after the onset of stroke. Furthermore, the functional outcome was improved upon nicotinic acid treatment as evaluated by three established sensorimotor tests (Figure 3.5). Therefore, HCA_2 and the anti-lipolytic drug nicotinic acid possess immense therapeutic potential in treating cerebral ischemia given that nicotinic acid is already in clinical practice to treat dislipidemia since decades.

4.3. Neuroprotective and anti-inflammatory role of infiltrating monocytes/macrophages

In response to injury, inflammation being an integral part of body's defense mechanism initiates cascades of events in order to set back homeostatic functions. To prevent tissue demise and loss of functions, coordinated titration of inflammation is critical. In the context of cerebral ischemia, inflammation has been viewed as one of the key contributors to pathophysiological conditions (Iadecola & Anrather, 2011). Monocytes/macrophages are immune effectors cells and appear in the ischemic brain 24-48 hours after ischemia (Iadecola, 1997). The plastic nature of macrophages sets them apart from other immune cell populations and allows them to efficiently adapt to environmental signals. The pro-inflammatory nature of monocytes/macrophages is critical for host defense provided that they are well balanced and regulated (Mosser & Edwards, 2008). Alternatively activated macrophages are anti-inflammatory in nature and linked to wound healing and repair mechanism (Gordon, 2003;

Lawrence & Natoli, 2011). The role of monocytes/macrophages in cerebral ischemia as well as in other neurodegenerative diseases is critical (Biju et al, 2010; Butovsky et al, 2012b; Prinz et al, 2011b). In an elegant study, Gliem et al. demonstrated how a specific population of monocytes/macrophages critically determines outcome of lesion-associated inflammation (Gliem et al, 2012a). They provided evidence that inflammatory monocytes are recruited within 24 hours of cerebral ischemia in a CCL2-dependent manner and transform locally into a non-inflammatory state which then plays a role in maintaining the integrity of the neurovascular unit following cerebral ischemia (Gliem et al, 2012a). Monocytes/macrophages are heterogeneous in nature having different subtypes attributed to specific roles in a specific tissue environment. For example, highly mobile Ly-6C^{hi}CCR2^{+} monocytes are inflammatory in nature and are rapidly recruited to inflamed tissues, whereas Ly-6C^{lo}CCR2^{-} monocytes are resident and believed to be important for patrolling blood vessels (King et al, 2009; Mildner et al, 2009; Prinz et al, 2011b). In the current study, we found an increased infiltration of CD11b^{+}Ly-6C^{hi} monocytes in the ischemic hemisphere where as ischemia had no significant effects on CD11b^{+}Ly-6C^{lo} monocytes (Figure 3.8).

Given the complexity of macrophage activation, a number of markers have been identified to investigate macrophage polarization and its ultimate functions (Gordon, 2003; Mosser & Edwards, 2008). An anti-inflammatory role of monocytes/macrophages (M2) in cerebral ischemia has been described in many different animal models, most of which have demonstrated an alleviation of the pro-inflammatory M1 phenotype while promoting the M2 phenotype in different stages of ischemia (Frieler et al, 2011; Fumagalli et al, 2013; Hu et al, 2012; Xu et al, 2012).

In our experimental paradigm, we observed an infiltration of $CD11b^+$ and $Iba1^+$ monocytes/macrophages in the brain 48 hours after cerebral ischemia (Figure 3.1D and 3.6). Treatment with nicotinic acid reduced the number of infiltrating monocytes/macrophages as revealed by reduction in $mRFP^+$ cells (Figure 3.6 B, C). By generating bone marrow chimers we confirmed that HCA_2 receptors present in peripherial monocytes/macrophages account for this effect. In conjunction with this project, Sajjad Muhammad investigated the effect of nicotinic acid treatment in CD11b-DTR mice after cerebral ischemia (unpublished). As reported previously, ablation of $CD11b^+$ cells by itself did not alter the infarct size (Gliem et al, 2012b). However, when monocytes/macrophages had been ablated, nicotinic acid no longer reduced the infarct volume, demonstrating that the neuroprotective effect of nicotinic acid depends on monocytes/macrophages.

We observed an increased expression of *Mrc1* and *Fizz1 (Retnla),* marker gene for M2 polarized macrophages when we activated HCA_2 receptors with its agonist nicotinic acid (Figure 3.9 A, B). This suggests a partial M2 polarization since other marker genes were unaffected (Table 3.2)

Mrc1 is part of a specific cluster of genes that are coordinately expressed mainly in macrophages and bone marrow-derived dendritic cells (Mabbott et al, 2010). We observed a regulated expression of many of the other member genes in brain upon nicotinic acid treatment (Table 3.2) including *Igf1* and *Ang* (Figure 3.9 C, D) which are well known to induce neuroprotection and angiogenesis, respectively (Butovsky et al, 2006; Butovsky et al, 2007; Kieran et al, 2008; Sebastia et al, 2009; van Es et al, 2011). We observed similar findings in macrophages isolated from the ischemic brain (Figure 3.10A, B). Therefore, in our

study activation of HCA_2 by nicotinic acid released neuroprotective factors and offered an anti-inflammatory environment by partially polarizing monocytes/macrophages after cerebral ischemia.

4.4. Cyclooxygenase 1 (COX-1) and prostaglandin D_2 (PGD_2) dependent neuroprotection by monocytes/macrophages

The pro-inflammatory mediator COX is involved in the progression of stroke associated damage (Ahmad et al, 2010). Owing to the predominant expression in microglia, the constitutively expressed isoform COX-1 was suggested to be the major player in mediating the inflammatory response (Choi et al, 2009). Pharmacological inhibition of this enzyme leads to reduced neuronal injury and oxidative stress during transient global cerebral ischemia (Candelario-Jalil, 2003) although Iadecola et al. reported increased susceptibility to focal cerebral ischemia upon genetic deletion of *Cox-1* (Iadecola et al, 2001b). Due to these discrepancies in mediating inflammatory cascade, COX-1 warrants further investigations detailing its crucial role in cerebral ischemia.

Using genetic deletion of *Cox-1* and pharmacological inhibition of hematopoietic prostaglandin D synthase (HPGDS) by the small molecule HQL-79, we provided evidence that the neuroprotective effect of HCA_2 activation depends on COX-1 and HPGDS (Figure 3.11 B, C). Our data suggest a new concept by which PGD_2 release from monocytes/macrophages mediates the neuroprotective effect of HCA_2 receptors.

HPGDS is downstream of COX-1 and responsible for producing prostaglandin D_2 from its precursor PGH_2. Hanson et al. and others reported that HPGDS is the key enzyme that synthesizes PGD_2 in response to nicotinic acid treatment (Hanson et al, 2010; Song et al,

2012). In the normal brain, microglia expresses HPGDS (Liu et al, 2009; Taniguchi et al, 2007b). However, it is also known to be expressed in macrophages, dendritic cells, Langerhans cells, mast cells, Th2 cells, and megacaryocytes (Gandhi et al, 2011; Joo & Sadikot, 2012). Using bone marrow chimers exhibiting enhanced green fluorescence protein (EGFP) expression in bone marrow/blood-derived monocytes/macrophages Liu et al. demonstrated that 3 days and 7 days after reperfusion infiltrating monocytes/macrophages expresses HPGDS. Treatment with the inhibitor of HPGDS led to larger infarct volume (Liu et al, 2009)

PGD_2 plays an inflammatory role in peripheral tissues. It induces airway inflammation, inhibits platelet aggregation and induces peripheral vasodilation, glycogenolysis, allergic reaction, and intraocular pressure reduction (Ahmad et al, 2010; Taniguchi et al, 2007b). However, it is produced in the brain and is known to mediate homeostatic functions in regulating sleep, body temperature, nociception, and neuromodulation (Ahmad et al, 2010). Neuroprotective effects of PGD_2 have been demonstrated. Taniguchi et al. showed that PGD_2 is protective in hypoxic ischemic injury and this effect is mediated by DP1, a G_s protein-coupled receptor expressed in neurons and endothelial cells (Taniguchi et al, 2007b). Genetic deletion of DP1 resulted in increased susceptibility to ischemic brain damage which could be prevented by pharmacologic activation of the DP1 receptor (Ahmad et al, 2010).

Meyers and colleagues reported that activation of HCA_2 stimulates PGD_2 release from macrophages which was supported by our findings since we also observed increased plasma PGD_2 concentrations when mice were treated with nicotinic acid (Figure 3.11.A) (Meyers et al, 2007a). In line with others, the infarct volume of vehicle-treated *Cox-1*$^{-/-}$ mice was similar

as that of *Cox-1*$^{+/+}$ mice although Iadecola et al. reported *Cox-1*$^{-/-}$ as more susceptible to ischemic insults (Cheung et al, 2002; Iadecola & Alexander, 2001; Zou et al, 2006). This discrepancy could be attributed to different time points of infarct measurement since Iadecola et al. measured the infarct volume at 24 hours after ischemia.

Production of PGD_2 and its downstream 'double-dehydration' product 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is favored over PGE_2 during the resolution phase of inflammation (Gandhi et al, 2011) . Previous work has shown that PGD_2 helps to resolve inflammation (Rajakariar et al, 2007). In addition, it has neuroprotective effects (Masuda et al, 1986; Taniguchi et al, 2007a). PGD_2 has a short half-life in tissues and is spontaneously converted into the cyclopentenone 15d-PGJ_2, which inhibits the IκB kinase (IKK), the main activator of the transcription factor NF-κB and a key player in ischemic brain damage (Herrmann et al, 2005b; Rossi et al, 2000). It can also dampen NF-κB dependent transcriptional activation by blocking nuclear translocation and DNA binding of NF-κB (Surh et al, 2011). 15d-PGJ_2 is also an endogenous agonist of PPARγ, a transcription factor with neuroprotective properties (Ridder & Schwaninger, 2012; Zhao et al, 2009). Via this mechanism nicotinic acid is able to stimulate PPARγ in human monocytes in vitro (Knowles et al, 2006). Macrophages express PPARγ profusely and upon differentiation of monocytes into macrophages its expression is quickly induced (Bouhlel et al, 2007). Activation of PPARγ could lead to the generation of a specific macrophage population with a M2 phenotype and could exert pronounced anti-inflammatory properties on M1 macrophages by reprogramming mononuclear precursor cells in vivo (Bouhlel et al, 2007). Furthermore, it has been reported that 15d-PGJ_2 can stimulate angiogenesis (Kim & Surh, 2008), providing a potential explanation for how nicotinic acid treatment enhanced angiogenesis in the ischemic brain (Chen et al, 2007).

4.5. Proposed model of HCA_2-mediated neuroprotection in cerebral ischemia

Based on the current findings described in this study, we propose the following mechanism by which HCA_2 receptors mediate its neuroprotective role in cerebral ischemia (Figure 4.1). After an ischemic insult, peripheral monocytes/macrophages infiltrate the brain through a leaky blood-brain barrier. Activation of HCA_2 receptors present in these peripheral monocytes/macrophages by agonist binding would lead to activation of phospholipase C-β (PLCβ) via βγ-subunit of the receptor. Inositol-1,4,5,-triphosphate (IP_3) produced by PLCβ would release intracellular calcium which induces the activation of phospholipase A_2 (PLA_2). Arachidonic acid (AA) is synthesized and eventually metabolized to PGD_2 by the action of COX-1 and HPGDS. PGD_2 itself may lead to neuroprotection and maintain an anti-inflammatory environment. However, PGD_2 is further metabolized to the cyclopentenone 15-d-PGJ_2, which is a ligand of PPARγ. Activation of PPARγ is neuroprotective. Furthermore, 15-d-PGJ_2 inhibits IKK, a key enzyme responsible for activating NF-κB and thereby maintains an anti-inflammatory environment by dampening pro-inflammatory signaling.

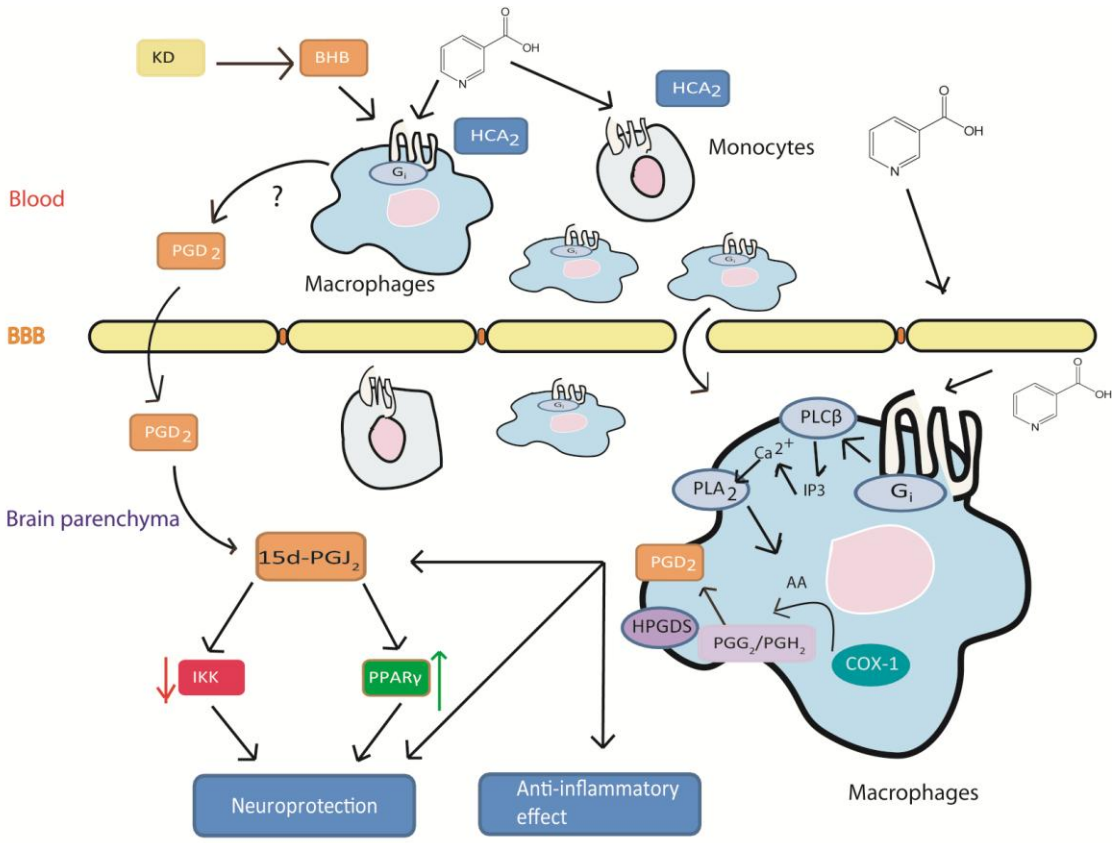


Figure 4.1. HCA_2-mediated neuroprotection in cerebral ischemia. *Agonist binding to HCA_2 leads to synthesis of AA from which PGG_2 is formed by the action of COX-1 and then HPGDS produce PGD_2 from is precursor. PGD_2 further metabolized to 15d-PGJ_2 which inhibits IKK. 15d-PGJ_2 also could activate PPARγ which is neuroprotective.*

4.6. Implication of HCA_2 activation

The current study demonstrated that HCA_2 activation induces a neuroprotective repertoire of monocytes/macrophages that can reduce ischemic brain damage. Infiltration of

monocytes/macrophages into the diseased brain has been noted in several neurodegenerative disorders (Prinz et al, 2011a), which suggests that the findings we obtained in a model of ischemic stroke may have implications that extend far beyond this specific disease. Importantly, the HCA_2 receptor is a good target for drug development, with some agonistic compounds already in clinical use to lower plasma lipids and others showing evidence of a superior potency or of fewer side effects than nicotinic acid during clinical trials (Lauring et al, 2012; Offermanns et al, 2011; Shen & Colletti, 2010). Thus, the vision of a 'ketogenic diet in a pill' may be within reach. A synthetic HCA_2 agonist with a favorable pharmacological profile may bring this therapeutic principle to the large population of patients suffering from stroke and neurodegenerative diseases. In conclusion, these data suggest a novel concept in which HCA_2 receptors provide the pharmacological basis for modulating monocyte/macrophage function and redirecting these important cells into a neuroprotective pathway.

4.7. Area of uncertainty

We found that ketogenic diet, BHB and nicotinic acid reprogrammed monocytes/macrophages into a phenotype that offered neuroprotection in cerebral ischemia. This protective effect was dependent on COX-1 and HPGDS-mediated PGD_2 production. However, there are certain issues that are still illusive and are not explained by the current study and warrant further investigations. Although we demonstrated that Cox-1-dependent PGD_2 production is responsible for neuroprotection, it was not clear whether infiltrated mononuclear cells express COX-1.

In our experiments, we documented increased PGD_2 production in plasma. It is possible that brain PGD_2 could play a key role as well. Therefore, measurement of brain PGD_2 needs to be performed to identify the critical source of PGD_2.

While COX-1 is constitutively expressed in almost all tissues, the COX-2 isoform is an inducible form that is induced by inflammatory stimuli (Choi et al, 2009). We demonstrated in our study that nicotinic acid-induced PGD_2 production was COX-1-dependent. However , we cannot exclude a possible role of COX-2 since COX-2 has also been reported to be expressed constitutively in the brain, especially in hippocampal and cortical glutamatergic neurons, and to play a role in neurovascular coupling during functional hyperemia (Choi et al, 2009). Thus, it is important to investigate the role of COX-2 in brain since COX-2 could also be linked to anti-inflammatory and neuroprotective properties (Choi et al, 2009).

Abbreviations

AA	Arachidonic acid
AcAc	Acetoacetate
AC	Adenylyl cyclase
AChE	Acetylcholinesterase
AD	Alzheimers diseases
ALS	Amyotrophic lateral sclerosis
AMV	Avian Myeloblastosis Virus
ANOVA	Analysis of variance
APC	Allophycocyanin
ATP	Adenosine tri phosphate
BAC	Bacterial artificial chromosome
BBB	Blood-brain barrier
Bcl2	B- cell lymphoma 2
BHB	β-hydroxybutyrate
BlK	Blank
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD11b	Cluster of designation 11 b
cDNA	Complementary deoxyribonucleic acid
CETP	Cholesteryl ester transfer protein
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DAMP	Damage-associated molecular pattern molecules
DAPI	4´,6-Diamino-2-phenylindole
DMEM	Dulbecco´s modified eagle medium
dNTP	Deoxyribonucleotide triphosphate
dPBS	Dulbecco's phosphate-buffered saline
EIA	Enzyme immunoassay
FDA	Food and Drug Administration
FFA	Free fatty acid
Fizz1	Found in inflammatory zone 1
GFAP	Glial fibrilatory acedic protein
GM-CSF	Granulocyte macrophage - colony stimulating factor
GPCRs	G protein-coupled receptors
GPR109A	G protein-coupled receptor 109 A
HCA_2	Hydroxyl-carboxylic acid receptor 2
HDL	High density lipoprotein
HIF-1α	Hypoxia inducible factor 1
HM74 A	Human receptor 74 A

HPGDS	Hematopoietic prostaglandin D synthase
HQL-79	4-(Diphenylmethoxy)-1-(3-2*H*-tetrazol-5-yl)propyl]-piperidine
HSL	Hormone sensitive lipase
Iba 1	Ionized calcium binding adaptor molecule 1
IC	Ischemic core
IFNγ	Interferon gamma
IGF-1	Insuline like growth factor-1
IKK	IκB kinase
IL-10	Interlukin-10
IL-13	Interlukin -13
IL-4	Interlukin-4
iNOS	Inducible nitric oside synthase
IP_3	Inositol-1,4,5-triphosphate
IκB	Inhibitors of κB
KD	Ketogenic diet
LH	Left hemisphere
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
M-CFU	Myeloid colcony forming unit
M-CSF	Macrophage colony stimulating factor 1
MDP	Macrophage and dendritic cell progenitor
MOX	Metoxime
Mrc1	Macrophage mannose rceptor 1
mRFP	monomeric red fluorescence protein
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
ND	Normal diet
NeuN	Neuronal nuclei
NFκ-B	Nuclear factor kappa B
NSB	Non specific binding
NT	Normal tissue
PBMs	Peripheral blood-mono-nuclear cells
PBS	Phosphate-buffered saline
PD	Perkinson's diseases
PE	R-Phycoerythrin
PFA	Paraformaldehyde
PGE_2	Prostaglandin E 2
PGF_2	Prostaglandin F 2
PGG_2	Prostaglandin G 2
PGH_2	Prostaglandin H 2

PGHS	prostaglandin endoperoxide H synthase
PGI_2	Prostaglandin I 2
PGJ_2	Prostaglandin J 2
PGs	Prostaglandins
PKA	Protein kinase A
PLA_2	Phospholipase A 2
PLC-β	Phospholipa se C beta
PPARγ	Peroxisome proliferator-activated receptor gamma
PtdSer	Phosphatidyl serine
PUMA-G	Protein-upregulated in macrophages by INFγ
RH	Right hemisphere
RT-PCR	Reverse transcriptase polimerase chain reaction
SD	Silver deficit
SEM	Standard error of mean
TA	Total activity
TG	Triglyceride
TGFβ	Transforming growth factor beta
TIM4	T cell immunoglobulin and mucin domain-containing milecule 4
TLRs	Toll like receptors
TNFα	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
VLDL	Very low density lipoprotein
$Vol_{infarct}$	Infarct volume
YM1	Chitinase 3-like-3

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Publications

Manuscript published

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2. Bargiotas P, Muhammad S, **Rahman M**, Jakob N, Trabold R, Fuchs E, Schilling L, Plesnila N, Monyer H, Schwaninger M (2012) Connexin 36 promotes cortical spreading depolarization and ischemic brain damage. Brain research 1479**:** 80-85

3. Rosell A, Agin V, **Rahman M,** Morancho A, Ali C, Koistinaho J, Wang X, Vivien D, Schwaninger M, Montaner J (2013) Distal Occlusion of the Middle Cerebral Artery in Mice: Are We Ready to Assess Long-Term Functional Outcome? Transl Stroke Res Volume 4, Issue 3 (2013), Page 297-307

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4. **Mahbubur Rahman**, Sajjad Muhammad, Hui Chen, Dirk A. Ridder, Barbora Pokorná, Tillman Vollbrandt, Jürgen G. Okun, Stefan Offermanns and Markus Schwaninger

   The β-hydroxybutyrate receptor HCA_2 induces a neuroprotective phenotype of macrophages

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5. Ann-Marie Heinonen, **Mahbubur Rahman**, Godwin Dogbevia, Stefan Wölfl, Rolf Sprengel, Markus Schwaninger

   Use of the 2A peptide for the virally mediated coexpression of two proteins in cerebral ischemia