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DISSERTATION

Necdin is a novel modulator in microglia proliferation in the murine brain following cerebral ischemia

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For my parents

Abbreviations

ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
APAF	Apoptotic protease activating factor 1
APC	Allophycocyanin,
Bcl-2	B-cell leukemia 2
BDNF	Brain derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
BBB	Blood brain barrier
BV	Pacific Blue
CAT	Catalase
CBF	Cerebral blood flow
CD	Cluster of differentiation
CDC2	Cell division cycle 2
CI	Confidence interval
CNS	Centre Nervous System
CREB	cAMP response element binding protein
CX3CR1	CX3C chemokine receptor 1/fractalkine receptor
CX3CL1	Chemokine (C-X3-C motif) ligand 1
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DIV	Day in vitro
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
DRG	Dorsal root ganglion
DMEM	Eagle's minimal essential medium
EDTA	Ethylendiaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
E2F	E2 (adenoviral protein) factor

EdU	5-ethynyl-2´-deoxyuridine
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDAC1	Histone deacetylase 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HRP	horseradish peroxidase
Iba1	ionized calcium binding adaptor molecule 1
ICAM1	Inter-cellular adhesion molecule 1
IPW	Imprinted in Prader-Willi syndrome
iap	Intracisternal A particle repeat
iNOS	Inducible nitric oxide synthase
IL-1	Interleukin-1
LDH	Lactate dehydrogenase
LVPs	Lentiviral particles
MACS	Magnetic activated cell sorting
MAP2	Microtubule associated protein 2
MABP	Mean arterial blood pressure
MAPK	Mitogen-activating protein kinase
MAGE	Melanoma antigen gene expression
MHC	Major histocompatibility complex
MHD	MAGE homology domain
MCAo	Middle cerebral artery occlusion
mRNA	Messenger ribonucleic acid
MSc	Master of Science
NEDD8	Neuronal-precursor-cell-expressed developmentally downregulated protein-8
nNOS	Neuronal nitric oxide synthases

NaCl	Sodium chloride
Necdin	Neurally differentiated embryonal carcinoma-derived proteIn
NP-40	Nonidet P-40
NFκb	Nuclear factor kappa B
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NGF	Nerve growth factor
OGD	Oxygen-glucose deprivation
PLL	poly-L-lysine
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PE	Phycoerythrin
PMN	Polymorphonuclear leukocyte
PWS	Prader-Willi syndrome
PC	Phase contrast
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
PKA	Protein kinase A
Rb	Retinoblastoma protein
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
rtPA	Recombinant tissue plasminogen activator
rCBF	Regional cerebral blood flow
SOD	Superoxide dismutase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNRPN	small nuclear ribonucleoprotein N
Sirt	Sirtuin gene
SUMO	Small ubiquitin-like modifier
TCR	T cell receptor
TGF-beta	Tranforming growth factor-beta
TNF-alpha	Tumor necrosis factor-alpha
TLR	Toll-like receptor

TPP2	Tripeptidyl-peptidase II
UBL	Ubiquitin-like protein
VEGF	Vascular endothelial cell growth factor
ZNF	Zinc finger

1 Summary

Necdin is a member of the MAGE (melanoma antigen) family genes. The deletion of the necdin gene is implicated in Prader-Willi syndrome and is maternally imprinted in human and mouse. To date, expression and physiological function of necdin is associated with neuronal cell cycle of the central nervous system (CNS). In this study, we identify a previously unknown role of necdin for microglial proliferation.

In resident microglia of mouse brain, necdin was expressed at low levels, but increased significantly following brain ischemia, seen both in vitro and in vivo. Downregulation of endogenous necdin expression (via intra-arterial injection of lentiviral particles coding microRNA targeting necdin) resulted in increasing proliferation of ionized calcium binding adaptor molecule 1 (Iba1)-positive cells. In addition, necdin mediated proliferative Iba1-positive cells were restricted to a subtype of resident microglia which assumed a reactive phenotype. This reactive subpopulation of resident microglia was recognized as $CD11b^{+}/CD45^{high}$ expressing cells. Furthermore, a putative posttranslational modification of necdin was identified in microglial cells. Taken together, necdin is a modulator that negatively regulates the proliferation of reactive resident microglia following ischemic conditions.

There is some debate as to whether the presence of reactive microglia in higher amount at the lesion sites are beneficial or detrimental, thus further study is required to elucidate the impact of necdin in brain ischemia. Furthermore, necdin may serve as a therapeutic target in modulating microglial proliferation in the pathophysiological cascade after brain ischemia.

2 Introduction

2.1 Cerebral Ischemia

2.1.1 Prevalence, mortality, treatment options and prognosis

Stroke is the third leading cause of death and major cause of permanent disability worldwide. Ischemic stroke accounts for 80% of the total cases, and the rest is represented by the hemorrhagic strokes (1). Ischemic stroke is caused by a transient or permanent reduction in regional cerebral blood flow, which is essential in supplying oxygen and nutrients to the brain. The occlusion that renders declining blood flow is a result of either an embolus or local thrombosis (2). This pathological condition renders ultimately to the dysfunction and/or death of brain cells, which is manifested by long-term neurological impairments that reflects the severity of the insult and vulnerability of the neurons in the affected brain regions. To date, the only FDA (US Food and Drug Administration) approved treatment is the thrombolytic therapy, whereby recombinant tissue plasminogen activator (rtPA) is administered to re-open occluded blood vessels. However, the application of rtPA is restricted to a narrow therapeutic time-window of 4 and a half hours after stroke onset (3), which is the limiting factor of the treatment, thus failing to reach most of the patients. Therefore, a thorough understanding of underlying mechanisms and molecular players in damaging cascades after stroke are instructive, as well as imperative, in the identification of novel drug targets to allow the emergence of new avenues for therapeutic intervention in cerebral ischemia.

2.1.2 Experimental Stroke Models in mice

In humans, ischemic stroke occurs most frequently in the brain region of the middle cerebral artery (MCA) (4). Thus, rodent models of focal cerebral ischemia are being used to make new discoveries on novel therapeutic approached. Permanent or transient occlusion of MCA is performed in mice or rats in order to simulate human stroke patients. There are generally two durations of MCAo widely used for transient stroke model, one reflecting a milder ischemic injury with a 30 min occlusion and the other with 60 min occlusion for a severe ischemic insult (5). Depending on the model used, certain mechanisms may predominate as described in detail below.

Selective neuronal vulnerability has been described and characterized in a 30 min transient MCAo, a model in which caspase-dependent delayed apoptotic death predominates (6, 7) but is associated with intact energy metabolism in medium spiny neurons of the striatum (6). Katchanov et al have shown that there is a substantial loss of medium spiny projection neurons at day 3 within the ischemic lesion. In contrast, the numbers of cholinergic, GABAergic, and somatostatin-containing interneurons in the ischemic striatum were not different from those in the contralateral hemisphere at 3 and 14 day (6). In contrast to this selective parenchyma necrosis of distinct neuronal populations more severe episodes of ischemia (60 min occlusion time followed by reperfusion) leads to an aggravation of the infarct area and necrosis of all neurons within the core of the infarct (7). Under these conditions, the evolution of injury is predominated by a failure of the cerebral energy state.

2.1.3 Mechanisms of ischemic brain injury

The "neurovascular unit" consists of neurons, astrocytes, pericytes, extracellular matrix (ECM) and microvascular endothelium which are the anatomical substrate of the blood brain barrier (BBB). The complex and dynamic interplay between these different cell types in the neurovascular unit is very essential for proper functioning of the brain under physiological conditions, as well as in determining the extent of neuronal damage in the pathogenesis of brain ischemia. The reduction in blood flow compromises functional neuronal activity in the ischemic brain. Moreover as ischemia progresses, metabolic activity is suppressed to maintain the structural integrity of the cells (8). This leads to a complex sequence of pathophysiological events that impairs neurological functions mediated by necrosis, excitotoxicity, periinfarct depolarizations, oxidative stress, inflammation and apoptosis (2, 4). The pathogenic events evolve in an overlapping manner, occurring over minutes, while others over hours to days (Figure 1), which contributes to the demise of neurons, glia and endothelial cells (2, 9, 10).

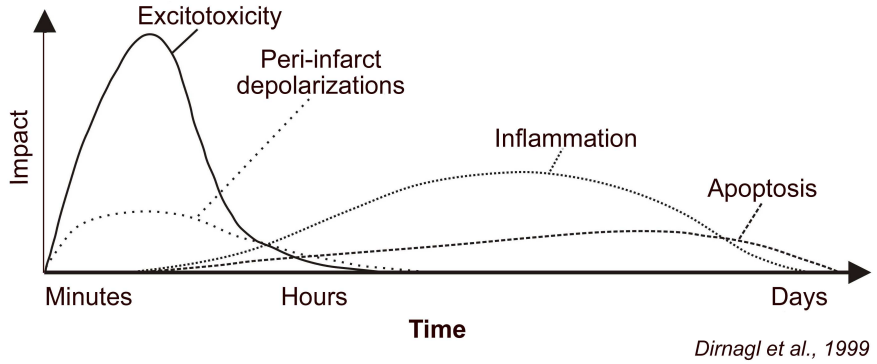
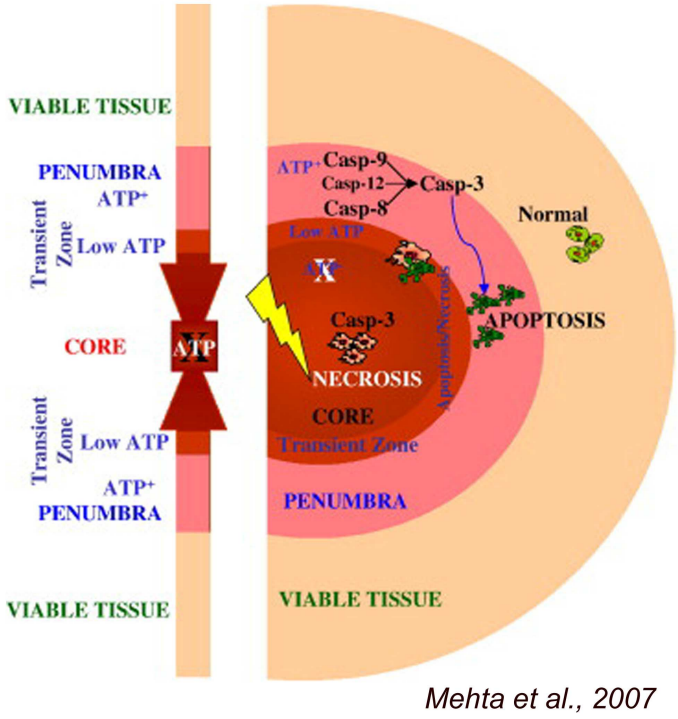


Figure 1 Putative cascade of damaging events in focal cerebral ischemia. The x-axis reflects the evolution of the cascade overtime, while the y-axis aims to illustrate the impact of each element of the cascade on final outcome.

Under physiological conditions, the normal cerebral blood flow is around 50 to 60 ml/100 g/min. However during cerebral ischemia, the blood flow at the ischemic core (infarction) region drops to <7 ml/100 g/min, which leads to irreversible cellular damage that leads to the death of the regional brain tissue. This is the region where excitotoxicity is first triggered and consists mainly of necrotic cells. The ischemic core is surrounded by the penumbra region, where blood flow ranges from 7 to 17 ml/100 g/min. This is the region where collateral blood flow is able to buffer the full-blown ischemic effect and remains metabolically active but electrically silent (11). Nevertheless, the outcome of the cells in the penumbra region is determined by the intensity and duration of the ischemic insult. Given that remnants of ATP are still presence, the penumbra region is viable although functionally impaired. It is a site where a delayed mode of cell death predominates, mainly via mechanisms such as apoptosis and inflammation (2, 10). Therefore, the penumbra region is a potentially salvageable tissue region, and this is where the ultimate goal of neuroprotection lies. Nevertheless, the core region can gradually spread to the penumbra region over time without treatment, owing to ongoing excitotoxicity or to secondary damaging phenomena, such as spreading depolarization, inflammation and apoptosis. Existing evidences demonstrate that a penumbra region exists in ischemic stroke patients. However, the extent and temporal dynamics of this area is not well defined, it could exist for a shorter time period and smaller in humans (12). The pathogenic events that follow ischemia are discussed below in more detail.



Mehta et al., 2007

Figure 2 A scheme of brain damage during cerebral ischemia. The core is a region where cells undergo necrosis and is surrounded by another region called ischemic penumbra, a site of delayed mode of cell death (apoptosis) due to availability of ATP. Moreover, a transient zone occurs between the core and penumbra region is likely to merge to the core if the cerebral blood flow is not restored early enough. The penumbral region is surrounded by a region of viable tissue.

2.1.4 Energy failure and Excitotoxicity

Following brain ischemic insults, the first pathogenic event to be triggered within minutes is excitotoxicity. Moreover, excitotoxic mechanisms can cause acute cell death (necrosis) and initiate the subsequent molecular events, such as the delayed mode of cell death (apoptosis), and post-ischemic inflammation. With energy depletion due to regional failure of substrate delivery to the brain, in particular oxygen and glucose, resulted in impairment of ATP dependent ionic gradient pumps (Na^+/K^+-ATPase and Ca^{2+}-ATPase), which is important in maintaining the membrane potential. Subsequent depolarizations of plasma membranes mediates influx of sodium (Na^+) and calcium (Ca^{2+}), and outflow of potassium (K^+), which consequently triggers the release of neurotransmitters (13). Glutamate is the major excitatory neurotransmitter which functions as a key mediator in intracellular communication, plasticity, growth and

differentiation of the central nervous system (CNS). These neurotransmitters are released excessively in the mammalian brain following ischemia (14). The excessive presence of glutamate at the extracellular matrix during pathological conditions, such as focal ischemic stroke leads to overactivation of glutamate receptors. The activation of ionotropic glutamate receptors, including N-methyl-D-aspartate (NMDA), α-amino- 3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptor subtypes, eventually leads to Na^+, K^+ and/or Ca^{2+} permeability. The NMDA receptor is a major route for intracellular calcium influx. Under physiological functions, a mild depolarization mediated by AMPA and kainate receptors dislodged the magnesium ion (Mg^{2+}) from voltage-dependent NMDA receptors (15), which subsequently led to the influx of Ca^{2+}. Besides ionotropic receptors, the metabotropic receptors that mediate their action through G proteins can trigger the release of Ca^{2+} from the intracellular calcium storage (16, 17). This in turn leads to the activation of calcium dependent proteases, lipases and endonucleases causing cell death at the ischemic core.

2.1.5 Oxidative and nitrosative stress

Influx of Ca^{2+} through NMDA receptors may lead to the activation of a Ca^{2+}-dependent enzyme neuronal nitric-oxide synthase (nNOS) and subsequent synthesis of NO, which results in the formation of superoxide (O_2^-), peroxynitrite ($ONOO^-$) and hydroxyl (OH^-) radicals (18) that is deleterious to the ischemic brain tissue (19). Although reactive oxygen species (ROS) are important for normal physiological functions, however brain tissue is extremely vulnerable to ROS in excess, which is in part due to the high lipid content. Mitochondria are a major source for ROS production. During oxidative stress in cerebral ischemia, rapid overproduction of ROS easily overwhelms the scavenging capacity and the relatively low levels of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx) and non-enzymatic antioxidants (vitamin E, C and glutathione) (19, 20). Additionally, oxidative stress causes the oxidation of proteins that facilitates electron transport in the inner mitochondrial membranes, leading to H^+ extrusion and ATP production (21). Subsequently, mitochondrial transition pores are formed that caused the leakiness of mitochondrial membrane and thereby promoting mitochondrial swelling, cessation of ATP production and induction of cellular apoptosis (22, 23).

Another source of ROS production is derived from the reperfusion under thrombolytic therapy, which further generates O_2^- and H_2O_2 from the oxidation of xanthine and hypoxanthine by xanthine oxidase, thereby mediating neuronal injury (24, 25). The damage caused by the surge

of oxidative and nitrosative stress with delayed reperfusion in part set the narrow therapeutic time limit of 3 hours.

2.1.6 Peri-infarct depolarizations

After the onset of a focal reperfusion deficit, ischemic neurons and glia undergo an anoxic depolarization in the core region. With energy depletion, these cells are unable to repolarize again. In contrast, cells in the penumbra region (where some perfusion is preserved due to collateral arteries) have the ability to repolarize, however at the expense of further energy consumption. Thus, as the cycle of depolarization and repolarization increases, the infarcts can grow larger without therapeutic interventions (26). The ability of repetitive peri-infarct depolarization at the penumbra zone is the marker that distinguished it from the core region.

2.1.7 Apoptosis

Transient or permanent deficits in cerebral blood flow trigger either cellular necrosis or apoptosis. Necrosis is the predominant mechanism in the ischemic core region minutes after the onset of stroke. On the contrary, as shown in figure 1, apoptosis is a relatively delayed process following ischemic brain injury, predominating at the penumbra region due to ATP preservation from sustained mild perfusion (10). Apoptosis can be triggered by ionic imbalance, oxygen free radicals, death receptor ligation, and DNA damage as well as protease activation. The intrinsic apoptotic cascades become activated when cytochrome C is released from the outer membrane of mitochondria, an event mediated by members of the bcl-2 protein family, thereby activating an apoptosome complex [apoptosis-activating factor (APAF) plus pro-caspase 9] in the presence of dATP (27). Caspases exists as zymogens in cells, and they function as aspartate-specific cysteine proteases. It has been identified that caspases 1 and 3 out of a total of 12, have a pivotal role in ischemia-mediated apoptosis. The genes for caspases and genes that suppress (i.e. Bcl-2, IAP) or augment (i.e. Bax, p53) cell death are expressed and activated in both early and late stages after ischemic onset. Activated caspases are proteases that modify crucial homeostasis and repair proteins that, in turn, kill cells. Furthermore, emerging data shows that genetic

manipulation or drugs that block caspase-family members is neuroprotective during the pathogenesis of ischemic injury (28).

On the other hand, the extrinsic apoptosis pathway is initiated by inflammatory signals, such as ligands from TNF family, in ischemia (29, 30). Subsequent events consist of activated downstream effector caspases targeting the substrates that dismantle the cell by cleaving homeostatic, cytoskeletal, repair, metabolic and cell signaling proteins (31).

2.1.8 Inflammation

Post-ischemic inflammation is a delayed mechanism that contributes to the demise of brain tissue following focal perfusion deficit, which may last for days and weeks (2, 32). It is characterized by a rapid activation of resident microglia and by infiltration of neutrophils and macrophages in the ischemic tissue, as demonstrated in animal models (33, 34) and in human stroke patients (35-37). Additionally, Ca^{2+}-related signalling of intracellular second-messenger systems, increase in ROS and hypoxia per se, triggers expression of proinflammatory genes, such as transcription factors i.e. nuclear factor-κB (NF-κB) (38), hypoxia inducible factor 1 (39), interferon regulatory factor 1 (IRF1) (40) and signal transducer and activator of transcription 3 (STAT3) (41), which leads to the production of platelet-activating factor (PAF), tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) by the injured brain cells (42). Consequently, increased levels of cytokines and chemokines enhance the expression of adhesion molecules, such as intercellular adhesion molecules 1 (ICAM-1), P-selectins and E-selectins on microvascular endothelial cells (35, 43, 44). Upregulation of ICAM-1 results in adhesion and transmigration of infiltrating polymorphonuclear (PMN) leukocytes, such as bone-marrow derived neutrophils and followed by monocytes macrophages, through the compromised BBB into the injured brain parenchyma. Moreover, it has been demonstrated that chemokines, such as interleukin 8 and monocyte chemoattractant protein 1, are produced by the injured brain cells and have a role in guiding the blood-borne circulating leukocytes towards their destination (45, 46). The accumulation of these cells in the cerebral capillaries may further impair the blood flow and extravasates the injured brain. Whereas production of neurotoxic substance, such as pro-inflammatory cytokines, chemokines and oxygen/ nitrogen free radicals, by these cells share the same consequences (47).

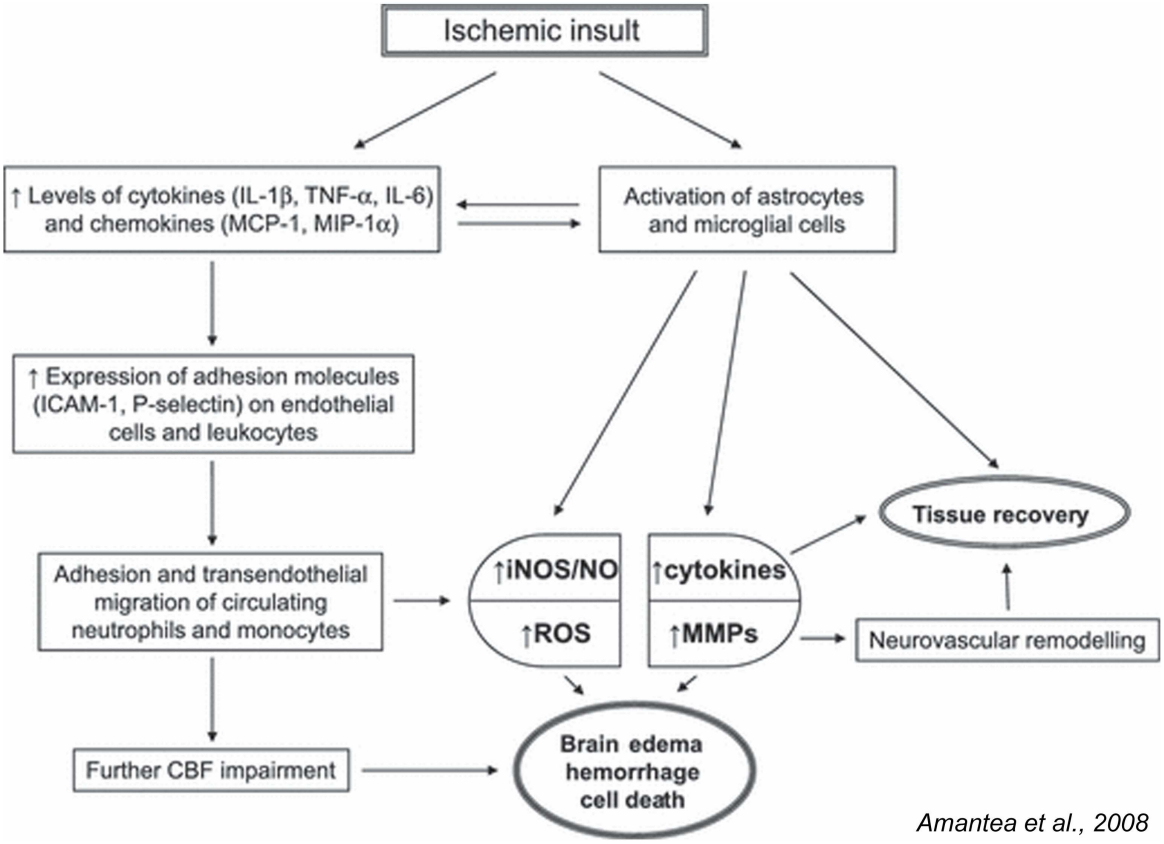


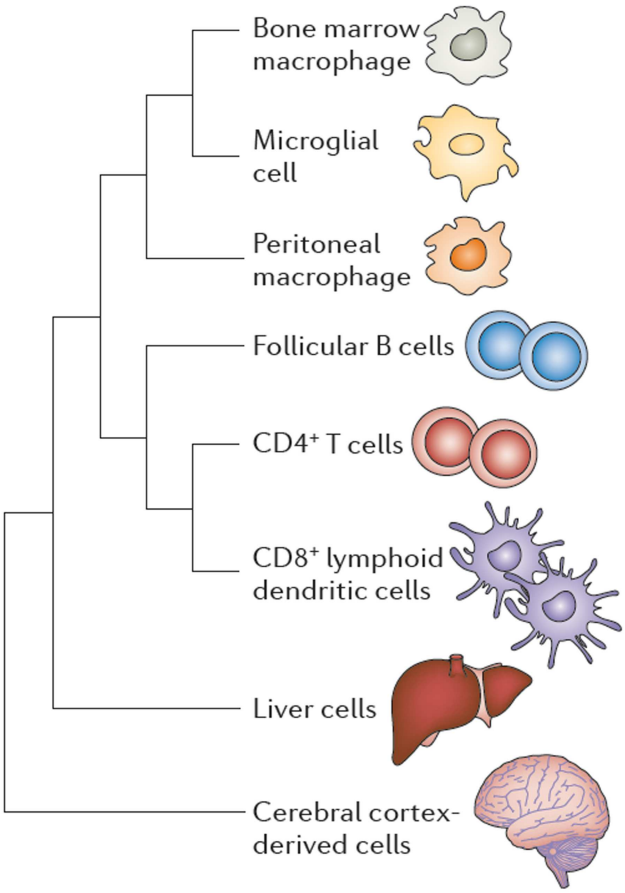
Figure 3 Main mechanisms/ pathways of post-ischemic inflammation. CBF, cerebral blood flow; MIP-1α, macrophage inflammatory protein-1 alpha; ROS, reactive oxygen species.

2.2 Innate immune system in the CNS

2.2.1 Microglia

Microglia are specialized macrophages of the CNS that were recruited and differentiated in the central nervous system (CNS) during embryogenesis (48, 49). These specialized cells are distinguished from other glial cells such as oligodendrocytes, astrocytes and neurons, by their origin, morphology, gene expression pattern and functions. After a long-lasting debate, the ontogeny of microglia has finally reached a consensus that it was of myeloid lineage. This is confirmed by their absence from the CNS of PU.1 (hematopoietic-specific transcription factor)-null mice and the repopulation of microglia in the brain of these mice following bone marrow

transplantation is performed (50). However, the local key factors that influenced the progenitor cells into microglia remains to be elucidated. Moreover, the lineage relationship between microglia and macrophages is well established, as determined by genome-wide microarray analysis (Figure 4). Despite the technical limitation in isolating microglia in this analysis which may lead to cell activation, microglia is most closely related to bone marrow-derived macrophages and followed by thioglycollate-elicited macrophages. Nevertheless, microglia can be distinguished from macrophages by several features. i) One of the striking features of microglia is their particular downregulated phenotype with fine and long protrusions. These so-called 'ramified' resting microglia, are actually constantly screening their local microenvironment in the CNS (49, 51) as demonstrated by in vivo imaging (52, 53). In contrast to most other macrophages, the downregulated 'ramified' phenotype of microglia is profoundly influenced by their local microenvironment which is deprived of serum proteins due to the integrity of BBB (54). One of the stimuli that could lead to the activation of microglia is the presence of serum constituents, such as fibrinogen. ii) Furthermore, in contrast to peripheral macrophages, microglial cells are in immediate contact with the other cells types in the CNS. Thus, expression of receptor-ligand pairs on the microglia and other adjacent cells will influence their phenotype and status of activation. For instance, CX3CR1 is expressed by all monocytes, including microglia, in humans as well as in mice. It has been suggested that microglia are actively repressed from inhibitory signal through the CX3CL1 receptor, CX3CR1 (55). CX3CL1 is a chemokine that is released from CNS neurons through the action of a disintegrin and metalloproteinase domain-contraining protein (ADAM) proteases. Thus, the loss of the CX3CL1 inhibitory signal through systemic inflammation or injured neurons will lead to the activation of microglia in the local microenvironment. iii) Neurotransmitters are the medium of communication between neurons and with other glial cells in the CNS, therefore altered synaptic activity altering the availability of neurotransmitters can affect microglia in a more diverse and pervasive manner. In contrast to peripheral macrophages, in which the regulation by neurotransmitter signalling is more intermittent, as demonstrated from the cholinergic anti-inflammatory response (56).

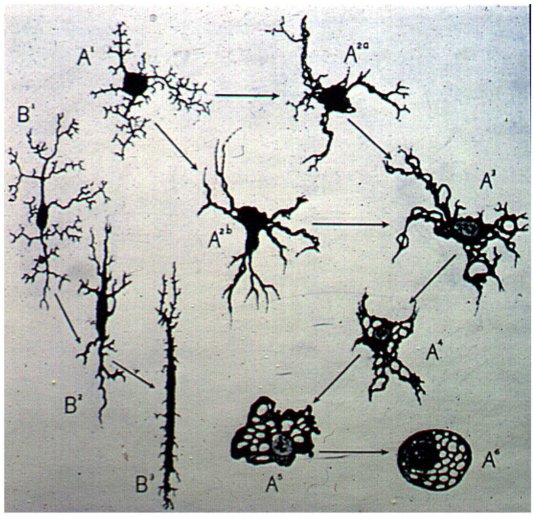


Saijo and Glass, 2011

Figure 4 Relationship of resident microglial cells to the other myeloid-lineage cells. The figure depicts the molecular relationships between different haematopoietic lineage cells, liver cells, brain cells and microglia isolated from wild-type mice based on gene expression as determined by genome-wide microarray analysis (BioGPS website). The relationship between microglia and bone marrow-derived macrophages are more closely related compared to the thioglycollate-elicited macrophages. In contrast, microglia are more distantly related to other haematopoietic and non-haematopoietic cell types.

2.2.2 Microglia in ischemic brain injury

Upon injury or infection, microglia are the first line of defence in the CNS (57, 58), which become rapidly activated by adopting an 'ameboid' reactive phenotype, producing a plethora of pro-inflammatory compounds such as cytokines, chemokines, ROS and nitric oxide (59), higher proliferative capacity, chemotaxis, phagocytotic, upregulated expression of immunomodulatory surface antigens (60, 61), such as MHC class II to enable them to present antigen to T cells through T cell receptor (TCR) (62).



drawings from Pío del Río Hortega

Figure 5 Diversity of microglia phenotype, ranging from 'ramified' phenotype with long branches (protrusions) to reactive 'ameboid' phenotype.

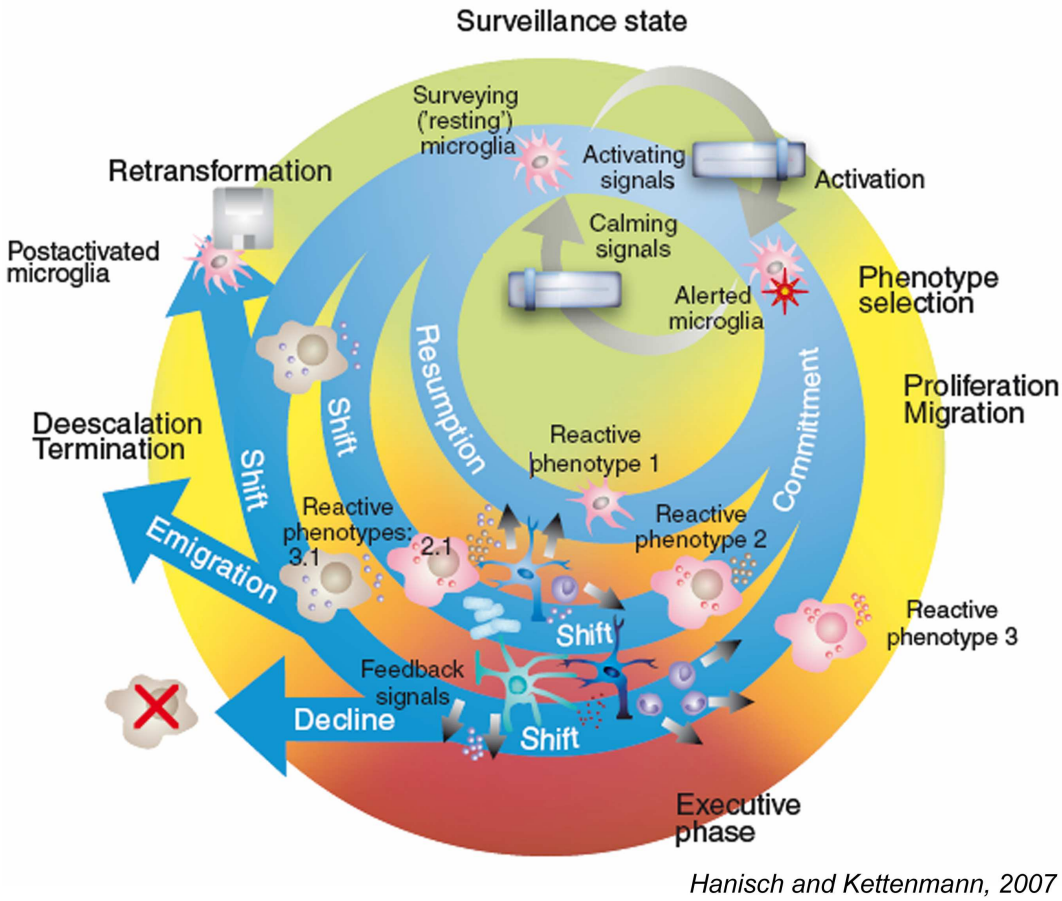
Four to six hours after the onset of brain ischemia, astrocytes become hypertrophic, followed by the activation of resident microglial cells, resulting in the production of a wide range of proinfammatory mediators, which contribute to pathogen clearance (63). Twenty-four hours after MCAo, a fully developed microglial reaction is particularly found in the core and predominantly in the penumbra region, and within days most microglia became phagocytotic (32, 64, 65). Moreover, the proliferative capacity of microglia surges and peaks at 48-72 h and may last for several weeks after the initial injury (66, 67).

Infiltration of blood-borne macrophages in the injured brain parenchyma due to the impairment of the BBB, are not distinguishable from the reactive resident microglial cells (68), thus it is impossible to determine their distinct contribution to the ischemic stroke injury. Therefore, GFP

bone marrow chimeric mice are used in experimental studies to distinguish the two cell populations following ischemic injury, and reveal that resident microglia dominate over blood-borne macrophages by cell count during the first 3-4 days of cerebral ischemia (33, 34, 64).

The role of microglia in ischemic stroke remains controversial. Existing data demonstrates that microglia can be detrimental, as well as beneficial in the ischemic brain tissue. Several studies proved that minocycline confers neuroprotection by inhibiting the activation and proliferation of resident microglia in stroke model (69-71). On the other hand, other studies showed that accumulation of microglia can be beneficial in cerebral ischemia. In a study, a transgenic mouse system expresses a mutant form of the herpes simplex virus thymidine kinase driven by myeloid specific CD11b promoter (*CD11b-TK*^{mt30} mice) was utilized, whereby proliferating microglia/macrophages is selectively ablated by ganciclovir treatment. These animals were treated with ganciclovir 48 hours before subjection to 60 min transient MCAo, resulting in a significant increase in the infarct size (66). Furthermore, neurotrophic growth factors such as Insulin-like growth factor 1 (IGF-1), were produced by proliferating microglia and thus help reduce inflammation. This study indicates that proliferating microglial cell population confers neuroprotection in ischemic injury. On another approach, a series of studies using transplantation of isolated microglia in the ischemic brain proves to be neuroprotective. In one study, cultured microglia (4000-50,000) were injected into the ventricles of adult rats after 2 hours of transient MCAo, significantly smaller infarct size were observed at the third day, as well as improved behavioural outcome at day 8 (72). In another study, Imai and co-workers injected 10^6 of isolated microglia intra-arterially from brain cultures in Mongolian gerbils before ischemia or after reperfusion. Neuronal death and functional deficit could be reduced when challenged by global ischemia induced by two-vessel occlusion. Moreover, the administrated exogenous microglia triggered a higher expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor, which promotes neuronal survival (73).

The production of neurotoxic and neuroprotective factors are of importance in determining the neuronal survival and regeneration in response to ischemic injury. Thus, microglia and macrophages may contributes to brain tissue recovery by mediating plasticity and by scavenging the necrotic debris (47). Furthermore, the protective role of microglia in eliminating excess excitotoxins may be in part through phagocytosis of infiltrating neutrophils (67) and through production of various neurotrophic factors, such as neurotrophins and growth factors (fibroblast growth factors, TGF-ß1) (74-77).



Hanisch and Kettenmann, 2007

Figure 6 Diversity of microglial activity states throughout the activation process. The traditionally termed 'resting' state of resident microglial cells are constantly screening for potential threat to CNS homeostasis. Stimuli causing activation of microglial cells include infection, trauma or cell impairment, as well as the loss of constitutive 'calming' signals. Initial response of microglia activation may further commit to distinct reactive phenotypes, constituted by transcriptional profiles and nontranscriptional changes, and enter their executive phase (for example, release of cytokines and chemokines, phagocytotic activity). In the scheme, three examples illustrating the main stream of phenotypes (1,2,3) after initial activation, however the diversity could be wider. Subsequently, reactive microglia may further change, as depicted by phenotypes 2.1 and 3.1, depending on the alteration (by elimination or fading) of the initial activating signals as well as signals received from the resident cells from the microenvironment and invading immune cells (feedback signals). The changes of reactive microglia may shift and eventually leading to a more repair-orientated profile, whereas others may emigrate to the blood system or die (indicated by an 'X' over cell, others may revert to a 'resting' state). Alternatively, postactivated microglia which have acquired some experience (indicated as memory in the figure by a floppy disk icon) may not retransform back to completely naive state, thereby keeping subtle changes, such as in transcriptional activity that affect their sensitivity to constitutive (calming) signals or alter responses to subsequent stimulation.

2.3 Necdin

2.3.1 Location of human necdin gene and its pathological implications in human and animal model

Nicholls and coworkers showed that human necdin (Neurally differentiated Embryonal Carcinoma-Derived proteIN) gene is mapped to chromosome 15q11-q13 (78). Human and mouse necdin genes are maternally imprinted and transcribed only from the paternal allele. As shown in figure 2, necdin is one of the four imprinted genes (SNRPN, ZNF127, IPW) mapped to the deleted region implicated in the Prader-Willi syndrome (PWS). This is a neurodevelopmental disorder caused by genetic defects (paternal deletions, maternal disomy, or imprinting mutations), resulting in the loss of necdin expression. Symptoms manifested by PWS patients are variable, which include transient hypotonia, respiratory distress, hypogonadotropic hypogonadism, and cognitive failure to strive, hyperphagia leading to severe obesity, somatosensory deficits, behavioral abnormalities and mild to moderate mental retardation (79, 80). Mice deficient in necdin gene (i.e. necdin knockout mice) mimic aspects of this disease, including neonatal respiratory depression, behavioral defects, reduced luteinizing hormone releasing hormone- and oxytocin-producing neurons in the anterior hypothalamus, as well as congenital deficiency of central respiratory drive in neonatal mice (81-83).

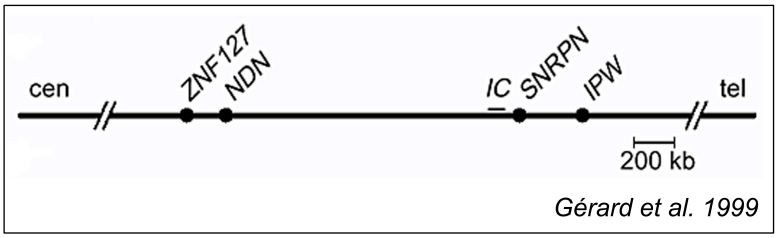


Figure 7 Genetic map of human chromosome 15q11−q13. The mouse genome contains a region of conserved synteny on chromosome 7. The four genes indicated are either deleted or not expressed in PWS patients. IC, imprinting centre.

2.3.2 Classification of Necdin

Necdin is a 325 amino acid residue protein. It was first identified in 1991 by isolation from neuronally differentiated P19 embryonal carcinoma cells (84). The necdin gene is reported to lack introns in the mouse and consist of a single exon (Figure 4)(85). The necdin gene shares about 30% homology with other MAGE (Melanoma Antigen Gene Expression) protein family. It belongs to the basic type II MAGE group, which can be found in various normal tissues (86, 87). The physiological roles of most of the proteins in this class are unknown except for necdin, which is implicated in cell cycle control. The MAGE family proteins share certain homologous region, including a 200 amino acid MAGE homology domain (MHD). Each member of the MAGE family possesses a unique N- and C-terminal region surrounding a large central and conserved region termed MHD (86).

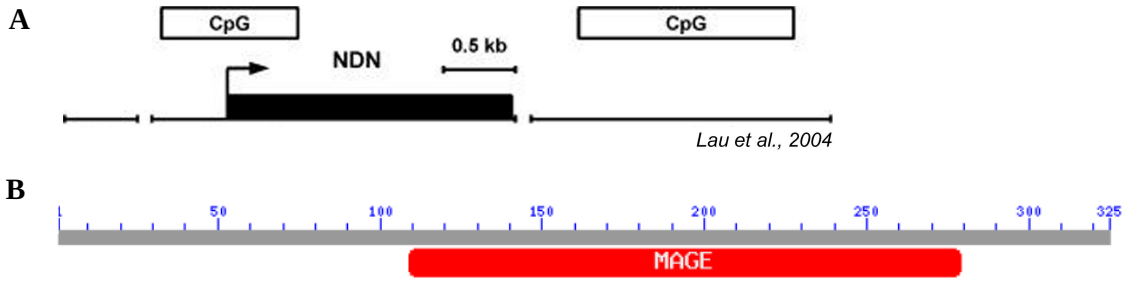


Figure 8 Schematic diagram of necdin. (**A**) The coding region of necdin indicates an intronless structure. (**B**) Necdin contists of 325 amino acids and shares a conserved MAGE domain which is indicated by the red box. In addition, this region serves for interaction with other proteins.

2.3.3 Implication of necdin in physiology and pathology of the CNS

The necdin gene is expressed predominantly in postmitotic neurons throughout the central nervous system (CNS) and peripheral nervous system (PNS) (85, 88), as well as in non-neuronal cells such as skeletal myocytes, chrondrocytes, adipocytes and skin fibroblasts (81, 89-91). To date, there were no direct studies of necdin in neurodegenerative animal models, such as Alzheimer's disease, Parkinson's disease, brain trauma and stroke. However, necdin has been

demonstrated to be a multifunctional protein, as well as a major key molecule in mitotic arrest, promoting differentiation and survival of postmitotic neurons in in vitro studies using cell lines and primary neuronal cultures. Furthermore, inappropriate activation of cell cycle regulators is implicated in the pathophysiology of a variety of CNS diseases, including both acute and chronic neurodegenerative disorders.

Accumulating evidence indicates that necdin mediates growth arrest. For instance, ectopic necdin restricts proliferation in NIH3T3 embryonic fibroblast cells (92), and SAOS-2 osteosarcoma cells by interacting and thereby inhibiting transcription elongation factor E2F1 (93). E2F1 is a member of a family of transcription factors which are essential for cell cycle progression (94). Additionally, necdin can act as a cell cycle regulator by interacting with cell cycle promoting protein, such as the SV40 large T antigen, adenovirus E1A and transcription factor E2F1, by trans-activating relevant genes in a RB-regulated pathway (93). A separate study demonstrated that necdin restricts proliferation in hematopoietic cells during hematopoietic regeneration (95).

Necdin can serve as a neuroprotectant, by promoting cell survival through inhibition of p53-induced apoptosis in osteosarcoma U2OS cells (96), and E2F1-induced apoptosis of differentiated neuroblastoma cells was inhibited by ectopic necdin (97, 98). Furthermore, endogenous necdin mediates survival of postmitotic primary cerebellar granule neurons by suppressing the E2F1-Cdc2 apoptotic pathway, which is activated under various neuropathological conditions (99). The localization of necdin is mainly in the cytoplasm of postmitotic neurons and shown to be translocated to the perinuclear compartments under certain conditions, possibly to enable its transcriptional activities (100). In addition, apoptosis induced by DNA damage can be rescued by necdin, forming a stable complex with p53 and Sirt1, which downregulates p53 acetylation levels (101). On another approach, necdin mediated the terminal differentiation and survival of neurotrophin growth factor (NGF)-dependent dorsal root ganglion (DRG) neurons and that necdin-deficient nascent neurons were destined to undergo caspase-3 dependent apoptosis (102).

Kuwajima and colleagues demonstrated that the differentiation of GABAergic neurons was facilitated by the association of necdin with Dlx homeodomain protein via MAGE-D (103). The differentiation of GABAergic neurons in the mouse embryonic brain was rather selective since no appreciable changes in the distribution or pattern of the number of glutamaergic neurons was observed, which indicates that the effect of necdin on maturation and differentiation is only in selective subtypes of neurons.

Taken together, necdin is a multifunctional protein, as well as a major key molecule in mitotic arrest, promoting differentiation and survival of postmitotic neurons in in vitro studies using cell lines and primary neuronal cultures. To date, the role of necdin is primarily related to neurodevelopmental processes of the CNS, however, this protein proves to be neuroprotective through suppression of several apoptotic pathways which are predominant in neurodegenerative disorders.

2.3.4 Posttranslational modification: SUMO and NEDD8

The posttranslational modification via NEDDylation (NEDD8) has been shown to be essential for cellular function. Furthermore, it is involved in cell cycle progression, cell growth and survival (104, 105). NEDD8 belongs to one of the ubiquitin-like proteins (UBLs) family (106). Human deneddylase 1 (DEN1), also known as NEDD8-specific protease (NEDP1) or SENP8, is responsible for processing the precursor form of NEDD8 into the mature form by removing 5 amino acid residues from its C-terminal tail, as well as deconjugating NEDD8 from its modified substrates. NEDD8 is an 81–amino acid protein with 9 kDa relative molecular mass and is 60% identical and 80% homologous to ubiquitin.

Another class of UBLs is the small ubiquitin-related modifier (SUMO) conjugation system. The SUMO conjugation pathway plays a key role in the regulation of a diversity of cellular processes. There are at least four isoforms exist in vertebrate (SUMO 1-4), their activity is generally enhanced in response to stimuli, such as heat shocks, ischemic injury and high levels of oxidative stress. Furthermore, SUMO can be modulated by altering the expression levels or activities of the E1, E2 and E3 ligases (enzymes) in the pathways. Precursor form of SUMO can be catalyzed to its mature form by sentrin specific proteases (SENPs) in humans. Moreover, Senps belongs to cysteine proteases that consists of six members (SENP1-3 and SENP 5-7) are able to deconjugate SUMO from its modified protein substrates (107).

These UBLs are structurally related to biquitin and similarly form conjugate with a broad range of different substrates through discrete E1-E2 (and sometimes E3) cascades.

2.4 Hypotheses

The goal of this doctoral thesis is to validate the following hypotheses:

The penumbra region is the salvageable area of the ischemic brain, whereby partial blood flow is maintained and thus delayed modes of cell death predominate, such as apoptosis and inflammation.
We were interested in necdin, which is expressed predominantly in postmitotic neurons. It is a multifunctional protein involved in cell cycle regulation, and acts as a potent growth suppressor. Apart from being present in neurons, necdin has not been characterized in other cell types of the CNS. Since microglia originate from the bone marrow, and necdin has been shown to regulate the proliferation of hematopoietic cells, we hypothesized:

1.1 Necdin may be expressed by, and is the molecular substrate that regulates the activation and proliferation of reactive resident microglia, in the ischemic brain in vitro and in vivo.

Posttranslational modification pathways may alter the function of a protein target in a diverse way, from relocalization or half life of the protein, to cell survival. Thus, it plays a pivotal role in cellular homeostasis. We identified a higher molecular weight necdin protein band (~10 kDa), that is immunoreactive to the necdin antibody.

1.2 We identified a putative posttranslational modification of the necdin protein in microglial cells.

Cerebral ischemia triggers the activation of microglia along with infiltrating macrophages, which accumulate at core of the lesion site and in the penumbra region. As the blood brain barrier (BBB) is impaired, these accumulated cells at the lesion site represent a mixture of intrinsically activated microglia and infiltrating blood cells. Both populations can be distinguished via bone marrow chimera with GFP expressing cells entering the brain. So far, it is not clearly understood whether these two cell populations may have differing impacts on stroke outcome. Taken together, the number of microglia and invading macrophages might be crucial for the size of the ischemic lesion and it is therefore of high biological relevance to understand the endogenous mechanisms underlying the cell cycle control in

microglia/macrophages in the ischemic tissue. Since we are interested in necdin as the molecular regulator of microglia,this leads us to our next hypothesis/ question to be solved.

2 Necdin is expressed and regulated in one of these two subpopulations of monocytic cells (resident microglia versus blood-borne macrophages).

3 Necdin expressing cells belong to one of these subpopulations of monocytic cells (i.e. 'ramified' phenotypic microglia, reactive microglia and infiltrating macrophages)

3 Materials and methods

3.1 Materials

3.1.1 Cell culture media and materials

Product	Supplier
B27 Supplement	Gibco (Germany)
Collagenase	Serva (Germany)
Collagen-G solution	Biochrom (Germany)
D-(+)-glucose	Sigma (Germany)
Dulbecco's Phosphate Buffered saline (PBS)	Biochrom (Germany)
Dulbecco's phosphate-buffered saline (DPBS)	Biochrom (Germany)
Eagle's minimal essential medium	Biochrom (Germany)
Fetal calf serum (FCS)	Biochrom (Germany)
Fetal calf serum, Gold (FCS, Gold)	PAA (Austria)
Glutamate	Sigma (Germany)
HEPES	Biochrom (Germany)
L-Glutamine	Biochrom (Germany)
Lipofectamine 2000	Invitrogen (Germany)
MEM non essential amino acids	PAA (Austria)
Neurobasal medium (NBM)	Gibco (Germany)
PEG-IT	System Biosciences (USA)
Percoll	GE Healthcare (United Kingdom)
Penicillin/ Streptomycin	Biochrom (Germany)
Poly-L-Lysine	Biochrom (Germany)
Roswell Park Memorial Institute (RPMI)-1640	Biochrom (Germany)
Roswell Park Memorial Institute (RPMI)-1640 without phenol red	Biochrom (Germany)
Sodium pyruvate	PAA (Austria)

3.1.2 Chemicals

Product	Supplier
Acetone	Roth (Germany)
Ampicillin (50 µg/ml)	Roth (Germany)
b-mercaptoethanol	Merck (Germany)
Bovine albumin (BSA)	Sigma (Germany)
Bromphenol blue	Sigma (Germany)
Coomassie brilliant blue G	Fluka (Germany
Dimethylsulphoxide (DMSO)	Sigma (Germany)
DMSO	Sigma (Germany)
DTT	Bio-mol (Germany)
Dual color Precision Plus protein standards	BIO-RAD (USA)
EDTA	Roth (Germany)
EGTA	Roth (Germany)
Ethanol (99.9%)	Sigma (Germany)
Ethidium Bromide	Sigma (Germany)
Glycerol	Merck (Germany)
Glycine	Sigma (Germany)
Hydrogen peroxidase (H_2O_2) 30%	Roth (Germany)
Hydrochloric acid (HCl)	Sigma (Germany)
Isofluorane (Forene)	Abbott (Switzerland)
ImmunoFluor Mounting Medium	ICN Biochemicals (USA)
Isopentane	Roth (Germany)
Kanamycine (50 µg/ml)	Sigma (Germany)
Kodak Biomax Light-1 X-ray films	Sigma (Germany)
LumiGLO reagent and peroxide	NEB (Germany)
Lauryl sulphate (SDS)	Sigma (Germany)
Magnesium chloride ($MgCl_2$)	Serva (Germany)
Methanol	Roth (Germany)
Milk powder (blocking grade)	Roth (Germany)
Monosodium phosphate (NaH_2PO_4)	Sigma (Germany)
Monopotassium phosphate (KH_2PO_4)	Sigma (Germany)
Nonidet P 40 (NP40)	Sigma (Germany)

Paraformaldehyde (PFA)	Merck (Germany)
Phenylmethanesulfonylfluoride (PMSF)	Sigma (Germany)
Potassium chloride (KCl)	Sigma (Germany)
Propidium iodide	Sigma (Germany)
PAGEr Precast Gel 4-20%	Lonza (USA)
Prolong gold antifade reagent	Invitrogen (Germany)
Protease Inhibitor tablets	Sigma (Germany)
Sucrose	Sigma (Germany)
Sodium bicarbonate ($NaHCO_3$)	Sigma (Germany)
Sodium chloride (NaCl)	Roth (Germany)
Sodium deoxycholate ($C_{24}H_{39}NaO_4$)	Sigma (Germany)
Sodium fluoride (NaF)	Sigma (Germany)
Sodium orthovanadate (Na_3VO_4)	Sigma (Germany)
Sodium pyruvate ($CH_3COCOONa$)	Sigma (Germany)
Sulphuric acid ($H_2SO_{4)}$	Roth (Germany)
TEMED	Sigma (Germany)
Tris	Sigma (Germany)
Triton-X	Roth (Germany)
Trizol	Invitrogen (Germany)
Trypsin/EDTA	Biochrom (Berlin, Germany)
Tween-20	Sigma (Taufkirchen, Germany)
Unmasking Fluid C, Citrate based, pH 6	Bioprime (Germany)
Xylol	J. T. Baker (Netherlands)

3.1.2 Antibodies, reagents and kits

Product	**Supplier**
Alexa Fluor dyes-488, donkey anti-rabbit IgG	Invitrogen (Germany)
Alexa Fluor dyes-488, donkey anti-sheep IgG	Invitrogen (Germany)
Alexa Fluor dyes-488, donkey anti-mouse IgG	Invitrogen (Germany)
Alexa Fluor dyes-647, anti-EdU	Invitrogen (Germany)
Alexa Fluor dyes-594, donkey anti-guinea pig	Invitrogen (Germany)
Biotinylated anti-rabbit	Invitrogen (Germany)
Biotinylated anti-mouse	Invitrogen (Germany)

Block-iT™ Pol II miR RNAi Expression Vectors Kits	Invitrogen (Germany)
Bradford Protein Assay	BIO-RAD (USA)
diaminobenzidine (DAB) staining (SigmaFast™DAB)	Sigma (Germany)
CalPhos™-Kit	Clontech (Germany)
CD11b microbeads	Miltenyi Biotech (Germany)
Click-iT® EdU Alexa Fluor® 647 Imaging Kit	Invitrogen (Germany)
Donkey anti-mouse HRP-linked	Amersham (USA)
Donkey anti-rabbit HRP-linked	Amersham (USA)
Donkey anti-guinea pig HRP-linked	Abcam (United Kingdom)
ECL Plus Western Blotting Detection	GE Health Care (United Kingdom)
Guinea pig anti-necdin (GN1, GN2)	Dr. Yoshikawa (Institute for Protein Research, Japan)
Hoechst 33258, bis-benzimide	Sigma (Germany)
LumiGlo, enhanced chemiluminescence (ECL) Reagent	Cell Signalling (USA)
Mouse anti-EGFP (SC9996)	Santa Cruz (USA)
Mouse anti-GAPDH (MAB374)	Millipore (Germany)
Protease inhibitor cocktail	Thermo Scientific (Germany)
Phosphatase inhibitor cocktail	Thermo Scientific (Germany)
Qiagen plasmid maxi kit	Qiagen (Germany)
Qiagen PCR purification kit	Qiagen (Germany)
Rabbit anti-necdin (NC243)	Dr. Yoshikawa (Institute for Protein Research, Japan)
Rabbit anti-HDAC1 (SC7872)	Santa Cruz (USA)
Rabbit anti-CD11b (AB75476)	Abcam (United Kingdom)
Rabbit anti-GFAP	Dako (Netherlands)
Rabbit anti-Iba1	Wako (Japan)
Rat anti mouse CD45 PE-linked	BD Pharmingen (Germany)
Rat anti mouse CD11b BV-linked	BD Pharmingen (Germany)
Sheep anti-NEDD8	Abcam (United Kingdom)
Sheep anti-SUMO 2 (BML-PW0510)	Enzo (United Kingdom)
Streptavidin anti-biotinylated antibody	Invitrogen (Germany)
ViraPower™ Lentiviral Expression Systems	Invitrogen (Germany)

3.1.3 Tools and equipment

Product	Supplier
autoMACS separator	Miltenyi Biotech (Germany)
Blotting chamber Trans-Blot Semi-Dry Transfer Cell	Biorad (Germany)
Biofuge Fresco Centrifuge	Heraues (Germany)
Centrifuge, Eppendorf 5810R	Eppendorf (Germany)
Confocal microscope, Leica LFSA	Leica (Germany)
Cryostat, Cryo-Star HM 560	Microm (Germany)
Criterion cassettes	Bio-rad (Germany)
Cytospin 3 Centrifuge	Shandon (Germany)
Electrophoresis chamber	Biorad (Germany)
Eppendorf Mastercycler epgradient S	Eppendorf (Germany)
FACSDiva analyzer	BD Biosciences (Germany)
Flasks, multi-well plates, tubes	Cornington(Netherlands)
Fluorescent microscope, DMRA2	Leica (Germany)
Fuchs-Rosenthal cell count chamber	Roth (Germany)
FUSION chemiluminescence system	Peqlab (Germany)
Hera Cell 240 incubator	Heraeus (Germany)
Hera Safe laminar flow hood	Heraeus (Geramny)
Inverted epifluorescence microscope, IX81 Olympus	Olympus (Germany)
Julabo waterbath SW 22	Julabo (Germany)
Magnetic resonance Imaging (MRI)	Pharmascan 70/16 (Germany)
Microtome, HM330	Microm (USA)
Nanodrop® ND-1000 Spectrophotometer	Montchanin (USA)
Polyvinylidene Fluoride membrane (PVDF) transfermembrane	Roth (Germany)
OGD chamber, Concept 400	Ruskinn Technologies (United Kingdom)
pH meter, pH100	VWR International (Germany)
Power supply, Power Pack 200	Bio-rad (Germany)
Sonicator, Sonorex Super 10P	Bandelin Electronic (Germany)
Table centrifuge, 5417R	Eppendorf (Germany)
Whatman paper	Biometra (Germany)
Vortex Genie 2	Bohemia (USA)

3.1.4 Animals

Animals	Suppliers
C57Bl/6N mouse	Charles River Laboratories (Germany)
C57Bl/6N Postnatal day 3 (P3)	Charles River Laboratories (Germany)
EGFP transgenic mice	self-made/generated
Bone marrow chimeras	self-made/generated

3.1.5 Cell lines

Cell line	Supplier
HEK 293 FT	American Type Culture Collection (USA)

3.2 Methods

3.2.1 Primary cell cultures

3.2.1.1 Mixed glial culture

Culture plates were pre-treated by incubation with poly-L-lysine (5 µg/ml in PBS w/o). After at least 2 hours of incubation time at room temperature, the plates were rinsed twice and mixed glial cells were seeded in either 24-well plates (for immunocytochemistry) or in 6-well plates (for western blotting analysis) in wells with medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 µg/ml Streptomycin].

Mixed glial cultures were prepared from newborn mice brain (postnatal day 3, P3) of C57BL6/N wild type mouse strain, using techniques as described previously (108). Briefly, meninges, bulbus and (hippocampus/corpus callosum) were removed from the cortical tissue. Tissue was cut into small pieces and trypsinized for 15 min at 37°C. The cells were then washed twice with media and once in PBS. The cells were in media suspension before the dissociation step was carried out with a pipette. The suspension was centrifuged for 5 min at 161 x g, pellet was resuspended and filtered with 70 µm filter. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml Streptomycin, kept at 37℃ and maintained at 5% CO_2. Next day, the cultures were washed once with PBS and maintained in complete media. All following experiments were done between DIV 10 - 15 in culture. Prior usage, the condition of the cultures was assessed by light microscopy.

3.2.1.2 Microglial cells

Microglial cells were isolated from the astrocytic layer of the mixed glial cultures by gentle shaking of the flask for 1 hour at 250 rpm. The cells were then seeded on 24 well plates at a density of 5 x 10^4 to 10^5.

3.2.1.3 Primary peritoneal macrophages

About 5 ml of medium [Roswell Park Memorial Institute (RPMI)-1640, 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml Streptomycin] was intraperitoneally injected into each mice, followed by a few minutes of short belly massage. Harvested suspensions were centrifuged and cultured for 2 hours before being washed with media without serum. The cells were returned with complete medium cultured for a day before obtaining the cell lysates for western blotting analysis or fixed with 4% Paraformaldehyde (PFA) for immunocytochemistry.

3.2.2 Lentiviral particles coding for control and necdin microRNA

3.2.2.1 Construction and screening of microRNA target sites

The target sequences within murine necdin (NM_010882) at the starting point of 132, 444 and 855 were designed using an online software system (Invitrogen). The pre-microRNA sequencing oligonucleotides were: microRNA_132: TGC TGT CAA CAT CTT CTA TCC GTT CTG TTT TGG CCA CTG ACT GAC AGA ACG GAG AAG ATG TTG A; microRNA_444: TGC TGA TTT CTT GTA GCT GCC CAT GAG TTT TGG CCA CTG ACT GAC TCA TGG GCC TAC AAG AAA T; microRNA_855: TGC TGT AAT TCT GCT GGA CGA ACT CCG TTT TGG CCA CTG ACT GAC GGA GTT CGC AGC AGA ATT A, and *Lac Z* as a non-targeting negative control. Third-generation lentiviral particles were generated as described previously (109-111) with the following modification: A 216 bp DNA fragment with necdin targeted microRNAs or non-targeting control microRNA (*Lac Z*) were subcloned from pcDNA6.2-GW-EmGFP (Invitrogen) using XhoI-BsrGI into the SalI-BsrGI site to the parental lentiviral transfer plasmid (pRRL-SIN-cppt-PGK-GFP-WPRE), attained from the Trono lab (Addgene plasmid 12252). Positive clones were sequence verified and tested for the highest interfering efficiency in 293FT cell line via western blotting analysis. Transfection was done using Lipofectamine 2000 (Invitrogen).

3.2.2.2 Lentiviral particles production

Two of the pcDNA containing the highest interfering efficiency of pre-microRNA cassette were recombined into a lentiviral expression plasmid (PGK vector) respectively, with a specific cloning strategy. The lentiviral expression plasmid containing the pre-microRNA_132 and pre-microRNA_855 cassette were co-transfected with packaging plasmids psPAX and pMD2G into 293FT cells using CalPhos™-Kit (BD-Biosciences).The two harvests of viral particles supernatants from the 293FT culture medium were accumulated after 48 and 72 hours of transfection. The supernatants were centrifuges at 1790 xg for 15 min and filtered with 0.45 µm PVDF membrane. The viral particles were concentrated with Peg-it solution overnight at 4°C, aliquoted in PBS and stored at -80°C for later use. Mixed glial cultures were transduced on days in vitro (DIV)-8. Transduction efficiencies and multiplicities of infection were determined and calculated from serial dilutions in neuronal cultures using enhanced green fluorescent protein (EGFP) fluorescence as a reporter after 96 hours.

3.2.3 Injury paradigms

3.2.3.1 Oxygen-glucose deprivation (OGD) as injury paradigm in vitro

Culture medium was removed, rinsed twice with warmed PBS and replaced by a deoxygenated balanced salt solution containing (in mmol/L): Na^+ 143.8, K^+ 5.5, Ca^{2+} 1.8, Mg^{2+} 0.8, Cl^- 125.3, HCO^{3-} 26.2, $(H_2SO_4)^{2-}$ 1.0, $(SO_4)^{2-}$ 0.8, glycine 0.01 at pH 7.4. The same solution with additional of 25 mmol/L D-glucose under normoxia served as control. The cultures were exposed to oxygen and glucose deprivation (OGD), by replacing the culture medium with deoxygenated balanced salt buffer and by placing in the hypoxia chamber for different time points, such as for 90 min and 180 min, respectively. Oxygen–glucose deprivation was terminated by returning the cultures the conditioned medium and the normoxic incubator. After 24 hours of reoxygenations, cells were lysed for western blotting analysis or fixed for immunocytochemistry.

3.2.3.2 Transient middle cerebral artery occlusion (MCAo) as injury paradigm in vivo

Animal experiments were performed in accordance with the institutional and international guidelines. All surgical procedures were approved by the local authorities (animal proposal number: G0385/08). Mice were anaesthetized with 1.5% Isofluorane for induction and maintained in 1.0% Isofluorane in 70% N_2O, and 30% O_2 with the use of a vaporizer and a face mask. The mouse stroke model with focal middle cerebral artery occlusion (MCAo) was performed as described by Endres and colleagues (112, 113). Briefly, focal MCAo was induced with a silicone-coated 8.0 nylon monofilament. The filament was introduced into the left internal carotid artery up the anterior cerebral artery, thereby occluding the middle cerebral artery and anterior choroid arteries. Filaments were withdrawn after 1 hour of occlusion to allow reperfusion. To ensure equivalent levels of ischemia in all animals, regional cerebral blood flow (rCBF) was measured using laser-Doppler-flowmetry (Perimed, Jarfälla, Sweden). An rCBF that is lower than 20% of the baseline measurement prior occlusion during the procedure was considered as successful, and it was returned to approximately 100% within 5 min after reperfusion. Core temperature during the procedure was maintained at 36.5°C ± 0.5°C with a heating pad.

Animals were sacrificed for histological analysis at 24, 72, and 96 h after MCAo/ reperfusion, the brains snap-frozen in isopentane on dry ice and stored at -80°C until further use. For 5-bromo-2-deoxyuridine (BrdU) labeling experiments, animals received intraperitoneal injections of 50 mg BrdU/kg body weight at a concentration of 10 mg/mL BrdU (Sigma, St. Louis, MO) dissolved in sterile 0.9% NaCl solution.

3.2.4 Bone marrow transplantation

Bone marrow (BM) transplantation was performed as described previously (114). BM cells were obtained from EGFP transgenic 8-12-week-old male C57BL/6. All recipient mice received 5 x 10^6 cells by tail vein injection. Four weeks after BM transplantation, ischemia was induced in chimeric mice by filament occlusion of the middle cerebral artery.

3.2.5 Real Time RT PCR

Total RNA was extracted using the Invisorb RNA Kit II (Invitek, Berlin, Germany) and real-time-RT-PCR was performed as described previously (115). Primers were designed to amplify mouse transcripts of necdin and tripeptidyl-peptidase II (TPP2). Amplification products were controlled by melting curves. No-RT- and non-template controls were run for all reactions. The values were normalized to the stably expressed TPP2.

3.2.6 Western blotting

For total cellular protein extraction, cells were lysed in TNN lysis buffer (150 mM NaCl, 10% glycerol, 0.5% Triton-X-100, 1 mM DTT, 0.5 mM PMSF, 5 mM orthovanadate, protease inhibitor and phosphatase inhibitor) for 20 min incubated on ice and clarified at 12 000 ×g for 10 min at 4°C. Supernatants were collected and stored at -80°C for later use.

For extraction of nuclear proteins, cells were lysed in cytoplamic lysis (CL) buffer [10 mM HEPES, 2 mM magnesium chloride, 1 mM EDTA, 1 mM EGTA, 10 mM potassium chloride, 1 mM dithiothreitol (DTT), 10 mM sodium fluoride, 0.1 mM sodium vanadate, 1% Nonidet P-40, protease inhibitor cocktail (Roche)] (200 µl CL buffer was used per 6 well plate), incubated on ice for 15 min and clarified at 12 000 ×g for 1 min. Supernatants were collected as cytoplasmic protein fraction and stored at -80°C. Pellets were further used for extraction of nuclear proteins in nuclear lysis (NL) buffer [25 mM HEPES, 500 mM sodium chloride, 5 mM magnesium chloride, 10 mM sodium fluoride, 1 mM dithiothreitol (DTT), 10% glycerol, 0.2% Nonidet P 40, protease inhibitor cocktail (Roche)], incubated on ice for 10 min, sonicated for 3 x 10 sec on ice and clarified at 12 000 ×g for 5 min. Supernatants were collected and stored at -80°C.

Twenty to fifty micrograms of cytoplamic or nuclear lysates were denatured by boiling at 95°C in equal volume of sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl pH 6.8) for 5 min. Samples were electrophoretically separated using pre-cast 4–20% SDS-PAGE, transferred onto nitrocellulose membranes and blocked in blocking buffer (5% w/v milk in PBS) for 1 hour at room temperature. Primary antibodies (0.2 to 1 µg/ml) were incubated in blocking buffer overnight at 4 °C on a rotary platform with gentle agitation. Membranes were subsequently probed with secondary HRP-

conjugated anti-rabbit IgG or anti-mouse antibodies (diluted 1:2500) in blocking buffer for 1 hour at room temperature on a rotary platform with gentle agitation. Equal loading was confirmed by probing the membranes with anti-GAPDH (1:2000; Santa Cruz) or anti-HDAC1 (1:50; Santa Cruz) antibodies. Detection was carried out using the enhanced chemiluminescence assay (Cell Signaling Technologies). The image was captured with a FUSION chemiluminescence system (PEQLAB) and processed using Adobe Photoshop.

3.2.7 Immunocytochemistry

For immunocytohemical analysis of mixed glial, isolated microglia or peritoneal macrophages, cells were fixed with 4% PFA at room temperature for 15 min, before permeability step in blocking solution (10% goat serum and 1% Triton X-100 in PBS) for 30 min at room temperature. Fixed cells were incubated with primary antibody (10% serum and 0.1% triton in PBS) raised against necdin (GN1; 1:500), Iba1 (1:500), GFAP (1:200), NEDD8 (1:500), SUMO 2/3 (1:500), respectively overnight at 4°C. Next day, cells were rinsed and developed with fluorescence dye-conjugated secondary antibodies (Alexa Fluor® Dyes: 488, 594 or 647; 1:1000) for 1 hour at room temperature. Slides were mounted with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI). Microscopic images were taken with an inverse IX81 Olympus epifluorescence microscope equipped with an ORCA camera (Hamamatsu) using Cell M™ software with the same settings for all pictures of the same session. Alternatively, isolated microglial cells were taken using upright confocal microscope (Leica LFSA). All images were processed using Adobe Photoshop.

3.2.8 Fluorescence Immunohistochemistry

Paraffin embedded brains sections were prepared from naive or MCAo mice. The brains were fixed by transcardial perfusion of 4% PFA solution in 0.1 M phosphate buffer (pH 7.4). The post-fixated brains were transferred to 30% sucrose in 0.1 M phosphate buffer, followed by dehydration, and paraffin embedding steps. The brain sections (3-4 µm thick) were mounted on objective slides coated with gelatine and followed deparaffinized steps. Subsequently, the sections were stained with antibodies raised against necdin (GN1; 1:500 or NC243; 1:1000),

Iba1 (1:500), EGFP (1:500), EdU (1:4) or BrdU (1:200), diluted in 10% goat serum and 0.1% Triton X-100 in PBS (except for EdU staining), for overnight incubation at 4°C. The following day, sections were rinsed and incubated with fluorescence dye-conjugated secondary antibodies (Alexa Fluor® Dyes: 488, 594 or 647; 1:1000) for 1 hour at room temperature. Sections were mounted with cover slips in ProLong Gold antifade reagent with DAPI. Microscopic images were taken at the cortical area with an inverse IX81 Olympus epifluorescence microscope equipped with an ORCA camera (Hamamatsu) using Cell M™ software with the same settings for all pictures of the same session. All images were processed using Adobe Photoshop.

3.2.9 Cytospin preparation and DAB staining

Cytospin preparations were prepared (Shandon) after FACS, a total of 4000-5000 cells per objective slides were fixed with methanol/acetone (1:1) for 10 min before rinsing. Subsequently, the cells were immensed in blocking buffer (0.3% H_2O_2, 10% goat serum and 0.1% triton in PBS) for 2 hours at room temperature before incubation with primary antibody against necdin (GN1; 1:500), overnight at 4°C. Next day, immunostaining followed the peroxidase method with biotinylated secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) and diaminobenzidine (DAB) staining (SigmaFast™DAB; Sigma). Sections were stained against DNA (DAPI dye) and mounted with cover slips in polyvinyl alcohol with diazabicyclooctane (DABCO) as anti-fading agent. Images were recorded using a Leica fluorescence microscope with a 40× oil immersion objective and a digital camera.

3.2.10 MACS-sorted CD11b positive cells

Cells obtained from mice brains (naive or MCAo) were prepared as previously described (116). Briefly, brains were homogenized mechanically using a scalpel and followed by enzymatic digestion with RPMI-1640 medium containing 5% fetal bovine serum, 0.16% DNase and 1% collagenase (10 mg/ml) for 30 min at 37°C. The reaction was ceased with the addition of 10 mM EDTA. The cell suspension was filtered with 70 µm nylon filter and clarified at 252 xg for 10 min at 4°C. Leukocyte cell fraction is separated by Percoll™ reagent. The obtained leukocytes cells were rinsed once with PBS, incubated with CD11b microbeads for 15 min at

4°C and followed by positive selection via magnetic separation with the autoMACS™ Separator. Next, the CD11b-magnetic activated cell sorting (MACS) sorted cells were either lysed for western blotting analysis or stained for fluorescence activated cell sorting (FACS) analysis.

3.2.11 Staining and Cell sorting by flow cytometry

Freshly isolated brain cells were prepared as aforementioned and incubated with pacific blue (BV)-labeled antibodies to CD11b (20 µg/ml) and/or fluorescein isothiocyanate phycoerythrin (PE)-labeled antibodies to CD45 (20 µg/ml) (BD Pharmingen), or with isotype-matched control antibodies, for 30 min at 4°C. Immunostained cells were sorted using a FACSDIVA analyzer (BD Biosciences).

3.2.12 Magnetic Resonance Imaging

Infarct volumes were measured using a T2-weighted MR imaging (MRI). MRI was performed using a 7 Tesla rodent scanner. A ^{1}H-RF quadratur Volumeresonator with an inner diameter of 20 mm was used. Data acquisition and image processing were carried out by using the Bruker software Paravision 4.0. During the imaging procedure, constant body temperatures (37°C) of mice were stabilized by a circulating-water blanket. Anaesthesia was induced by 2.5% and maintained by 1.5-2.0% isoflurane delivered in an O_2/N_2O mixture (30/70%) via a facemask. A T2-weighted 2D turbo spin-echo sequence was used for imaging the mouse brain (imaging parameters TR/TE = 4200/36 ms, rare factor 8.4 averages). There was 20 axial slices with a thickness of 0.5 mm/slice, a field of view (FOV) of 2.6 x 2.6 cm and a matrix of 256 x 256 were positioned over the brain from olfactory bulb to cerebellum. As for Image Analysis, calculation of lesion volumes was carried out by the program Analyze 5.0. The T2 signal hyperintensity in the ischemic areas were assigned as a region of interest in the program. This enables a threshold selection for image segmentation by connecting all pixels within a specified range of the selected seed pixel and resulted in a 3D object map of the whole stroke region. The total volume of the whole object map was automatically calculated.

3.2.13 Statistical evaluation

Data are presented as dot-plot and mean ± 95% confidence interval (CI). For comparison of two groups with parametric data a two tailed student's t-test was applied (i.e. for stroke volume measured by MRI). A Mann Whitney Rank Sum test was applied as indicated for non-parametric data (SigmaSTAT statistical software). All experiments were performed at least in triplicate unless otherwise stated. P-values < 0.05 were considered statistically significant. For in vivo experiments, a prior probability on having statistically significant reduced infarct volumes in the treatment group was based on following defined parameters: effect size: 0.25, mean standard deviation=0.32 and α=0.05 and $\beta = 0.2$ $(1 - ß \geq 0.8)$. With these defined parameters, a calculated number of animals needed per group is 28. An effect size of <0.25 is not relevantsince the biological impact is questionable. Assumptions on the mean standard deviations are derived from past data.

4 Results

4.1 Expression and regulation of necdin in microglial cells in vitro

4.1.1 Necdin expression levels increase in microglial cells following oxygen-glucose deprivation (OGD) subjection

Accumulating evidence shows that necdin is predominantly expressed in postmitotic neurons. The physiological function of necdin includes promoting neuronal survival, differentiation and restricts proliferation in hematopoietic stem cells (95, 99, 103). Thus, the aim of this study was to investigate the involvement of necdin expression in cell types of the brain other than neurons and whether the expression pattern of necdin was altered following OGD. For this purpose, primary microglial cells were isolated from mixed glial cultures on in vitro day (DIV)-12, prepared as described in Methods, and subjected to OGD on DIV-1 (after isolation) for 3 hours. After 5 hours of reoxygenation, the cells were fixed and immunostained for necdin, Iba1 (ionized calcium binding adaptor molecule 1) and DNA. Necdin expression was identified in primary microglial cultures by western blotting and immunocytochemistry. The purity of the isolated microglial cultures was confirmed by Iba1 staining (>99% $Iba1^+$ cells) (Figure 9A). The specificity of the necdin antibody (GN1) was verified as shown in figure 9B, in which immunocytochemistry with the GN1 antibody resulted in a marginal staining after preabsorption of the antibody with necdin recombinant protein prior to usage.

In line with the immunocytochemistry results, western blotting analysis showed that necdin protein levels were increased 24 hours following transient OGD (Figure 9C). Fractionated protein samples were collected at 5 and 24 hours reoxygenation time points and further analyzed with SDS-PAGE protein electrophoresis, followed by western immunoblotting using antibodies for necdin and p-Histone H3. Phosphorylation of Histone-H3 is extensive during mitosis, thus an indicator of dividing cells. Necdin levels increased in both cytoplasmic and nuclear fraction after OGD in comparison to the control sample (Figure 9D). Histone deacetylase 1 (HDAC1) shown in western blotting analysis served as a loading control.

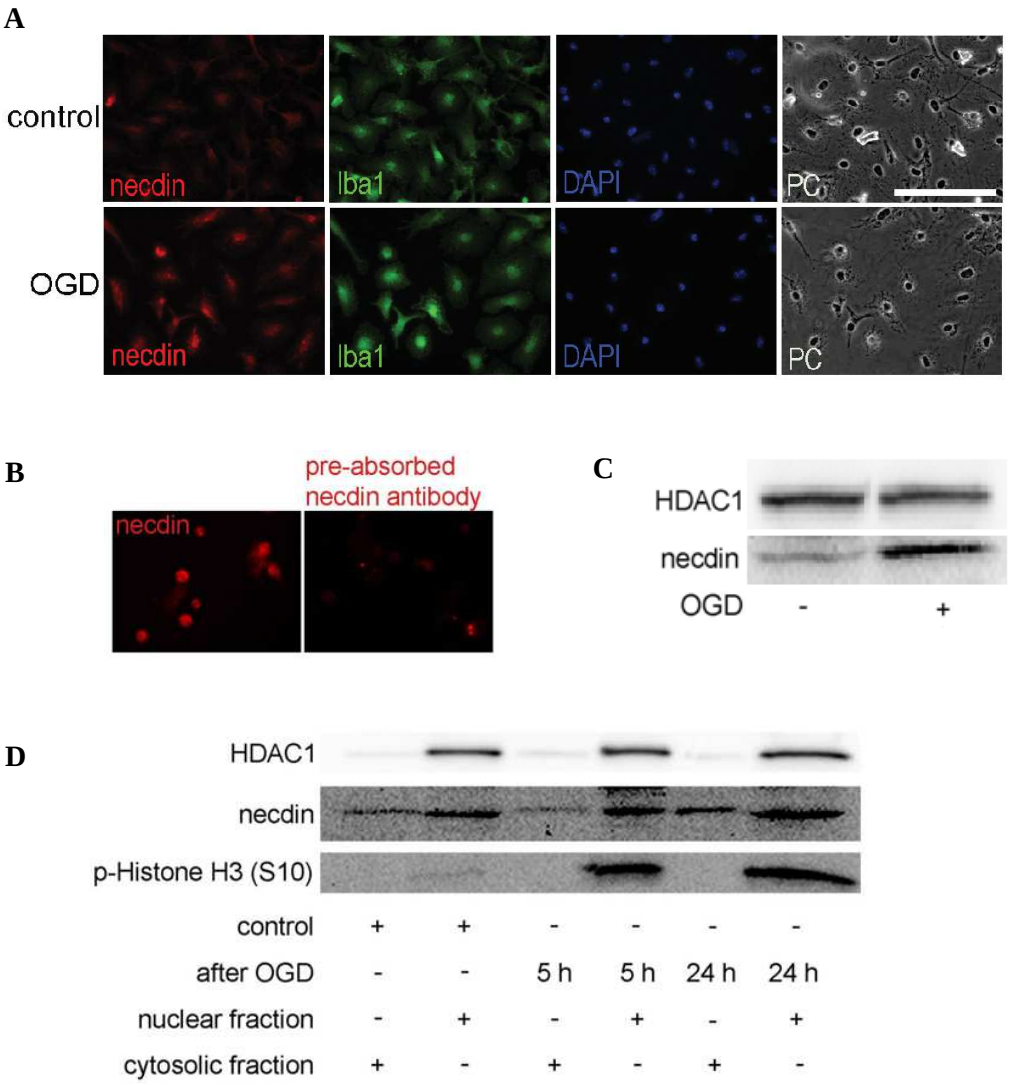


Figure 9 Necdin expression in microglia in vitro at baseline and after oxygen-glucose deprivation (OGD). (A, B, C, D) Primary microglial cells were subjected to OGD for 3 hours. (A) At 5 hours of reoxygenation time point, the cells were fixed and immunostained for necdin (red), Iba1 (green) and DNA (blue), additionally phase contrast (PC) was taken. Scale bar: 100 µm. (B) Preabsorption with recombinant necdin protein or secondary antibody alone served as control staining. (C) 30 µg of protein was subjected to sodium sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and membrane was probed for necdin and HDAC1 antibodies. The images represent the results of three independent experiments. (D) Necdin protein distribution at nuclear and cytoplasmic fractions in microglia in vitro at baseline and after OGD. Mixed glial cultures on DIV-12 were subjected to OGD. At the indicated reoxygenation time points, cell fractionation were obtained and analyzed via western blotting and probed for necdin, phospho-Histone H3 and HDAC1.

4.1.2 Necdin is expressed in resident microglial but not in peritoneal macrophages at baseline and following OGD subjection

Regarding the lineage relationship between peripheral macrophages and microglial cells, it is known that both originate from a common myeloid progenitor. Subsequently, the recruitment and differentiation of resident microglia occurs in the CNS during embryogenesis (117). Since necdin has been reported to modulate hematopoietic stem cells (HSCs) (95), we intended to test whether it is expressed in peripheral macrophages. To determine the necdin mRNA and protein levels in resident microglia and peritoneal macrophages, we used primary microglia isolated from the mixed glial cultures prepared from murine brain (P1-P3) and peritoneal macrophages isolated from adult mice (6-8 weeks old). The mRNA levels of necdin were 400-fold higher in primary microglia in comparison to the peritoneal macrophages (Figure 10A). Consistently, in contrast to the peritoneal macrophages, necdin protein was detected by western blot analysis in isolated microglial cells (Figure 10B). In addition, necdin protein levels remained undetected in peritoneal macrophages at 24 hours reoxygenation after OGD subjection. Equal protein loading was confirmed by probing the membrane with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

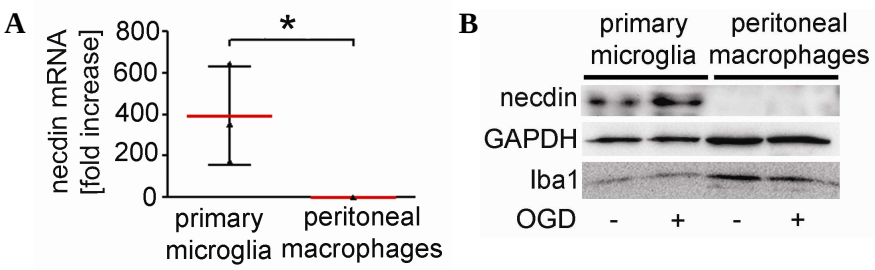


Figure 10 Differential necdin expression in microglia versus macrophages in vitro at baseline and after oxygen-glucose deprivation (OGD). (A) Quantitative analysis of necdin mRNA using real time PCR (normalized to TPP2 as a reference) against peritoneal macrophages using the ddCT method. Data were pooled from three independent experiments. Student's t-test $*P < 0.001$. (B) Necdin expression in primary microglial cells and peritoneal macrophages by analyzed via western blotting. Membranes were incubated with necdin, IbaI and GAPDH.

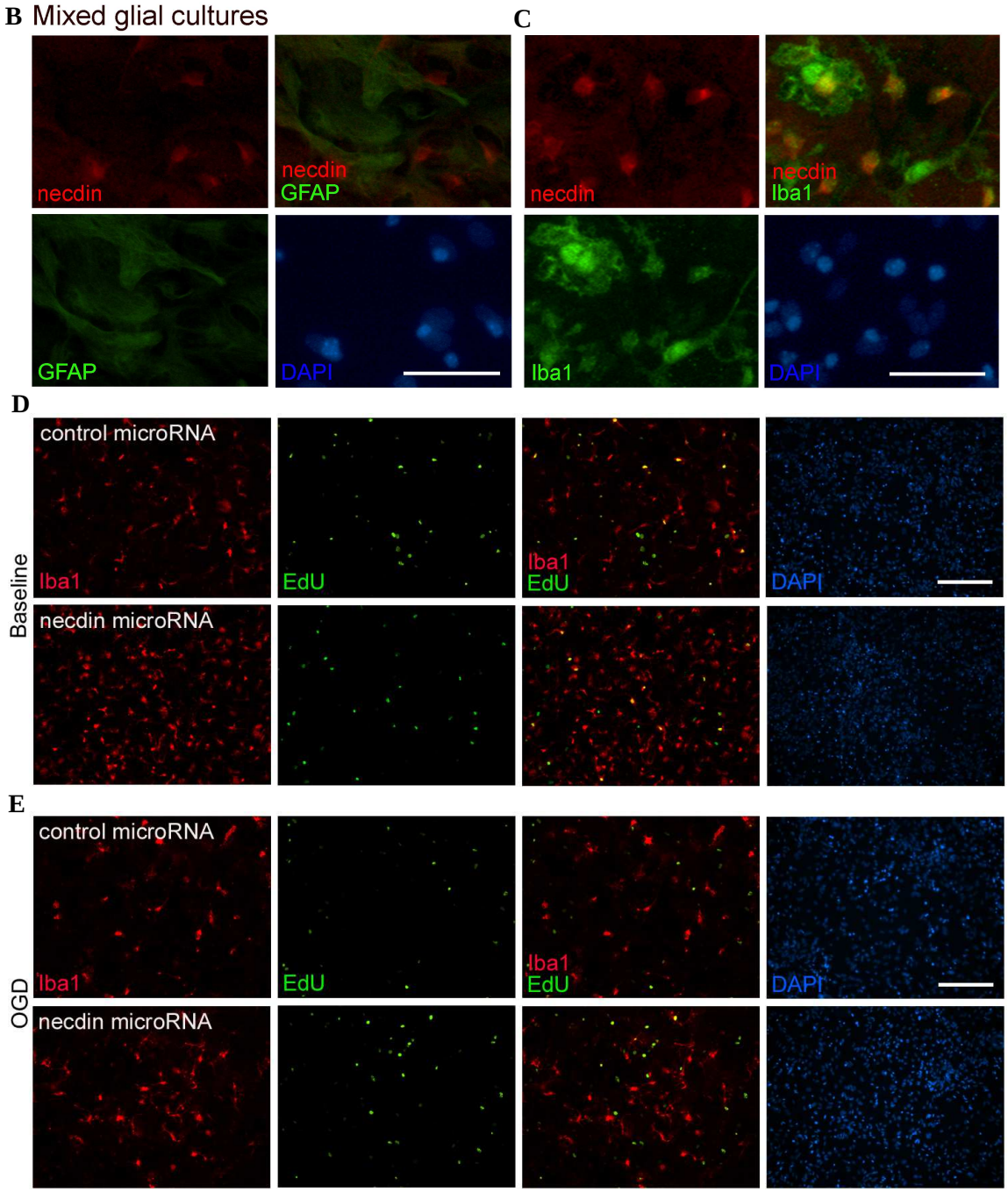
4.1.3 Necdin mediates growth arrest in primary microglia in vitro

To test the hypothesis that necdin may restrict cellular proliferation in glial cells, in particular microglia, a loss of function approach using lentiviral particles (LVPs) targeting necdin by microRNA expression along with a GFP reporter was established (Figure 11A). HDAC1 and enhanced green fluorescent protein (EGFP) protein levels were not altered while necdin microRNA interfered specifically to necdin protein in contrast to the non-targeting control microRNA.

Due to the laborious and costly method of using pure isolated microglial cells, we intended to test the function of necdin in mixed glial cultures. To test whether microglia were the only cells expressing necdin in the mixed glial culture, the cultures were fixed and immunostained for necdin, Iba1 or glial fibrillary acidic protein (GFAP). As shown in figure 11B, necdin positive cells were not immunoreactive to GFAP, which is a marker for astrocytic cells. In contrast, all necdin$^+$ cells were immunoreactive to Iba1 antibody. Furthermore, the necdin$^+$Iba1$^+$ double positive cells assumed an 'ameboid' phenotype.

We next evaluated a potential role of necdin expression for the survival and proliferation of the glial culture after OGD subjection. Mixed glial cells were inoculated with LVPs carrying either control or necdin microRNA. After 5 days of transduction, the cells were subjected to OGD and subsequent 24 hours reoxygenation. The Iba1$^+$ cells in necdin microRNA-transduced culture was significantly higher in numbers than that detected in control microRNA-transduced culture, at the baseline (3.1-fold) and after OGD subjection (2.5-fold), with negligible cell loss. Although, control culture exhibited a higher survival rate (1.2-fold), compared to the necdin microRNA-transduced culture, albeit statistically non-significant. Furthermore, application of thymidine analogues, EdU (5-ethynyl-2'-deoxyuridine), at 24 hours after reoxygenation and 2 hours prior to fixation demonstrated a higher number of cells entering S-phase, at baseline (4.8 ± 2.7 vs. 2.5 ± 1.4) and after OGD (0.5 ± 0.8 vs. 0.2 ± 0.4) in necdin versus control microRNA-transduced cultures, respectively (Figure 11C).


A
HDAC1
necdin
EGFP
control microRNA + -
necdin microRNA - +


B Mixed glial cultures
C
necdin
GFAP
DAPI
Iba1
D
Baseline
control microRNA
necdin microRNA
EdU
E
OGD

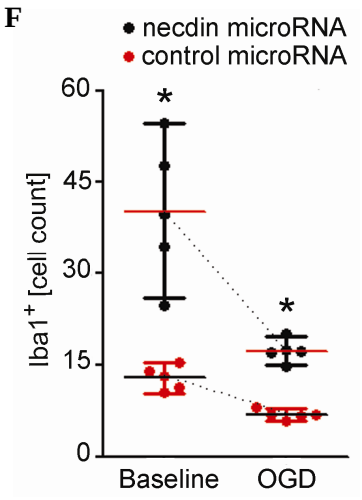


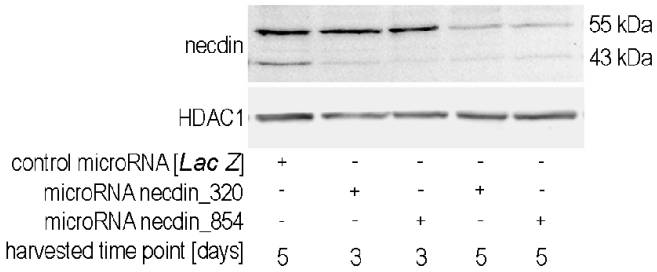
Figure 11 Assessment of the role of necdin via RNA interference. Mixed glial cultures were prepared as described in the method part. (A) The mixed glial cultures were transduced respectively with LVPs expressing necdin microRNA or control microRNA (*Lac Z*). Protein lysates were obtained at day 5 for western blotting analysis and probed with necdin and HDAC1. (B-C) Mixed glial cultures were fixed and immunostained for necdin (red) and GFAP (green) or Iba1 (green). (D-E) The cultures were stained for Iba1 (red), EdU (green) and DAPI (blue). Representative microscopic images of LVPs transduced mixed glial cultures, expressing necdin microRNA (lower panel) and control microRNA (*Lac Z*) (upper panel) at (D) baseline and (E) 24 hours after OGD. (F) Absolute numbers of $Iba1^+$ cells at baseline and after 24 hours of reperfusion. Data was presented as scattered dot-plot with mean ± 95% confidence interval (n=5). One Way RM ANOVA was applied; $*P < 0.001$. Scale bar: 100 µm.

4.1.4 Putative posttranslational modification of necdin by NEDD8 and SUMO2/3

Interestingly, an additional band of approximately 55 kDa was detected during western blotting (shown in Figure 12A). Moreover, by using microRNA-mediated necdin depletion, the downregulation of necdin protein followed a different kinetic, indicating a different stability levels of the protein, depending on the posttranslational modification (Figure 12A). Since the molecular weight difference was approximately 10 kDa, we investigated the co-localization of necdin with selected members of UBLs (Figure 12B).

Co-localization of necdin with NEDD8 and SUMO2/3 was detected in the microglial cells (Figure 12C, D). As for the SUMOylation site, necdin demonstrated an inverted SUMOylation motif with exact fit to SUMO sites (highlighted green; Figure 12B).

A



B

Putative NEDDylation (highlighted yellow) and SUMOylation (highlighted green) sites in necdin protein:

Necdin [Mus musculus] protein sequence was obtained from National Center for Biotechnology Information (NCBI) as shown below (NCBI reference sequence: NP_035012). The highlighted sequences were the corresponding putative posttranslational modification sites found in necdin.

```
MSEQSKDLSD PNFAAEVPDC EMQDSDAVPV GIPPPASLAA NLAGPPCAPE GPMAAQQASP

         70          80          90         100         110         120
PPEERIEDVD PKILQQAAEE GRAHQPQSPA RPIPAPPAPA QLVQKAHELM WYVLVKDQKR

        130         140         150         160         170         180
MVLWFPDMVK EVMGSYKKWC RSILRRTSVI LARVFGLHLR LTNLHTMEFA LVKALSPEEL

        190         200         210         220         230         240
DRVALNNRMP MTGLLLMILS LIYVKGRGAR EGAVWNVLRI LGLRPWKKHS TFGDVRKIIT

        250         260         270         280         290         300
EEFVQQNYLK YQRVPHIEPP EYEFFWGSRA NREITKMQIM EFLARVFKKD PQAWPSRYRE

        310         320
ALEQARALRE ANLAAQAPRS SVSED
```

Published motifs:

NEDDylation motif: [IL][VIT][RQ][IS][MLV]K[MAS][RHE] (118)

Inverted SUMOylation motif: [ED]XK[VILFP] (119)

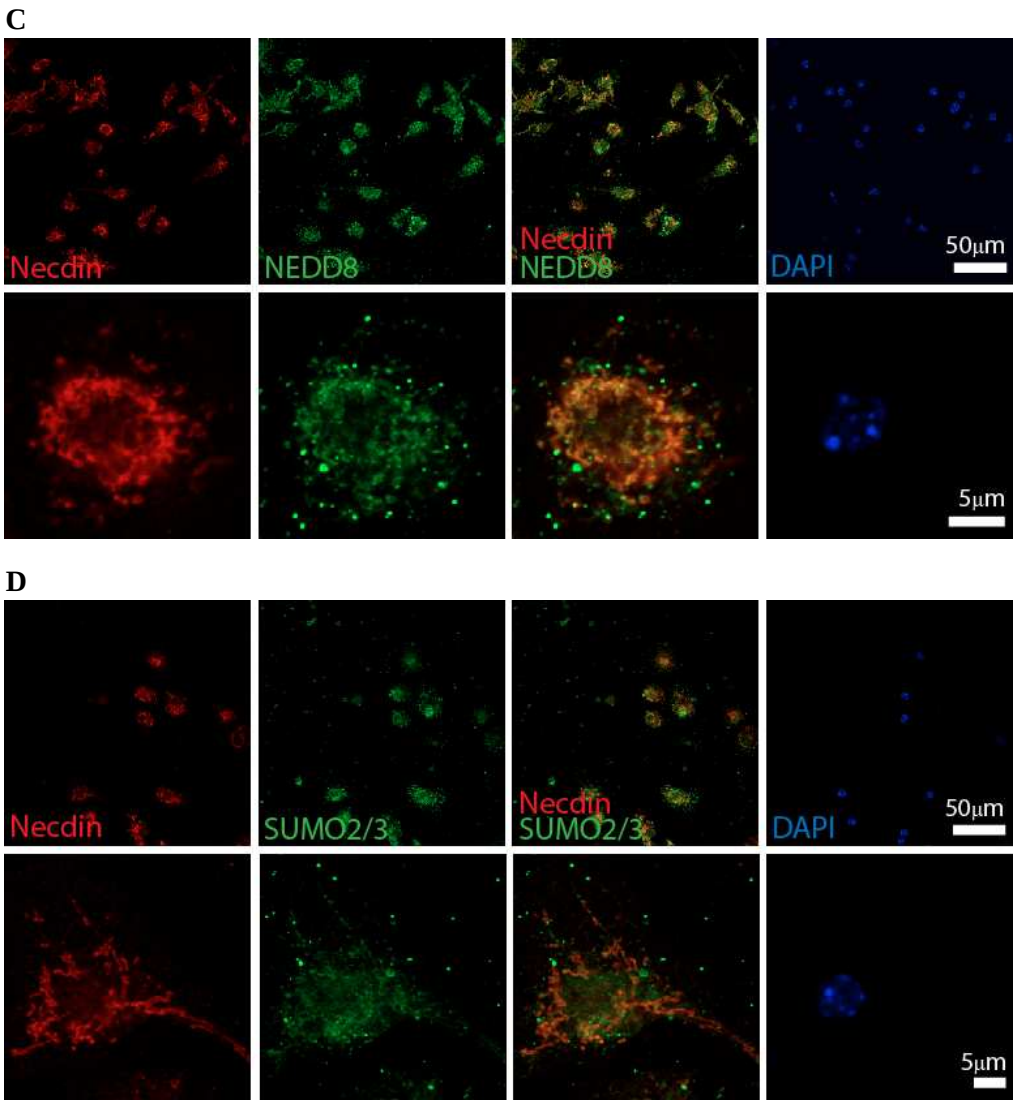


Figure 12 Posttranslational modification of necdin in microglia. (A) Putative upper necdin band detected in western blotting. The membrane was probed for necdin (NC243) and HDAC1 served as a loading control. (B) SUMO modified proteins contain an acceptor Lys next to a Ψ, which is a hydrophobic residue. (C) Representative confocal microscopy images of microglial cells. The primary microglial cells were fixed and stained with NEDD8 (green), necdin (red) and DNA (blue). (D) The primary microglial cells were fixed and stained with SUMO2/3 (green), necdin (red) and DNA (blue).

4.2 Expression and regulation of necdin in microglial cells in the ischemic brain in vivo

4.2.1 Necdin expression levels at different reperfusion intervals after MCAo

To test whether necdin protein levels were elevated at a particular time point after MCAo in the leukocyte population of the brain parenchyma, an enriched $CD11b^+$ cell population were obtained from NaCl perfused brains and necdin protein levels were further analyzed by western blotting.

We investigated necdin expression at 24, 48, and 72 hours after reperfusion. There were 10 to 12 brains collected for each time point, which were separated into ischemic (ipsilateral) and non-ischemic (contralateral) hemispheres. Brains of the respective time points were pooled and the leukocyte fraction was isolated via Percoll gradient and further sorted for $CD11b^+$ cell population using magnetic-activated cell sorting (MACS). The isolated cells were lysed to obtain whole cell lysates for western blotting analysis.

As expected, CD11b protein expression was increased specifically in the ischemic hemispheres at the indicated time points, which was expected as CD11b is an activation marker for leukocytes (121-123); the protein levels increased from 24 to 48 hours and remained stably expressed at 72 hours. A faint necdin band could be detected in naive brains and contralateral hemispheres at 24, 48 and 72 hours after MCAo. In the ipsilateral hemispheres, necdin expression could be significantly detected at all reperfusion time points. The protein level dramatically rose at 72 hours after MCAo.

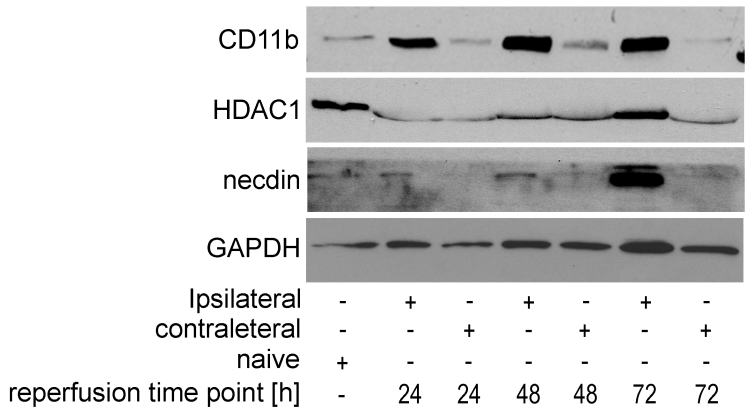


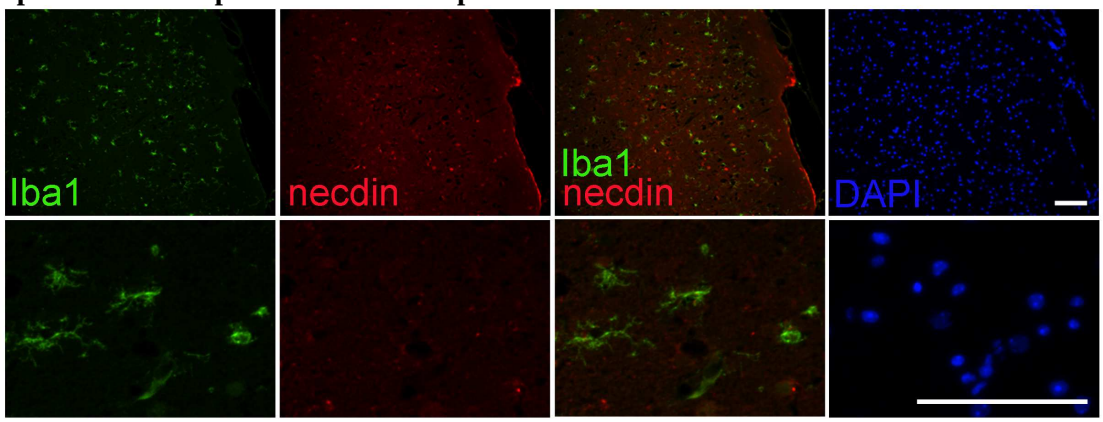
Figure 13 Necdin expression level at different reperfusion time points after MCAo. Ipsilateral and contralateral hemispheres of 10-12 mice per group were pooled prior to leukocyte isolation and magnetic-activated cell sorting with CD11b at the indicated time points after MCAo. Protein lysates were analyzed via western blotting and probed against CD11b, necdin, HDAC1 and GAPDH (latter two as loading controls).

4.2.2 Morphology of necdin positive cells in an ischemic mouse model (MCAo) and necdin expression level at different reperfusion intervals

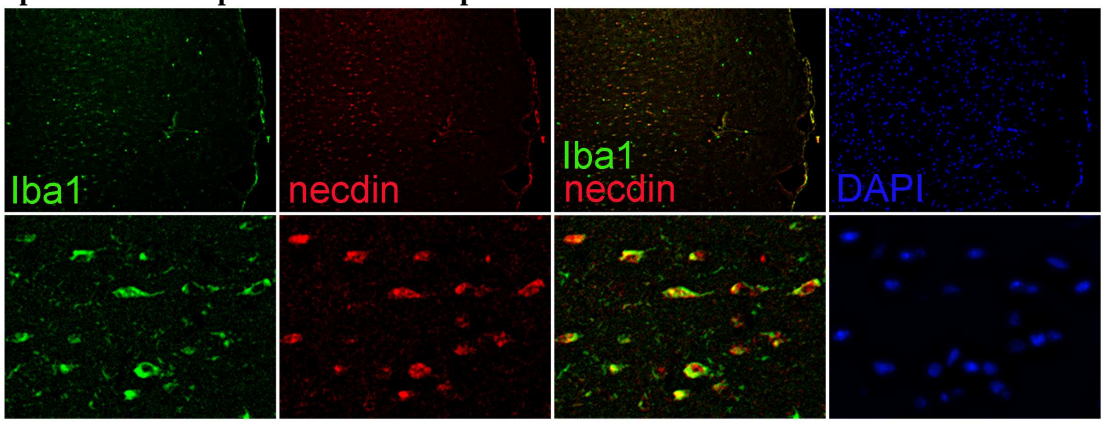
After having demonstrated that there was a surge of necdin expression at a specific reperfusion time point of 72 hours following 60 min MCAo, we investigated the specific cell type that was immunoreactive to necdin. By using immunohistochemistry, brain sections with different reperfusion intervals following transient MCAo were immunostained for necdin, Iba1 and DNA. Consistent with the western blotting analysis (figure 13), necdin expression was barely detectable at 24 hours reperfusion in the ischemic hemisphere (Figure 14A). Seventy-two hours following ischemic injury, the number of necdin$^+$Iba1$^+$ cells increased. Moreover, necdin$^+$ cells were typically 'ameboid-shaped', the presumed reactive form of microglia/macrophages (Figure 14B). At a later time point of 120 hours, necdin$^+$Iba1$^+$ cells were only sparsely detectable (Figure 14C). As for the contralateral hemispheres, necdin immunoreactive cells were not observed at all indicated reperfusion time points. Images were shown from the 24 hours after reperfusion time point of the contralateral hemispheres (Figure 14D).

Interestingly, ramified microglia (i.e. the 'resting/surveillance' form) did not express necdin in both ischemic and non-ischemic hemispheres (Figure 14A, C, D). Images from 72 and 120 hours reperfusion time points depicted the same observations (data not shown).

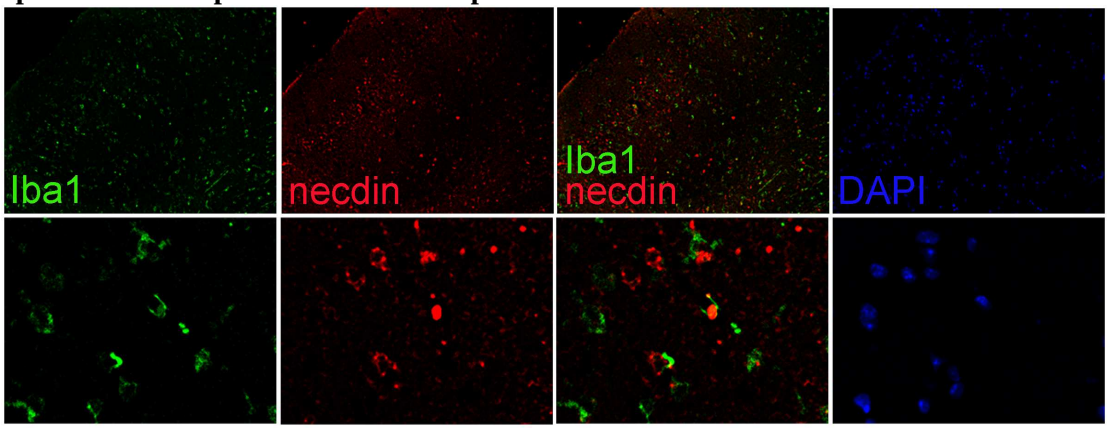
A Ipsilateral hemispheres: 24 hours reperfusion after MCAo



B Ipsilateral hemispheres: 72 hours reperfusion after MCAo



C Ipsilateral hemispheres: 120 hours reperfusion after MCAo



D Contralateral hemispheres: 24 hours reperfusion after MCAo

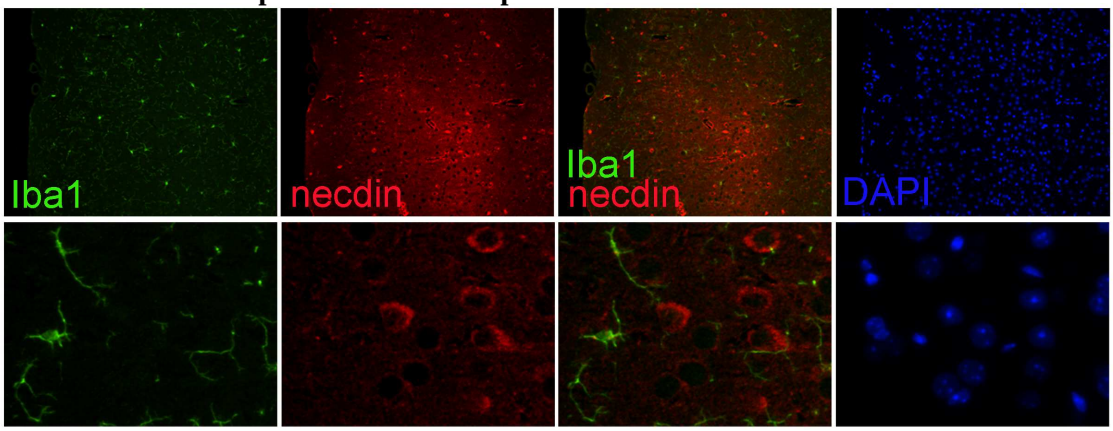
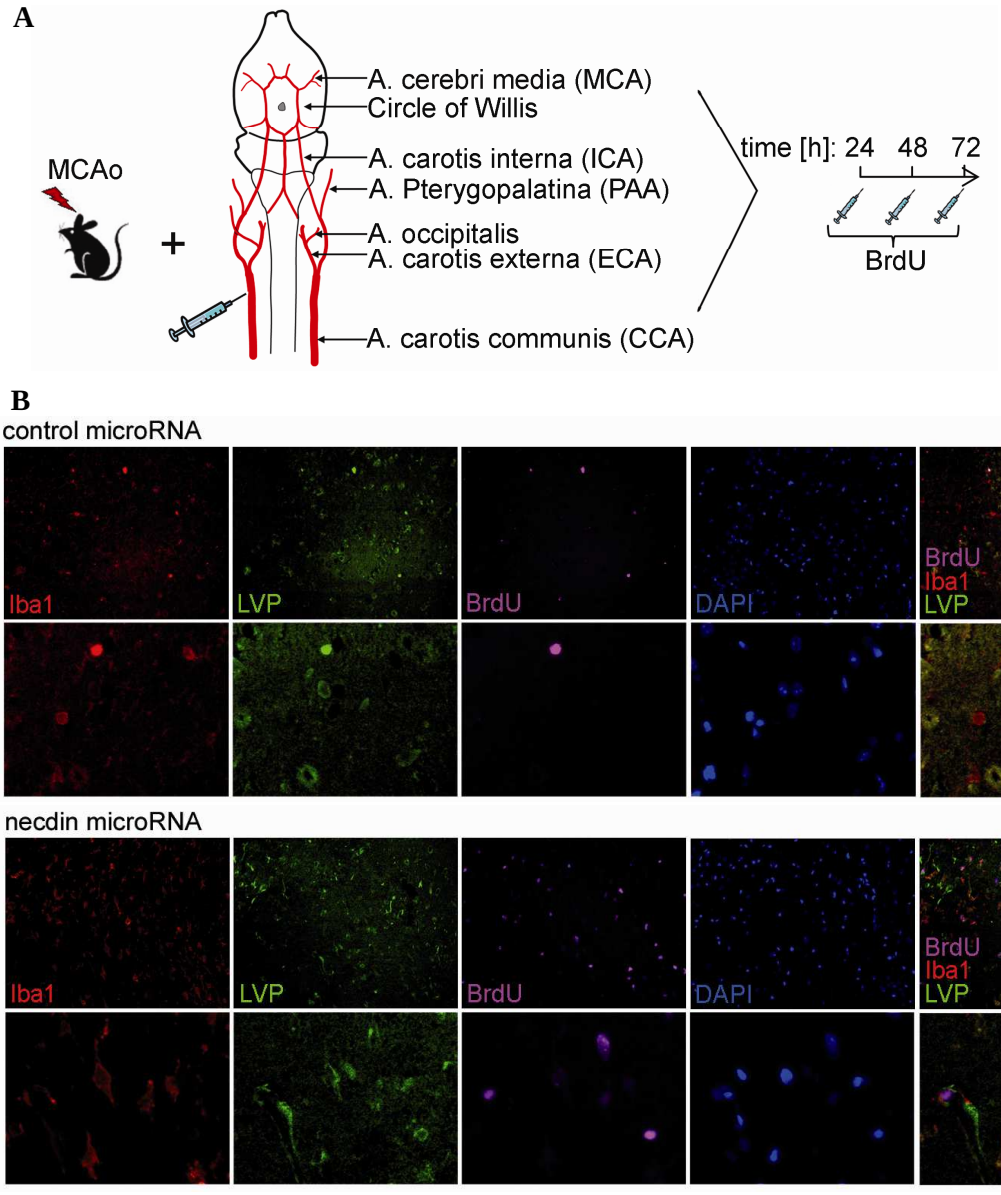


Figure 14 Necdin expression in subpopulation of microglia/macrophages. Animals were subjected to 60 min MCAo. (A) Brains were fixed at 24 hours reperfusion time point. Paraffin embedded brains sections (3 µm) were fixed and stained with Iba1 (green), necdin (red) and DNA (blue). (B) Immunostaining of ipsilateral hemispheres at 72 hours reperfusion time point. (C) Immunostaining of ipsilateral hemispheres at 120 hours reperfusion time point. (D) Immunostaining of contralateral hemispheres at 24 hours reperfusion time point. Scale bar: 100 µm.

4.2.3 Necdin mediates growth arrest in primary microglia in vivo

Having demonstrated that necdin plays a role as a negative regulator in microglia proliferation at baseline and after subjection to ischemic-like stress using OGD, we next investigated necdin proliferation on microglial cells in the ischemic brain in vivo. To test this hypothesis, LVPs carrying either control or necdin microRNA were intra-arterially injected following MCAo, which therefore acted predominantly on the ischemic hemispheres due to the compromised BBB. Note: LVPs-transduced $EGFP^+$ cells were not observed in the contralateral hemisphere and the number of $EGFP^+$ circulating leukocytes was low as shown by FACS analysis (data not shown). Briefly, LVPs were intra-arterially injected into the common carotid artery (CCA) and further advanced to the proximal internal carotid artery (ICA) immediately after retraction of the filament following transient MCAo. During 3 days reperfusion, mice were pulse-labeled with BrdU to analyze the kinetics and fate of proliferating cells marked by the thymidine analogue which incorporates into DNA during S-phase (Figure 15A; for detailed experimental paradigm).

The coronal brain sections were fixed and immunostained for Iba1, EGFP (LVPs) and BrdU as shown in the representative images (Figure 15B). We counted transduced microglial cells ($Iba1^+EGFP^+(LVPs^+)$) and proliferating transduced microglial cells ($Iba1^+EGFP^+BrdU^+$) within the cortical layers of the ischemic hemispheres. There was a significant 1.9-fold increase in number of proliferating microglial cells transduced with necdin microRNA compared to the control microRNA-transduced cells (Figure 15C). Notably, this effect was partially mediated since only approximately half of these $Iba1^+/BrdU^+$ cells were co-stained for the EGFP reporter. Transduction rate of $Iba1^+$ cells were 0.23 ± 0.10 (control microRNA) and 0.16 ± 0.05 (necdin microRNA).



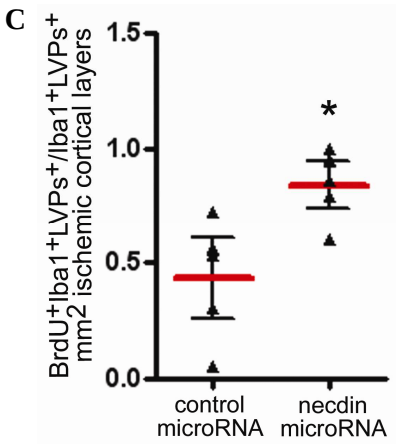
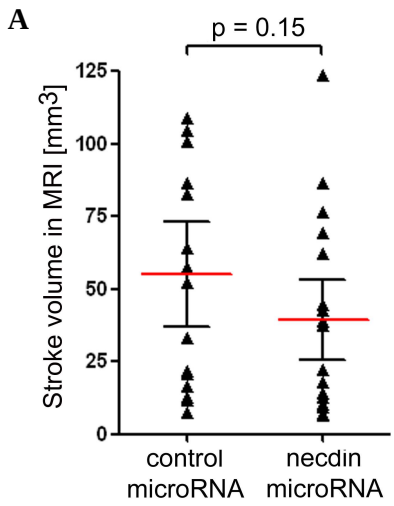


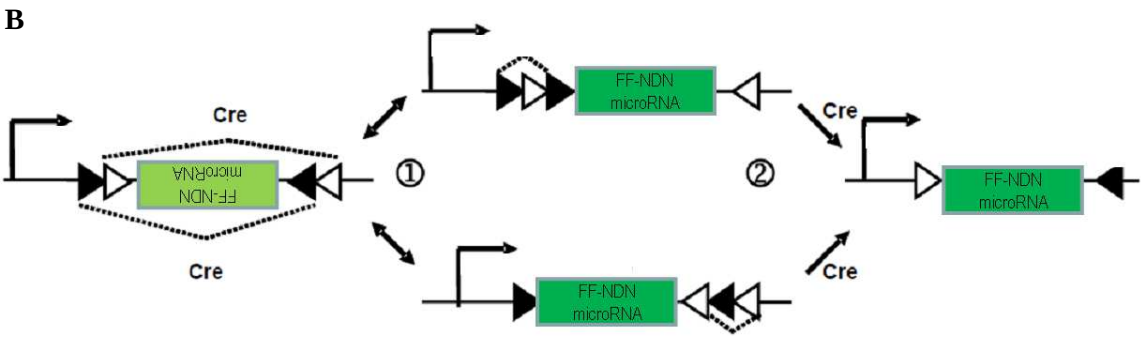
Figure 15 Assessment of the role of necdin in the ischemic brain via RNA interference. (A) A scheme on the experimental setup in investigating the role of necdin in microglia. (B) Representative images and quantitative analysis on cell proliferation after downregulation of necdin via RNA interference with lentiviral particles (LVPs) (Upper panel: microRNA with necdin; lower panel: non-targeting microRNA). Paraffin embedded brains sections (3 µm) were fixed and stained with BrdU (magenta), Iba1 (red), LVPs (green) and DNA (blue). Scale bar: 100 µm. (C) Cells were then counted as Iba1+LVPs+ double positive and BrdU+Iba1+LVPs+ triple positive cells. Animals underwent a 72 hours of reperfusion time point, with daily BrdU injection (Upper panel). For comparing two groups with parametric data, a two tailed student's t-test was applied. Data was presented as aligned dot-plot with mean ± 95% confidence interval (10 high power fields derived from 3 - 4 representative sections, 5 animals per group). Power for performed test with alpha = 0.05: 0.71; *P < 0.001; scale bar: 50 µm.

4.2.4 Loss of necdin following focal brain ischemia in vivo

After demonstrating that loss of necdin promoted proliferation of $Iba1^+$ cells in the ischemic brain, we next tested whether the higher cell count of proliferative $Iba1^+$ cells confers neuroprotection in an in vivo model of cerebral ischemia. Animals were subjected to 60 min MCAo and followed by intra-arterial injection of LVPs carrying either control or necdin microRNA during reperfusion. MRI was used to determine lesion volumes at day 5 after MCAo. There was no statistic difference between the control and necdin microRNA groups (figure 16A). The mortality rate was 0.25 for all three rounds of experiments (data not shown). Having demonstrated that there was no significant difference in lesion volumes between control and

necdin microRNA which were driven by ubiquitous promoter, we designed and generated constructs which allow selective expression of microRNAs in a cell type specific manner. As shown in figure 16B, the nucleotide sequence of control- or necdin-microRNA are inverted and flanked by loxP sites. In the presence of Iba1-Cre recombinase, loxP sites flanked-microRNA will be excised and flipped to allow transcription (124). Thus, by using this FLEX (flip and excision) system containing either control or necdin-microRNA in combination with Iba1-Cre recombinase, transcription is only expressed by Iba1^{+} cells. EGFP serves as a reporter for ectopic necdin expression, whereas mCherry serves as a reporter for microRNA (control or necdin) expression (figure 16C). Western blotting demonstrated that ectopic necdin was depleted by necdin microRNA in the presence of Iba1-Cre but not with control microRNA (figure 16D).





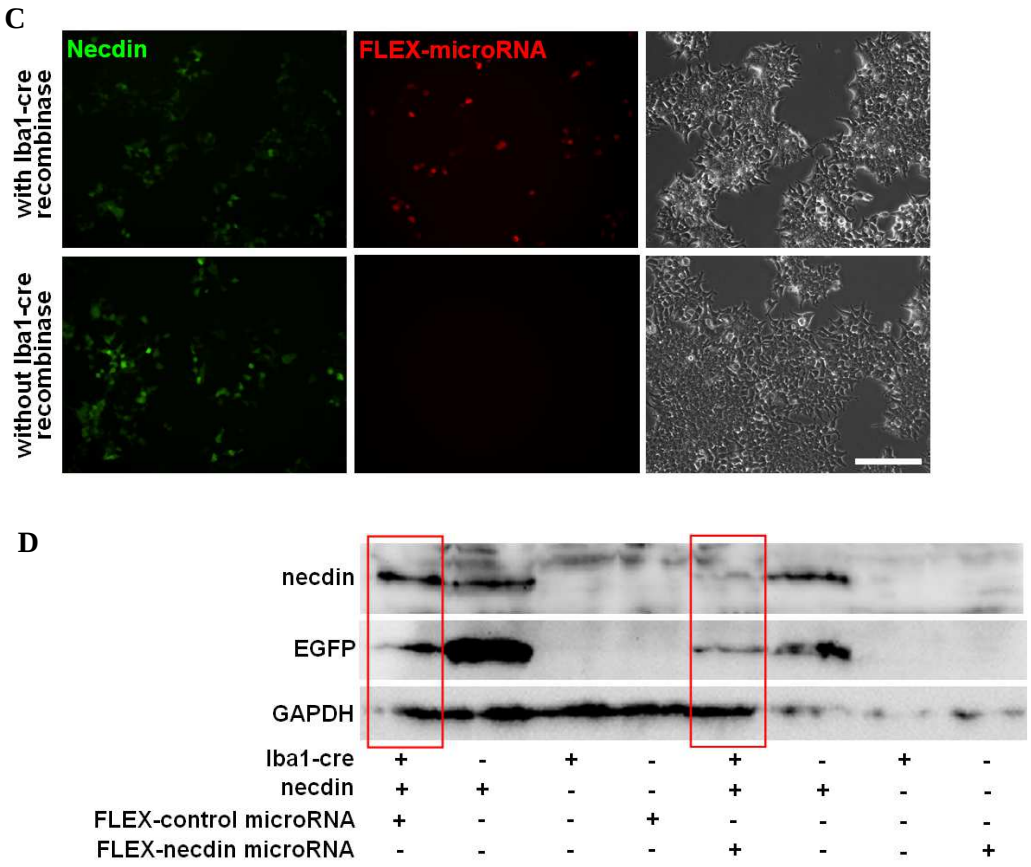


Figure 16 Ubiquitous- and microglia-specific promoter expressing necdin targeting microRNA. (A) For comparing two groups with parametric data, a two tailed student's t-test was applied. Data was presented as aligned dot-plot with mean ± 95% confidence interval, n = 20 vs. 22 (control microRNA vs. necdin microRNA). Power for performed test with alpha = 0.05: 0.17. (B) Schematic diagram on the FLEX system (C) Representative images of FLEX-microRNA expression (non-targeting- or necdin-microRNA) in the presence (upper panel) and absence (lower panel) of Iba1-Cre recombinase (upper panel) and absence of the Iba1-Cre. Expression of FLEX-microRNA (red) and extopic necdin (green) in HEK293 cells. Scale bar: 200 µm. (D) Protein lysates were obtained at day 5 for western blotting and probed with necdin, EGFP and GAPDH (loading control).

4.3 Endogenous necdin expression in a subpopulation of microglia/mcarophages in cerebral ischemia

4.3.1 Necdin is expressed in resident microglia but not in infiltrating macrophages in MCAo mice model

We have demonstrated that the expression of necdin was abundant in microglia and barely detectable in peritoneal macrophages at protein levels (Figure 10B). Nevertheless, the relationship between peritoneal macrophages is not as close to microglia as to peripheral macrophages, however, it is a good model for assessing certain protein expression and function. The lack of specific markers for discrimination between microglia and peripheral macrophages at the histological level prevents the precise identification of cellular subpopulations. To overcome this problem, bone marrow (BM) chimeric mice were utilized in this experiment, since it is the only means available to distinguish resident microglial cells from the infiltrating macrophages, which were tagged with EGFP.

Following 60 min MCAo and subsequent 72 hours reperfusion, brain sections were immunostained for necdin. In consistence with the western blotting data, infiltrating macrophages marked by EGFP did not express necdin (Figure 17).

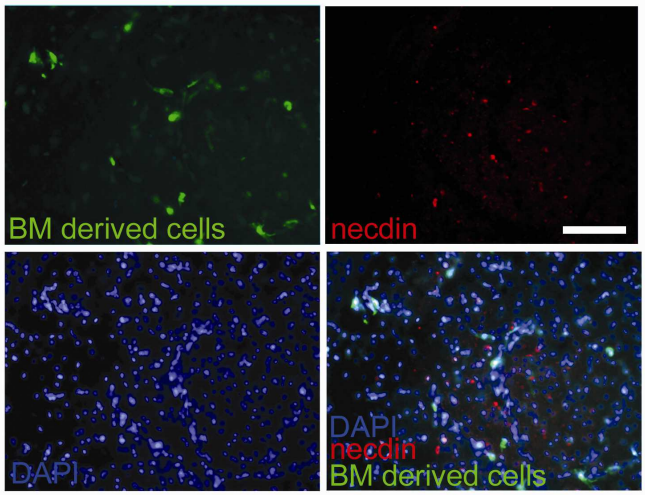


Figure 17 Necdin expression in resident microglia versus infiltrating macrophages in the ischemic brain. The chimeric mice were subjected to 60 min MCAo and subsequent 72 hours reperfusion. Free-floating brain sections (40 µm thick) were fixed and stained with necdin (red) and DNA (blue). Scale bar: 50 µm.

4.3.2 Preferential necdin expression in subset of microglia/macrophages in chimeric mice after MCAo subjection

To assess the transcriptional and translational levels of necdin in subsets of leukocytes, we used flow cytometry to sort for CD45 and CD11b to differentiate resting microglia from reactive microglia and infiltrating peripheral macrophages after transient MCAo (125, 126). After demonstrating that in contrast to BM derived macrophages, only a certain subset of resident microglial cells were immunoreactive to necdin, we attempted to utilize BM chimeras in combination with flow cytometry to further distinguish ramified ($EGFP^-/CD11b^+/CD45^{dim}$) from reactive microglial cells ($EGFP^-/CD11b^+/CD45^{high}$), where both cell populations were negative for EGFP after transient MCAo.

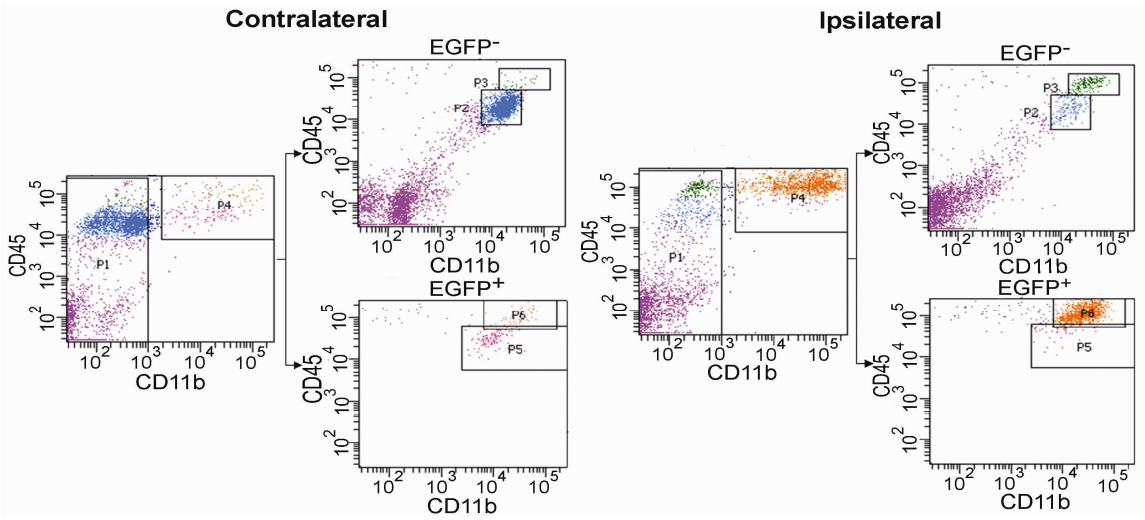
Mice were subjected to 60 min MCAo and subsequent 72 hours reperfusion before the brains were collected and separated into contralateral and ipsilateral hemispheres. Leukocyte fraction was obtained via Percoll gradient and further stained for CD45 and CD11b. The samples were FACS-sorted for $EGFP^-/CD11b^+/CD45^{dim}$ and $EGFP^-/CD11b^+/CD45^{high}$, $EGFP^+/CD11b^+/CD45^{dim}$ and $EGFP^+/CD11b^+/CD45^{high}$ (Figure 18A). Most of the $EGFP^+$ cells are BM derived cells which infiltrated the brain during the breakdown of BBB. In contrast to the contralateral hemisphere, the ipsilateral hemispheres demonstrated a prominent cell population of $CD11b^+/CD45^{high}$, irrespective of the origin of the cells (i.e. $EGFP^-$ or $EGFP^+$) (Figure 18A).

The collected FACS-sorted cells were used for assessing necdin mRNA levels. The necdin mRNA levels of all the FACS-sorted cell populations from the ipsilateral hemispheres were normalized to necdin mRNA from $EGFP^-/CD11b^+/CD45^{low}$ fraction. Cell populations that were below 150 events were excluded from the mRNA analysis, whereas for cytospin analysis more than 4000 cells were collected per fraction.

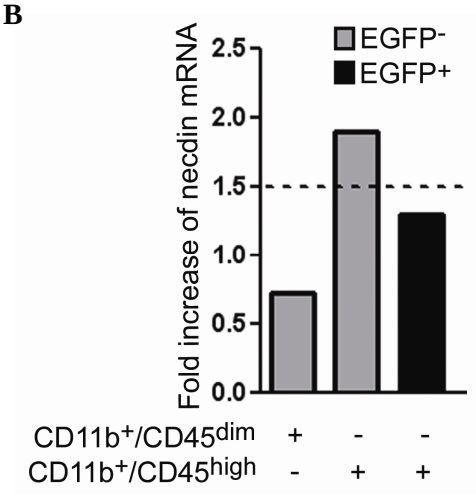
Of all the FACS-sorted cell populations from the ipsilateral hemispheres, necdin mRNA level of the $EGFP^-/CD11b^+/CD45^{high}$ cell population increased for 1.9-fold, whereas the other cell population were barely modified, 0.7-fold for $EGFP^-/CD11b^+/CD45^{dim}$ and 1.3-fold increment for $EGFP^+/CD11b^+/CD45^{dim}$ population (Figure 18B).

The collected FACS-sorted cells were cytospun and further fixed and immunostained for necdin and nuclear staining. In consistence with the immunostaining (Figure 14B) showing necdin^{+}Iba1^{+} cells adopted an ameboid phenotype, which was further confirmed by detection of necdin expression solely in the EGFP^{-}/CD11b^{+}/CD45^{high} cell population from the ipsilateral fraction (Figure 18C).

A



B



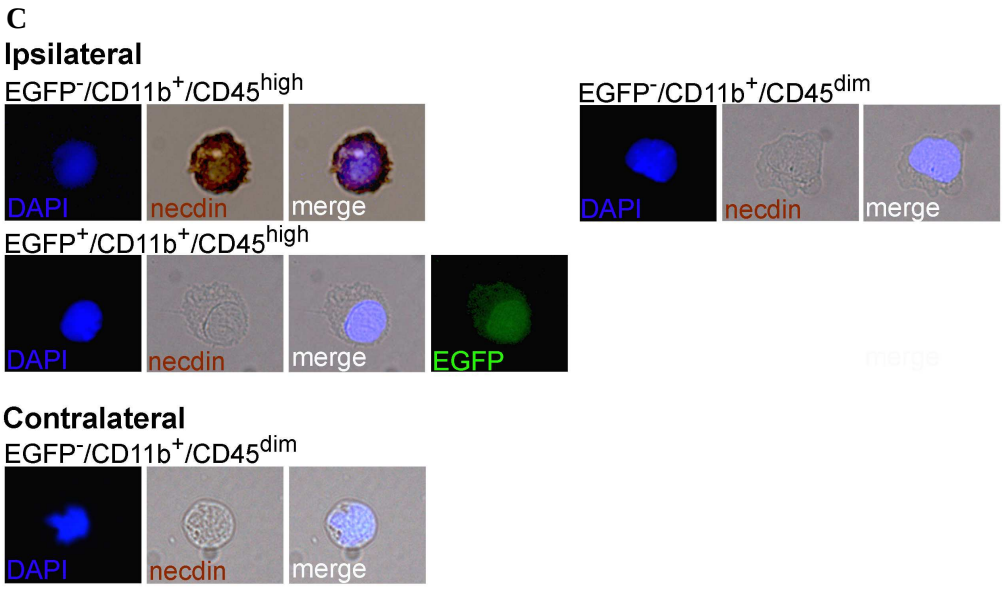


Figure 18 Preferential necdin expression in subset of brain inflammatory cells. (A) Representative dot-plot scatter analysis of cells isolated from ipsilateral ischemic hemispheres (left panel) and contralateral non-ischemic hemispheres (right panel) pooled from 5 mice. The EGFP expressing bone marrow derived cells along with the double staining for CD11b (BV conjugated) and CD45 (PE conjugated) allowed the identification of 3 populations: $EGFP^-/CD11b^+/CD45^{dim}$ (resting microglia, P2) and $EGFP^-/CD11b^+/CD45^{high}$ (reactive microglia and $EGFP^+/CD11b^+/CD45^{high}$ (bone marrow derived cells, P6). (B) Quantitative analysis of necdin mRNA using real time PCR (normalized to TPP2 as house keeping gene) against $EGFP^-/CD11b^+/CD45^{dim}$ (microglia, P2) from the contralateral hemispheres, using the ddCT method; n=5. (C) Representative microscopic images of stained cytospin preparations with necdin (DAB), DNA (blue), EGFP (green) and phase contrast (PC).

5 Discussion

5.1 Expression and regulation of necdin in microglial cells in vitro

For the past few decades, numerous studies have been conducted on the role of microglial cells in neurodegenerative disorders. Yet, the role of microglia in the pathological brain remains controversial. Furthermore, distinct contribution of microglia to the pathophysiological cascades triggered by the ischemic brain is masked by the infiltration of blood-borne leukocytes predominantly during the breakdown of BBB and the unavailability of markers to discriminate these subpopulations.

Thus, the present work demonstrated the novel finding that necdin was expressed in resident microglial cells, and its expression was upregulated upon ischemic insults. There are three main parts of the discussion. Part one consists of in vitro data on the expression and regulation of necdin in microglial cultures. Furthermore, we demonstrated a posttranslational modification of necdin protein in microglial cells in vitro. This is followed by part two where in vitro findings (from part one) were corroborated by vivo data on necdin expression and regulation in MCAo model. In part three, we addressed the question if necdin was preferentially expressed in subpopulations of microglial cells.

5.1.1 Necdin expression levels increase in microglial cells following oxygen-glucose deprivation (OGD)

The loss of necdin due to genetic defect is implicated in the Prader-Willi syndrome (78). Thus, necdin plays a pivotal role during the developmental phase of the CNS. Moreover, necdin is a multifunctional neuronal protein that promotes cell survival, differentiation and cell cycle arrest. In particular, this protein is involved in the regulation of hematopoietic stem cells during hematopoietic regeneration (95).

The expression and function of necdin have previously been described in different cell types, i.e. neurons, muscle cells and adipocytes.

As for the CNS, necdin has only been described in neurons. Thus, we tested whether necdin was also expressed in other cell types of the CNS, i.e. glial cells, and whether it was regulated under ischemic conditions. Our immunocytochemistry and western blotting analysis in primary microglial cells demonstrated a novel expression of necdin. These observations were in line with a study showing the involvement of necdin in hematopoietic stem cells (95), since the origin of microglia is of the myeloid lineage (50). Additionally, besides the fact that necdin was expressed in isolated microglia cultures at baseline conditions, this protein was also induced in an ischemic-like stress in vitro model, i.e. OGD. Consistently, necdin was previously reported to have physical interaction with, as well as biological effect on hypoxia-inducible factor-1 alpha (HIF-1α) (127, 128). Moon et al demonstrated that necdin reduces the transcriptional activity and decreases HIF-1α protein levels (127). HIF-1 is a transcription factor regulated under hypoxic conditions (129). During hypoxia as in brain ischemia model, this protein stabilizes and promotes neovascularization (130). Furthermore, exposure to hypoxia induces microglia migration which is abrogated by blockade of HIF-1α (131), implying the novel role of necdin in negatively regulating microglia migration/proliferation via a reduction of HIF1-α under ischemic conditions.

Necdin is initially known as a nuclear protein that is expressed in differentiated neurons (84). However, it is now clear that necdin has multiple roles and interacts diversely with nuclear (96, 128, 132), cytoplasmic (89, 133, 134) and plasma membrane (135, 136) proteins. By using large-scale interaction screen analysis together with the combined network established from previously published interactions, the diverse role of necdin was further confirmed as a cytoplasmic adaptor, as well as a nuclear factor (137). In addition, Lavi-Itzkovitz and colleagues suggested that the subcellular localization of necdin protein is modulated via the utilization of multiple karyopherin-dependent pathways, and demonstrated that nuclear exclusion of necdin induces cell death (137). In line with the literature, protein fractionation on microglial cells revealed the presence of necdin in both cytoplasmic and nuclear subcellular localization. Furthermore, there was an increase of necdin in both cytoplasmic and nuclear fractions at 24 hours after OGD in comparison to baseline control and earlier time point. Although the functional outcome on necdin subcellular localization remains to be elucidated, the expression and increment of necdin in both subcellular fractions suggested that this protein was regulated upon ischemia and may have a functional role.

Cellular division and proliferation on the mixed glial cultures after OGD exposure was validated by upregulation of the phosphorylation of Ser10 of Histone H3 (p-Histone H3), which correlated with chromosome condensation during mitosis or meiosis (138-140).

5.1.2 Necdin is expressed in resident microglial but not in peritoneal macrophages and following OGD subjection

Both resident microglia and peripheral macrophages originate from myeloid lineage (50). Thus, after having shown that necdin was regulated in isolated primary microglial cells, we next investigated the expression of necdin in the blood-borne macrophages. Due to technical limitations in culturing blood-borne macrophages that infiltrate the ischemic hemispheres, we analyzed isolated peritoneal macrophages as an in vitro model. The limitation of using peritoneal macrophages is that these cells were not exactly similar to circulating monocytes in the blood stream. Peritoneal macrophages are monocytes recruited into tissues and differentiate in the absence of inflammation, whereas blood-borne monocytes were recruited into tissues triggered by inflammation (141)**.** Nevertheless, peritoneal macropahges are the best in vitro model in exploring the role of infiltrating macrophages in the ischemic brains, since these cells can be cultured for further subjection to ischemic-like injury paradigm. Besides, the molecular relationship between blood-borne and peritoneal macrophages is close (142).

Our data demonstrated a significantly higher transcriptional level of necdin in primary microglia in comparison to peritoneal macrophages. These observations were in consistence with the absence of necdin proteins in peritoneal macrophages at baseline, as well as after exposure to OGD. To date, there is no endogenous marker available in distinguishing resident microglia from blood-borne macrophages that infiltrates the brain during pathophysiological conditions. Thus, necdin may be a potential endogenous marker in diffentiating the infiltrating blood-borne macrophages from the resident microglia in the CNS.

5.1.3 Necdin mediates growth arrest in primary microglia in vitro

Several studies showed that cerebral ischemia elicits microglia activation and resulted in the accumulation of microglia at the site of injury from local proliferation and migration (73, 143). After demonstrating the potential role of necdin in regulating microglial cells, we determined the impact of this protein in microglia via a loss of function approach using lentiviral particles targeting necdin by microRNA expression. Mixed glial cultures were used in this study as microglial cells proliferate better in the presence of astrocytic layer. Additionally, the expression of necdin was restricted to $Iba1^+$ cells and not in $GFAP^+$ cells. Therefore, they were suitable to be utilized to test the impact of necdin specifically in microglial cells in mixed glial cultures.

We observed that these $necdin^+/Iba1^+$ cells adopted an 'ameboid' phenotype and found loosely attached on top of the astrocytic layers. These observations were in agreement with our data showing necdin expression in isolated microglia. Of note, the in vitro activation status of these microglia was not reflecting the resting type but rather a reactive type of microglia due to their reconstitution away from its microenviroment (49).

We next evaluated a role of necdin expression on the survival and proliferation of the glial culture after OGD subjection. Cell count of $Iba1^+$ and EdU incorporated cells suggested that necdin regulates negatively on $Iba1^+$ cell proliferation before and after exposure to ischemic-like stress, i.e. OGD, respectively. In addition, a negligible cell loss between the control and necdin microRNA-transduced cultures were determined, therefore we confirmed that the higher cell count was not due to lower survival rate of the cultures. Taken together, these data suggest a novel role of the MAGE family member necdin in suppression of microglial cell proliferation in vitro at baseline, as well as after OGD subjection. Our in vitro observations were in accordance with previous studies demonstrating necdin's role as a cell cycle arrest in NIH3T3 and SAOS2 cell lines (92, 93), as well as in white adipocyte progenitor (144) and hematopeictic stem cells (95). Furthermore, necdin is able to interact with nuclear transcription factors that regulate cell cycle progression i.e. p53 and E2F1, and thereby repressing their transcriptional activities (93, 96).

5.1.4 Putative posttranslational modification of necdin by NEDD8 and SUMO2/3

We have identified an immunoreactive band at a higher molecular weight (~10 kDa) than native necdin. Additionally, this protein band was downregulated by necdin microRNA. These observations indicated a high-molecular weight modification involving addition of other peptides/proteins (e.g., SUMOylation, NEDDylation, ubiquitination and ISGylation) to necdin. We ruled out ISG15 which has a 17 kDa molecular weight and focused on NEDDylation (8 kDa) and SUMOylation (~12 kDa), which have molecular weights similar to the shift in size between the putative posttranslationally modified necdin protein and the native protein. Futhermore, necdin contains predictive sites for SUMOylation and NEDDylation. This was further confirmed by confocal microscopy showing co-localization of necdin with both SUMO2/3 and NEDD8, respectively. We showed for the first time that SUMO2/3 and NEDD8 were expressed in microglial cells.

In agreement, both posttranslational modifiers have been implicated in ischemic models in vitro. For instances, SUMO2/3 upregulation is implicated in ischemia-like stress (111), and NEDD8 stabilizes HIF-1α in normoxia and further increased its level in hypoxia (145). Both posttranslational modifiers were reported to be increased in ischemic-like stress or through the conjugation with substrates, which coincide with our western blotting analysis showing a slower kinetic in the microRNA-mediated necdin downregulation of the posttranslationally modified band. Thus, the posttranslationally modified necdin may have a longer protein half-life.

5.2 Expression and regulation of necdin in microglial cells in the ischemic brain in vivo

Microglial culture is an important in vitro model for exploring the diverse aspect of microglial cell biology. However, the pitfall of this model is that the removal of these microglial cells from their native microenvironment leads to inherent changes in their phenotype (49). Thus, in vitro findings may be insightful but are neither entirely reflecting the in vivo situation nor easily translated into in vivo settings.

The following studies were aimed to corroborate our in vitro findings on the expression and regulation of necdin in microglial cells in the context of cerebral ischemia in vivo.

As aforementioned, mild and severe MCAo are widely used depending on the question to be addressed, as the duration of the occlusion will determine the infarct area it encompasses. Throughout the whole study, we used the severe 60 min transient MCAo model, whereby the infarct area stretches to the cortical layers (5).

5.2.1 Necdin expression levels at different reperfusion intervals after MCAo

Having shown that necdin is expressed in primary microglial cells in vitro, we next investigated necdin expression in the murine brain in vivo, as well as determining the kinetic of necdin expression subjected to transient MCAo. The 60 min MCAo model consists mainly of necrotic cell death in the infarct core area (7). Under these conditions, the evolution of injury is predominated by a failure of the cerebral energy state.

We proceeded with a 60 min MCAo mice model to examine the expression and function of necdin. Interestingly, unlike in the microglial culture, where necdin was constitutively expressed at baseline, necdin was only mildly expressed in $CD11b^+$ cell population derived from naive brains, as well as from ischemic hemispheres at early reperfusion time points, i.e. 24 and 48 hours, after MCAo.

In contrast to a later reperfusion time point after MCAo, i.e. 72 hours, necdin expression was robust and restricted to the ischemic hemispheres only. Consistently, Farber et al reported that the number of $Iba1^+$ cells peak at 72 hour reperfusion in a 60 min MCAo model (146), which coincides with the peak of necdin expression in our studies. Thus it is tempting to speculate that necdin may have a role as a negative regulator of the inflammatory $Iba1^+$ cells.

5.2.2 Morphology of necdin positive cells in an ischemic mouse model (MCAo) and necdin expression level at different reperfusion intervals

After having demonstrated that necdin expression was significantly expressed specifically at 72 hours after MCAo, we proceeded to investigate the phenotype of necdin$^+$Iba1$^+$ cells in the ischemic hemispheres. Given that the morphology adopted by resident microglia is highly diverse and is profoundly influenced by its local environment, it is interesting to observe the morphology adopted by Iba1$^+$ cells that express necdin.

In line with our western blotting results where necdin was abundantly expressed at a later time point after MCAo, i.e. 72 hours, frequent numbers of necdin$^+$Iba1$^+$ cells were observed and restricted to the ischemic hemispheres only. Furthermore, virtually all these cells adopted a reactive 'ameboid' phenotype. These observations imply a de novo expression of necdin protein in Iba1$^+$ cells upon ischemic insult, particularly in those reactive 'ramified' microglia/macrophages. Necdin expression was only observed in neurons in the contralateral hemispheres.

Even though necdin was discovered about 2 decades ago, the descriptions of necdin in an in vivo setting only address the pool of neurons in the CNS. Nevertheless, it was clearly detected in microglial cell culture. The reason for this discrepancy may reflect the fact that cultured microglial cells excluded from their microenvironment inside of the brain can only be maintained in their reactive form.

Taken together, we described a previously unknown expression of necdin in resident microglia in the ischemic brain. These studies indicated a role of necdin in anti-inflammation, as necdin was expressed in the ameboid phenotypic-reactive microglia only and thereby restricts proliferative Iba1$^+$ inflammatory cells from overwhelming the brain parenchyma.

5.2.3 Necdin mediates growth arrest in primary microglia in vivo

Albeit demonstrated in cultured primary microglial cells in vitro, we wanted to delineate if necdin plays a role in the modulation of proliferation of $Iba1^+$ cells after brain ischemia in vivo. To test this hypothesis, lentiviral particles coding for control or necdin microRNA were intra-arterially injected at the beginning of reperfusion. Intra-arterial injection resulted in transduced cells restricted to the ischemic territory of the MCA. Necdin microRNA-transduced microglia appeared to proliferate significantly more than the control microRNA-transduced cells in the cortical layers of the ischemic hemispheres

Of note, only roughly half of the pool of proliferating microglia/macrophages was directly induced by microRNA-mediated depletion of necdin, since the other half of the proliferating cells was not transduced with LVPs. These data suggest a role of necdin as a paracrine mediator of cell proliferation. Thus far, necdin is capable of interacting with interleukin-1 (IL-1) α and IL-1ß, whose attributes include regulating cell proliferation (91) and inflammatory response (147). Additionally, IL-1α and IL-1ß are pro-inflammatory cytokines that contribute prominantly to ischemic brain injury. We speculate that necdin mediates cell proliferation via paracrine signaling by interacting and thereby regulating cytokines in the ischemic brain.

Taken together, this result suggests that necdin specific RNA interference impacts on the proliferating fraction of $Iba1^+$ cells after brain ischemia in vivo, which was in consistence with our in vitro data, as well as with the literature (92, 93, 95, 96, 144).

5.2.4 Loss of necdin following focal brain ischemia in vivo

Having demonstrated that necdin microRNA-transduced cells proliferated significantly more than the control microRNA-transduced cells in the ischemic brain, we next determined the effects on the infarct volume of these cells by using MRI .

There was no significant difference in the infarct volume between mice injected with control and necdin microRNA following focal MCAo. Of note, statistical evaluation of this data set demonstrated that it was prone to a type II error (ß=0.83), indicating a high probability (83%) of

a false negative. Furthermore, a repetition of the experiment was quickly abandoned after recalculation of the number of cases needed according to the parameters obtained from this set of experiment (effect size = 28.9%, mean standard deviation = 75.2%, $\alpha = 0.05$, ß = 0.2, Power: 1-ß = 0.8).We ended up with a calculation of 135 cases per group by taking 25% of the mortality rate into account. A preclinical study with such a high number of animals would never have been approved by the animal welfare institutions. A flaw on the experimental paradigm was that it incurred a high variance on the infarct volumes. Thus, we decided to work on improving the experimental setup i.e. by using a cell type-specific intervention strategy. Our rationale for using this ubiquitous promoter in combination with a severe MCAo model was that even though necdin-expressing neurons were affected, neurons in the infarct core region are prone to undergo necrotic cell death. Thus, necdin targeting microRNA should only affect microglial cells invading into this area. Nevertheless, the usage of a ubiquitous promoter was a failed attempt, since unspecific RNA interference approach targeting both neuronal and non-neuronal brain cells might have masked a biological effect of proliferating microglia population. Therefore, it is essential to target microglia specifically, as neurons at the infarct region could be more susceptible to ischemic stroke since necdin has been reported to be neuroprotective (99, 101, 102, 136).

Although conventional necdin knockout mice have been previously used (82, 148, 149), however these transgenic mice do not allow a cell type specific analysis and may suffer from a higher vulnerability for other CNS-cell types in general. Thus, the ideal approach is to use selective RNA interference in specific cell type. Therefore, we generated i) FLEX-cassette consisting of RNA interference targeting necdin, whereby necdin microRNA will be conveniently excised and flipped to allow transcription to take place in the presence of Cre recombinase ii) Cre recombinase driven by Iba1 promoter (Iba1-Cre). The necdin microRNA-FLEX cassette in combination with Iba1-Cre recombinase will allow the expression of necdin microRNA only in $Iba1^+$ cells. These constructs have been successfully generated and established in cell culture models. In the presence of Cre recombinase driven by Iba1 promoter (Iba1-Cre), inverse necdin microRNA was transcribed and visualized by the expression of mCherry fluorescent protein. Furthermore, western blotting analysis demonstated that ectopic necdin was depleted by necdin microRNA and in the presence of Cre recombinase only. Taken together, for future experiments to assess stroke outcome from proliferating microglia, we have established tools for a microglia specific promoter in causing the loss of necdin protein.

5.3 Endogenous necdin expression in a subpopulation of microglia/mcarophages in cerebral ischemia

The breakdown of BBB during brain pathology allows the infiltration of blood-borne macrophages into the local brain parenchyma. Up till now, no immunomarker is available in discriminating resident microglia from peripheral macrophages that enters the brain. Thus the impact of the distinct population that may contribute to the pathogenesis in neurodegenerative disorders remains obscured. Microglia studies that require this discrimination is circumvent by using the bone marrow chimeras, whereby infiltrating leukocytes in the brain parenchyma, including peripheral macrophages will be marked by $EGFP^{+}$.

Additionally, distinct activation status of resident microglia can be distinguished by using BM chimeras in combination with flow cytometry. The ramified microglia were marked by $EGFP^{-}/CD11b^{+}/CD45^{low}$, whereas reactive microglia were $EGFP^{-}/CD11b^{+}/CD45^{high}$. Following studies demonstrated that necdin was preferentially expressed in subpopulations of microglial cells.

5.3.1 Necdin is expressed in resident microglia but not in infiltrating macrophages in MCAo mice model

BM chimeric mice are used in discriminating two cell populations (resident microglia vs. infiltrating blood-borne macrophages) in the brain parenchyma under pathophysiological conditions. However, the limitations of this method is that these mice are irradiated before receiving engraftment from the donor cells expressing EGFP, which inevitably causes artefacts (i.e abnormal proportion of the donor cells in the blood stream and damage of the vessels). Thus, the search of a marker is pivotal in order to determine the impact contributed by distinct cell population towards the CNS pathologies.

After having shown that both transcriptional and translational levels of necdin were in consistence and highly expressed in cultured microglia but not in peritoneal macrophages. These observations suggest a preferential expression of necdin in these two cell populations, which led us to investigate on the necdin expression levels in the in vivo context (i.e. resident

microglia vs. blood-borne macrophages). By using BM chimeric mice generated from EGFP transgenic animals, we observed that necdin expression was restricted to resident microglia only.

Thus, our in vitro and in vivo findings indicated that necdin was preferentially expressed in these two cell subpopulations, which may be a potential marker in discriminating these two cell populations. Since not all $Iba1^+$ cells were $necdin^+$, only a small subpopulation of the resident microglial cells can be distinguished from the infiltrating blood-borne macrophages.

In agreement, preferential necdin expression has been demonstrated in preadipocytes, brown preadipocytes have a higher expression levels than white preadipocytes (150). Additionally, necdin has been shown to affect differentiation of GABAergic neurons but not Glutamatergic neurons (103).

5.3.2 Preferential necdin expression in subset of microglia/macrophages in BM chimeric mice after MCAo subjection

As we have shown that necdin was expressed only by a proportion of the $Iba1^+$ cells, and immunohistochemistry showed that $necdin^+$ cells were restricted to the reactive 'ameboid' phenotype in the ischemic hemispheres. In addition, we have ruled out the infiltrating macrophages to be immunoreactive to necdin. Thus we inferred that $necdin^+$ cells were resident microglia which was in their reactive state in this particular time point after MCAo, i.e. 72 hours. To confirm this hypothesis, FACS sorting of leukocyte in the ischemic hemispheres of BM chimera were used to distinguish ramified phenotypic microglia ($CD11b^+/CD45^{dim}$) from the reactive microglia ($CD11b^+/CD45^{high}$). In line with our hypothesis, necdin mRNA and protein levels were in consistence and detected in reactive microglial cell population ($CD11b^+/CD45^{high}/EGFP^-$) only, whereas necdin protein levels were not observed in resting state 'ramified' microglia ($CD11b^+/CD45^{dim}/EGFP^+$) and granulocytes or infiltrating macrophages ($CD11b^+/CD45^{high}/EGFP^+$) populations. The role of resident microglia has mainly been attributed to morphologically reactive microglia (61). Recently, Vinet and coworkers showed that ramified microglia are neuroprotective in hippocampal excitotoxicity (151). However, no immunomarker has been described to distinguish reactive from resting/surveillance state microglia, except by using FACS to sort for CD45 and CD11b. Our

findings demonstrated that necdin^{+} cells represent a subpopulation of resident microglia which belongs to the reactive microglial subtype.

In summary, we identified a previously unknown expression of necdin in resident microglial cells in the CNS and report that necdin played a role in the proliferation of reactive resident microglia following cerebral ischemic conditions. Modulation of microglia proliferation might have an impact on long-term stroke outcome, although it is still under debate whether reactive microglia are beneficial or detrimental in the pathophysiology of brain ischemia. Thus, necdin may be used as an endogenous modulator in regulating the number of microglial cells or might interfere with their phenotypic characteristics in the brain and thereby regulate stroke outcome. In addition, future studies might identify endogenous microRNA profiles which impact on the translation of necdin mRNA and regulate the expression of necdin. This might impact on the differentiation of certain subpopulations of microglia during resolution of the inflammatory response after brain ischemia.

6 Conclusion

The most significant findings from this doctoral thesis are concluded as following:

1 Necdin, a neuronal protein was identified in resident microglia cells in vitro and was upregulated upon ischemic-like injury such as OGD. Furthermore, a putative posttranslationally modified necdin band at higher molecular weight was identified in microglial cells. By using RNA interference targeting necdin, a significantly higher number of proliferating $Iba1^+$ cells was observed. These in vitro data suggested that necdin is a negative modulator in microglia proliferation in ischemic-like injury in vitro.

2 The morphology of microglia is highly diverse, and their changes correlate with pathological conditions. We observed that $Iba1^+$ cells that are immunoreactive to necdin exhibited an ameboid phenotype reflecting the reactive state of microglia cells in cerebral ischemia. In contrast to microglial cells in vitro in which necdin was constitutively expressed, cerebral ischemia rendered de novo expression of necdin which peaks specifically in $CD11b^+$ cells at 72 hours. In line with in vitro studies, the loss of necdin in the context of ischemic injury triggers significantly higher proliferation of $Iba1^+$ cells in vivo. However, stroke volumes were not altered with higher cell number of proliferative $Iba1^+$ cells. To further address this question, contructs that allow selective expression of RNA interferance in the cell culture model i.e. a FLEX system in combination with Iba1 promoter driven Cre recombinase were established.

3 In contrast to isolated microglial cells, peritoneal macrophages did not express necdin. By using BM chimeric mice, whereby blood-borne monocytes which infiltrate the brain (marked by $EGFP^+$ cells) were distinguishable from resident microglia after cerebral ischemia, demonstrated that necdin was only expressed by resident microglia in vivo. Furthermore, necdin expression was expressed by a subpopulation of resident microglial cells, which are the $CD11b^+/CD45^{high}$ expressing cells (reactive microglia) in the context of cerebral ischemia. Taken together, necdin may be used as a marker for the identification of the subpopulation of reactive microglial cells in the brain.

Experiments on Human Beings

There were no experiments performed on human beings or human samples within this project.

Permission for Cell Culture and Animal Experiments

All cell culture experiments have been approved by LAGeSo (Landesamt für Gesundheit und Soziales) on TVA T0046/07 (Tötung von Wirbeltieren zu wissenschaftlichen Zwecken). An official authorization for all in vivo experiments was on TVA G0385/08.

Genetic Engineering Experiments

All lentiviral experiments have been approved by the LAGeSo on TVA G441/06, approval date: 30th November 2006, Project leader: Prof. Dr. Christoph Harms.

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Curriculum Vitae

Sabrina Lin Lin Lee

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

8 Erklärung

„Ich, Sabrina Lin Lin Lee, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Necdin is a novel modulator in microglia proliferation in the murine brain following cerebral ischemia“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den 14 Juni 2012

Sabrina Lin Lin Lee

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