The role of the complement system in ischemic stroke and neural plasticity

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- crystal structures of complement C3 and C3a - retrieved from RSCB.org (PDB ID: 2A73) and protepedia.org (ID: 4i6o), respectively
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- neuroblasts (red) migrating towards the infarct site – image by *Anna Stokowska*
- dendritic spines of a hippocampal neuron (green) - image by *Marta Perez Alcazar*

The role of the complement system in ischemic stroke and neural plasticity
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To my family

ABSTRACT

Evidence from experimental animal studies suggests that complement activation in the brain is a “double-edged sword” as it exerts beneficial or detrimental effects depending on the context. Here, we assessed whether complement activation in the systemic circulation could be a predictive biomarker of functional outcome after stroke. Further, we studied the role of the complement system in brain plasticity and recovery after ischemic stroke.

We found that acute and delayed phase plasma levels of C3 and C3a differ substantially among patients suffering from ischemic stroke of different etiology, and the association of plasma C3 and C3a levels with case/control status and with functional outcome is ischemic stroke subtype-dependent. In large vessel disease and cardioembolic stroke patients, C3 levels at 3-month follow up were associated with an unfavorable functional outcome at both 3 months and 2 years after stroke. However, in cardioembolic stroke patients moderate increase in plasma C3a/C3 ratio predicted favorable outcome after 2 years (Paper I and II). Furthermore, two single nucleotide polymorphisms (SNPs) in the *C3* gene were found to be associated with ischemic stroke independently of traditional risk factors and one of these SNPs was associated with cryptogenic stroke (Paper III). Also, two SNPs were associated with plasma C3a or C3 levels independently of age, sex and case/control status. Taken together, the role of the complement system in ischemic stroke is strongly dependent on stroke etiology.

We have also found that C3a overexpression in mice increased, whereas C3a receptor (C3aR) deficiency decreased the number of post-stroke-born neurons in the peri-infarct cortex without affecting the infarct size. Furthermore, the density of pre-synaptic puncta and GAP43-positive axonal growth cones in the cortex surrounding the infarct were lower in the C3aR-deficient compared to control mice, while in the C3a-overexpressing mice post-stroke axonal plasticity response was increased. Mice lacking C3aR showed a more pronounced sensorimotor functional deficit as assessed by behavioral testing (Paper IV). These results indicate that C3aR signaling should be considered as a target when designing therapeutic strategies to improve functional recovery after ischemic stroke.

To study complement-related neural plasticity in a non-pathological context, we performed electrophysiological recordings in the CA1 region of live hippocampal slices of young mice lacking C3 and control mice. We found that the C3-deficient mice had a decreased neurotransmitter release probability but dendritic spine density, and frequency and amplitude of miniature excitatory postsynaptic potentials were comparable in both groups of mice. Behavioral testing using the IntelliCage platform revealed that the C3-deficient mice performed better in the place and reversal learning tasks (Paper V). These findings may have implications for the management of disorders involving synapse elimination, such as Alzheimer’s diseases, autism or multiple sclerosis.

Keywords: ischemic stroke, complement system, neurogenesis, synaptic plasticity, hippocampus, learning and memory, functional outcome

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Stroke, eller slaganfall, är den ledande orsaken till handikapp hos vuxna i västvärlden och leder oftast till ett permanent assistansberoende. Ischemisk stroke, hjärninfarkt, är den vanligaste formen av slaganfall och orsakas av en blodpropp som förhindrar blodflöde till en del av hjärna. Som följd, på grund av syre- och näringsbrist, dör nervceller i det påverkade området. Detta leder också till inflammation och ytterligare skador på hjärnan. Idag är den enda behandlingen för stroke en upplösning av blodproppen genom en intravenös injektion av ett enzym som kallas tPA. Ett problem är dock att få patienter kvalificerar sig för denna behandling, då den bara är effektiv och relativt säker under en kort tidsperiod (4.5 timmar) från det att symptomen har börjat. För att kunna hjälpa fler patienter är det därför viktigt att utveckla nya behandlingar som kan användas i samband med en långsiktig rehabilitering.

Hjärnan har en begränsad förmåga att läka de områden som skadats svårt vid stroken. Dock har hjärnan har en fantastisk förmåga att anpassa sig till den nya situationen genom att skapa nya cellkontakter, synapser, och genom att ändra egenskaperna hos de fungerande cellkopplingarna så att de kan ta över funktioner som de förstörda kopplingar tidigare utförde. Detta kallas för ”neuroplasticitet”. Detta begrepp inkluderar även andra omformande processer i den friska hjärnan, t.ex. de som sker vid inlärning.

Immunförsvaret skyddar vår kropp från de skadliga effekterna av mikroorganismer (bakterier, virus m.m.) och förändrade celler som annars kan omvandlas till cancerceller. Komplementsystmet är en viktig del av det ospecifika immunförsvaret och består av mer än 30 proteiner (äggviteämnen) som verkar i kaskadform inne i blodet och andra kroppsvätskor och vävnader. Komplementsystmet är också involverat i de inflammatoriska reaktioner som sker i hjärnan efter en stroke. Dessa inflammatoriska reaktioner, om de blir okontrollerade,tror man är en bidragande faktor till att hjärnskadorna kan öka efter en stroke.

Levern är den huvudsakliga källan av komplementproteiner men överraskande nog har det visat sig att även hjärnans celler producerar komplementproteiner. Detta pekade på någon annan icke-immunologisk roll för komplementsystemet i hjärnan.

Eftersom det inte finns många studier kring de långsiktiga effekterna av komplementsystemets inverkan efter stroke, försökte vi förstå komplement-systemsreaktion hos strokepatienter. För att studera komplementsystemets roll i neuroplasticitet jämförde vi möss med förändrat komplementsystemet med normala möss och analyserade förändringar i deras hjärnor under normal utveckling och efter stroke.

Våra kliniska studier (på människor) visade att komplementnivåerna i blodet är förhöjd till olika grad i de olika ischemiska subtyperna av slaganfall efter stroke. Denna förhöjning var karaktäristisk för strokepatienterna och berodde inte på de traditionella riskfaktorerna (ålder, kön, hög blodtryck, sockersjuka, rökning och höga blodfettnivåer). Viktig nog höga blodnivåer av komplementproteinet C3 tre månader efter stroke korrelerade medmed högre grad av handikapp, men bara hos patienter med visa subtyper av stroke. Dock verkade måttlig aktivering av komplementsystemet (C3a/C3 kvot) ha ett något positiv effekt eftersom den associerades med mindre grad av handikapp efter

slaganfallet för patienter med kardioembolisk stroke (stroke orsakades av en vandrade blodpropp som formats i hjärtan). Vi fann även ett samband mellan några genetiska varianter av C3-genen och förekomsten av stroke, särskilt kryptogen stroke, den vars orsak inte kan identifieras trots extensiv utredning. Då rätt prognos kan underlätta specialanpassning av patientens rehabilitering, samt andra behandlingar, och bidra till en bättre återhämtning, så kan mätning av komplementsystemskomponenter i blodet vara ett bra diagnostiskt verktyg, åtminstone i vissa typer av ischemisk stroke.

I våra experimentella strokestudier har vi upptäckt att genetiskt förändrade möss som producerar komplementsystemspeptiden C3a i hjärnan i samband med ischemisk stroke har fler nyfödda nervceller och fler växande nervutskott i området runt den skadade regionen av hjärnan. I motsats till detta så har möss som saknar receptor, mottagare, för C3a färre nya nervceller och färre och mindre växande nervutskott runt strokeområdet.Dessa möss har också större funktionsnedsättning. Tillsammans pekar detta mot att C3a är viktigt för olika typer av neuroplastiska mekanismer som är involverade i återhämtning efter stroke.

För att fördjupa vår förståelse av komplementsystemets roll i hjärnan har vi också studerat neurologiska funktioner i hippocampus - en del av hjärnan som är viktig för inlärning och minne. Våra studier på möss visar att komplementsystemet spelar roll vid en typ av neuroplasticitet som är viktig för normala funktioner av hjärnan. Med hjälpen av elektrofysiologiska metoder fann vi att unga möss, som saknar det viktigaste komplementsystemsproteinet C3 har ökad synaptisk funktion i hippocampus. Detta beror troligen på att de har fler synapser i hippocampus. Som följd av detta, är dessa möss bättre på att lära sigatt utföra spatialminnesberoende uppgifter. Dessa resultat kan vara av betydelse för behandling av nervskadesjukdomar som orsakas av synapsförlust såsom Alzheimers sjukdom, autism och multipel skleros (MS).

Sammanfattningsvis visar våra resultat att komplementsystemet är en viktig vid stroke och att det är involverat både vid skadliga inflammationsprocesser och reparationsprocesser. Dessutom påverkar komplementsystemet även plasticiteten hos en frisk hjärna. Detta styrker åsikten att denna del av immunsystemet även är involverat i processer som inte är immunförsvarsrelaterade.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Stokowska, A**, Olsson, S, Holmegaard, L, Jood, K, Blomstrand, C, Jern, C, Pekna, M. Plasma C3 and C3a levels in cryptogenic and large vessel disease stroke: associations with outcome.
Cerebrovasc. Dis. 2011; 32:114-122

II. **Stokowska, A**, Olsson, S, Holmegaard, L, Jood, K, Blomstrand, C, Jern, C, Pekna, M. Cardioembolic and small vessel disease stroke show differences in associations between systemic C3 levels and outcome.
PLoS One. 2013; 8(8): e72133.

III. Olsson, S, **Stokowska, A**, Holmegaard, L, Jood, K, Blomstrand, C, Jern, C, Pekna, M. Genetic variation in complement component C3 shows associations with ischemic stroke.
Eur. J. Neurol. 2011; 18: 1272-1274.

IV. **Stokowska, A**, Atkins, AL, Barnum, SR, Wetsel, RA, Dragunow, M, Pekna, M. Receptor for complement peptide C3a stimulates neural plasticity after experimental brain ischemia.
Manuscript

V. Perez-Alcazar, M, Daborg, J, **Stokowska, A**, Wasling, P, Björefeldt, A, Kalm, M, Zetterberg, H, Carlström, K, Blomgren, K, Clementson Ekdahl, C, Hanse, E, Pekna, M. Altered cognitive performance and synaptic function in the hippocampus of mice lacking C3.
Manuscript submitted to *Hippocampus* 2013

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood brain barrier
bFGF	basic fibroblast growth factor
BNDF	brain-derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
CE	cardioembolism
CNS	central nervous system
CRP	C-reactive protein
CR1-4	complement receptors 1-4
CST	corticospinal tract
C3aR	C3a receptor
C5aR	C5a receptor
C5L2	C5a-like receptor 2
DAF	decay accelerating factor
DG	dentate gyrus
ELISA	enzyme-linked immunosorbent assay
EPSP	excitatory postsynaptic potential
EPSC	excitatory postsynaptic current
GABA	γ-amminobutyric acid
GAP-43	growth-associated protein 43
GAT	GABA transporter
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
GLT-1	glilal glutamate transporter 1
GPCR	G-protein-coupled receptor
IFNγ	interferon γ
IGF-1	insulin-like growth factor 1
IL	interleukine
i.p.	intraperitoneally
KO	knock-out
LPS	lipopolysaccharide

LTP	long-term potentiation
LVD	large vessel disease
MAC	membrane attack complex
MASP	MBL-associated serine proteases
MBL	mannose binding lectin
MCAo	middle cerebral artery occlusion
MCP	membrane co-factor protein
mGluR	metabotropic glutamate receptor
mRS	modified Rankin Scale
NGF	nerve growth factor
NSCs	neural stem cells
NSPCs	neural stem/progenitors cells
NMDA	N-methyl-D-aspartate
NT-3	neurotrophin-3
OB	olfactory bulb
OR	odds ratio
PBS	phosphate buffered saline
ROS	reactive oxygen species
RT	room temperature
SAHLSIS	the Sahlgrenska Academy Study on Ischemic Stroke
SGZ	subgranular zone
SNP	single nucleotide polymorphism
SSS	Scandinavian stroke scale
SVD	small vessel disease
SVZ	subventricular zone
TNFα	tumor necrosis factor α
TOAST	Trial of Org 10172 in Acute Stroke Treatment
VEGF	vascular endothelial growth factor
WT	wild type

BACKGROUND

STROKE

Clinical background

Stroke is currently the second most common cause of death and a leading cause of disability in adults worldwide (WHO, 2010). In Sweden more than 30 000 cases are diagnosed each year. Stroke is defined as a condition with sudden neurological symptoms of greater than 24 hours duration, occurring due to the interruption of blood supply to the brain and the subsequent shortage of oxygen and nutrients. If this situation is prolonged, it leads to metabolic breakdown, accumulation of toxic products, and brain cell death (infarction). The lack of blood supply can be caused by the obstruction of a blood vessel (ischemic stroke) or its rupture (hemorrhagic stroke). These two situations can also occur one after another in the condition termed hemorrhagic transformation of ischemic stroke. Ischemic stroke is the most common form of stroke and constitutes about 85% of all strokes, while hemorrhagic stroke accounts for about 15% (Donnan et al., 2008). Artery occlusion in ischemic stroke is most often due to a thrombus formation. The source of the thrombus may be local, when it is formed at the site of the occlusion, or remote, such as the heart or the surface of atherosclerotic plaque in large artery. In the latter case, the circulating clot is referred to as an embolus.

Currently, the only available treatments for ischemic stroke patients are early thrombolysis or thrombectomy. Intravenous administration of recombinant tissue plasminogen activator (rtPA) within 4.5 hrs from the ischemia onset has been found successful in improving the outcome of eligible stroke patients (Cronin, 2010). The rtPA works by converting plasminogen to plasmin that in turn degrades fibrin, resulting in clot dissolution. Unfortunately, this therapy is available only for a limited group of patients due to the narrow time window for administration (which is often missed). Age and symptom severity limits as well as restrictions on co-morbidities are additional reasons for exclusion. Therefore, large efforts are currently being made to develop therapeutic strategies that would be applicable in the later stage after ischemic stroke and could promote recovery of function. Such strategies include adjuvant therapies which stimulate neural plasticity, especially when applied in conjunction with relevant neurorehabilitation (reviewed in Pekna et al., 2012).

Etiological subtypes of ischemic stroke

The clinical presentation of ischemic stroke may differ depending on the location and size of the infarction, factors which also influence the prognosis. These differences are determined to a large degree by the heterogeneity of underlying pathophysiology, which forms the basis for etiological classification of ischemic stroke. The most commonly used classification criteria are derived from Trial of Org. 10172 in Acute Stroke Treatment and define the following subtypes: large vessel disease (LVD), small vessel disease (SVD), cardioembolism (CE), other determined cause of stroke and stroke of undetermined etiology (Adams et al., 1993). As for the purpose of this thesis the stratification of patients according to the etiology is of interest, a short characterization of the ischemic stroke subtypes is given below (summarized in Table 1).

Ischemic stroke due to large vessel disease (LVD)

LVD is the cause of around 15-20% of ischemic strokes although the proportion in the population may vary depending on age, sex and ethnicity (Kirshner, 2009). This type of stroke is diagnosed by identifying a significant stenosis or occlusion of a large or medium-sized pre-cerebral or cerebral artery due to atherosclerosis. As atherosclerotic plaques predominantly develop near the branching point of arteries, artery-to-artery embolization is the most common cause of ischemic stroke in this patients group. In terms of diagnosis, the mere presence of plaques is not sufficient for assigning LVD pathology as a cause of stroke and other clinical findings need to be consistent with the location of the atherosclerotic lesion, while a cardiac source of embolus needs to be excluded (Rovira et al., 2005).

Ischemic stroke due to small vessel disease (SVD)

SVD constitutes around 25% of all ischemic stroke causes. It manifests itself as the occlusion of end-arteries supplying deep brain structures such as basal ganglia, thalamus, brain stem and deep white matter, resulting usually in a small infarct (<15 mm on magnetic resonance image). Although SVD pathogenesis is not entirely clear, microatheroma and lipohyalinosis have been found to be associated with this type of ischemic lesions (de Jong et al., 2002). Clinically, SVD strokes present themselves as a

so called "lacunar syndrome", characterized by the absence of cortical symptoms or visual field deficits and include pure motor stroke, pure sensory stroke, ataxic hemiparesis or somatosensory stroke (Bamford et al., 1987). Other conditions that could also cause occlusion of a small brain vessel such as vasculitis, hematological diseases or embolism (from heart or large extracranial artery) need to be excluded for correct diagnosis (Arboix and Marti-Vilalta, 2004).

Ischemic stroke due to cardioembolism (CE)

About 25% of all ischemic strokes are caused by emboli originating from the heart. Infarcts in this stroke subtype are fairly large thus causing severe disabilities and the ischemic events are prone to recurrence. Atrial fibrillation is the major contributor to cardiac embolus formation by leading to atrial stasis that is associated with increased prothrombotic state (Rovira et al., 2005). Other major risk factors for cardioembolism include recent myocardial infarction, ventricular thrombosis, prosthetic valve endocarditis and patent *foramen ovale* although that latter cause is highly controversial (Ferro, 2003; Freeman and Aguilar, 2011).

Ischemic stroke of undetermined etiology

This category can be subdivided into two subcategories. If despite extensive investigation the cause of ischemic stroke remains undefined the stroke is classified as cryptogenic. This subtype constitutes about 30% of all ischemic strokes, however this number varies depending on the extent of investigation. Patients presenting with cryptogenic stroke are typically younger as compared to other etiologic subtypes. It has been suggested that this stroke subtype is in itself heterogeneous (Guercini et al., 2008; Jickling et al., 2012).

In some cases, more than one possible cause is identified or the investigation is cursory and then the stroke is classified as ischemic stroke due to an undetermined cause.

Stroke of other determined etiology

In addition to the major classes of ischemic stroke, other cause of ischemic stroke may be identified. The rare determined causes are: arterial dissection, vasculitis,

hypercoagulable states, hematological disorders or rare monogenic diseases (Levine, 2005; Ballabio et al., 2007; Ferro et al., 2010).

Features	LVD stroke	SVD stroke	CE stroke	Other cause	Cryptogenic stroke
Clinical					
Cortical or cerebellar dysfunction	+	-	+	+/-	+/-
Lacunar syndrome	-	+	-	+/-	+/-
Imaging					
Cortical, cerebellar, brainstem, or subcortical infarct >15 mm	+	-	+	+/-	+/-
Subcortical or brainstem infarct <15 mm	-	+	-	+/-	-
Tests					
Stenosis of an appropriate extracranial or intracranial artery	+	-	-	-	-
Cardiac source of emboli	-	-	+	-	-
Other abnormality on tests	-	-	-	+	-

Table 1. Features of TOAST classification of ischemic stroke subtypes. Adapted from Adams et al. (1993).

Risk factors for ischemic stroke

Several risk factors have been identified to be associated with ischemic stroke and they can be divided into non-modifiable and modifiable. The most important non-modifiable risk factors are old age, male sex, ethnicity, and family history of stroke, whereas some modifiable factors are hypertension, atrial fibrillation, diabetes mellitus, smoking, alcohol consumption, obesity and physical inactivity (Kirshner, 2009; Kokubo, 2012). In the recent years, factors such as chronic stress, increase in blood inflammatory and hemostatic markers as well as genetic polymorphism have been shown to be associated with increased risk of ischemic stroke, although causal role of these factors

remains to be determined (Hankey, 2006). Due to the already mentioned heterogeneity of pathophysiological mechanisms in ischemic stroke, it is conceivable that different stroke subtypes have different profiles of risk factors.

Pathobiology of ischemic brain damage

The brain requires a constant supply of oxygen and glucose to maintain its normal function, therefore if these demands cannot be met, the cellular “ischemic cascade” is initiated. These events produce an irreversibly damaged ischemic core and a potentially salvageable, hypoperfused adjacent region called penumbra (Hossmann, 1994). Cell death in the core is rapid (occurring within minutes) while the damage in penumbra develops more slowly owing to the collateral blood flow provided by anastomoses within the circle of Willis and leptomeninges (Ringelstein et al., 1992). However, if the normal levels of oxygen and glucose are not restored, tissue in that region will eventually die. The so called “ischemic cascade” consists of events that are secondary to the widespread death of neurons and glial cells and include mainly glutamate toxicity, oxidative stress and inflammation (Figure 1).

Glutamate toxicity

As a consequence of oxygen and glucose deprivation following ischemic stroke, a sharp decline in cellular ATP levels results in a dysfunction of membrane ion pumps. This causes a cellular efflux of K^+ and a consequent influx of Na^+, Ca^{2+}, and water (Martin et al., 1994). Increased intracellular levels of Ca^{2+} lead to depolarization of the affected neuronal membrane and the release of excitatory neurotransmitter glutamate. Glutamate binds to and activates N-methyl-D-aspartate receptors (NMDARs) and metabotropic glutamate receptors (mGluRs) on the neighboring neurons. Accompanying malfunction of glial amino acid transporters leads to failure in the re-uptake of extracellular glutamate and results in even further Ca^{2+} influx to the neurons (Manev et al., 1989). This accumulation of calcium ions leads to the activation of intracellular lipases and proteases causing cell damage and cell death. This series of events is referred to as excitotoxicity and has a critical role in the pathogenesis of ischemic stroke.

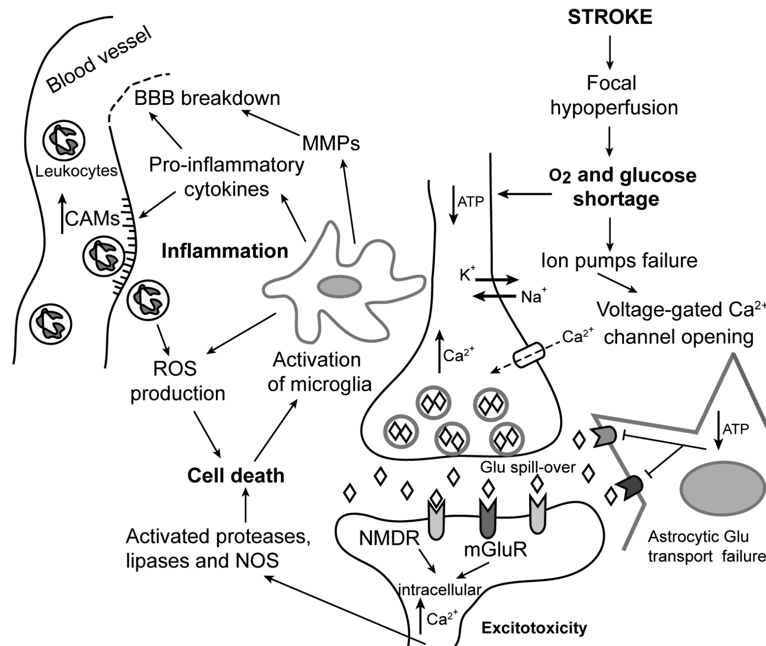

Figure 1. **Schematics of the ischemic cascade.** Due to energy shortage, ion imbalance results in depolarization of presynaptic neurons and uncontrolled release of glutamate which is not recycled by astrocytes, leading to glutamate "spill-over" to the perisynaptic space. Upon binding of glutamate to its receptors, mobilization of calcium ions from the intracellular storage sites occurs in post-synaptic neurons, leading to the release of toxic metabolites. Resulting neuronal cell death activates microglia which, through the release of inflammatory mediators, recruit leukocytes and promote their extravasation. This in turn can exacerbate inflammation and cause secondary tissue damage. Glu –glutamate.

Oxidative stress

Oxidative stress is defined as the condition occurring when the physiological balance between oxidants and antioxidants is disrupted in favor of the former with potential damage for the organism. Oxidative stress leading to ischemic cell death involves the formation of reactive oxygen species (ROS) and reactive nitrogen species through multiple injury mechanisms, such as mitochondrial inhibition, Ca^{2+} overload, reperfusion injury, and inflammation (Coyle and Puttfarcken, 1993). Brain ischemia generates superoxide (O_2^-), which is the primary radical from which hydrogen peroxide, the source of hydroxyl radical, is formed. Ischemia causes an increase in nitric oxide synthase

(NOS) type I and III activity in neurons and vascular endothelium, respectively. At a later stage, elevated NOS type II (iNOS) activity occurs in a range of cells including glia and infiltrating neutrophils (Lakhan et al., 2009). Large numbers of ROS are generated during an acute ischemic stroke and there is considerable evidence that oxidative stress is an important mediator of tissue injury in acute ischemic stroke (Cuzzocrea et al., 2001).

Post-ischemic inflammation

Inflammation is a physiological process that helps the body to eliminate pathogens and activate tissue regeneration. However, when uncontrolled, inflammatory processes can become excessive or chronic and can exacerbate tissue damage, and prevent recovery. Inflammation following cerebral ischemia is characterized by the accumulation of inflammatory cells and mediators in the ischemic brain. The first cells responding to ischemic insult are microglia which are the resident immunocompetent cells of the brain. Once activated, microglia undergo morphological transformation from the resting ramified state to the ameboid one (virtually indistinguishable from blood-derived macrophages) and they migrate to the site of injury to phagocytose apoptotic and necrotic cellular debris (Streit et al., 1999).

Microglia contribute to brain tissue damage by releasing inflammatory mediators such as ROS, proteases and pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor α (TNFα). These cytokines upregulate the expression of cell-adhesion molecules (CAMs) on endothelium of cerebral blood vessels (Wang and Feuerstein, 1995). This in turn promotes the adherence of circulating leukocytes to vessel walls followed by their migration into brain parenchyma with subsequent release of additional pro-inflammatory mediators and secondary injury. Neutrophils are the first white blood cells recruited from the periphery to the ischemic tissue (Stevens et al., 2002). They are followed by monocytes/macrophages and finally lymphocytes, which are believed to contribute to the delayed brain tissue damage mainly through the release of directly neurotoxic interferon γ (IFNγ) (Lambertsen et al., 2004). Oxidative stress and the inflammatory cascade alter the permeability of the blood-brain barrier (BBB). The activation of matrix metalloproteinases (MMPs) and the expression of various other proteases lead to BBB breakdown which exacerbates leukocyte extravasation.

Astrocytes, similar to microglia, are capable of secreting inflammatory factors such as cytokines, chemokines, and nitric oxide (Swanson et al., 2004). Chemokines are a class of cytokines that guide the migration of blood borne inflammatory cells, such as neutrophils and macrophages, towards the source of the chemokine. Consequently, they play important roles in cellular communication and inflammatory cell recruitment (Lakhan et al., 2009). Expression of chemokines such as monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and fractalkine following focal ischemia and the resulting increase in leukocyte infiltration is thought to have a deleterious effect ischemic brain (Kim et al., 1995).

One of the inflammatory mediators which also seems to play a major role after cerebral ischemia is the complement system. The activated complement cascade generates peptides with pro-inflammatory and chemotactic properties which among others functions upregulate the expression of CAMs thus promoting recruitment of inflammatory cells.

THE COMPLEMENT SYSTEM

The complement system was first discovered in the end of 19th century by Jules Bordet as a heat-sensitive factor in fresh serum that "complements" the effect of a specific antibody in the lysis of bacteria and red blood cells. Its importance as an effector of humoral immunity was extended with the early observations of opsonisation and participation in cellular immunity. The complement system is a general term attributed to a constellation of more than 30 soluble plasma and body fluid proteins and a number of cell receptors and control proteins found in the blood and tissues (Janeway et al., 2005). Their roles in innate immunity include the opsonisation and lysis of pathogens, elimination of soluble immune complexes, release of anaphylatoxins, stimulation of leukocytes chemotaxis and release of inflammatory cytokines. Complement affects adaptive immunity by regulating B and T lymphocyte function (Carroll, 2004). Complement activation provides a rapid and effective defense barrier against bacteria, viruses, virus-infected cells, parasites, and tumor cells. The predominant site of peripheral complement protein synthesis is the liver, where hepatocytes constantly produce and replenish circulating complement factors (Alper et al., 1969). Also monocytes and macrophages have been found to produce the majority of complement components especially upon stimulation with pro-inflammatory cytokines (Einstein et al., 1977; Cole et al., 1983). Activation of these circulating complement proteins in response to an injury or an infectious challenge results in a self-amplifying cascade of proteolytic reactions through one of the three major identified pathways, namely the classical, lectin or alternative pathways (Figure 2).

The third complement component (C3)

C3 is the central element of complement system and also the most abundant complement protein in plasma. The physiological concentration of C3 in human plasma is up to 1 mg/ml and increases during inflammatory states as C3 belongs to the acute phase proteins. The main source of C3 in the periphery is the liver, although it is also produced in other tissues. C3 is a large glycoprotein, consisting of α and β chains connected by a disulphide bridge. The α chain contains also an internal thioesther bond, which is hidden inside the inactive C3 molecule. The C3 α chain can be cleaved by a C3

convertase to generate two fragments, the small C3a and the larger C3b, which consists of the remaining part of the parental molecule (Sahu and Lambris, 2001). The cleavage causes a conformational change of C3b leading to the exposure of the thioesther bond that is susceptible to attack by electron donors such as hydroxyl or amine groups (Janssen et al., 2006). This reaction allows for the interaction of C3b with proteins and carbohydrates on the target surface by forming covalent ester and amide bonds. As a free thioesther bond is rapidly inactivated on contact with water, binding of C3b occurs only when it is generated in close proximity of the activating surface (Sahu and Lambris, 2001). C3b binds to the C3 convertase complex forming the C5 convertase (Figure 2). Apart from being a subunit of complement-related convertase, C3b is an opsonin and coats the circulating immune complexes recognized by the C3b receptor (CR3) on erythrocytes for their transport and neutralization in the spleen. C3b binds also directly to foreign or altered cells and thus promotes clearance and neutralization of pathogens apoptotic debris.

C3a participates in the recruitment and activation of leukocytes to produce and release inflammatory mediators and substances such as histamine which mediate the development of allergic reactions and it is therefore regarded as an anaphylatoxin (Klos et al., 2009). Unless it is bound to its receptor, C3aR, C3a is rapidly inactivated by plasma carboxypeptidases through the removal of C-terminal arginine, which results in the formation of $C3a_{desArg}$. This truncated molecule does no longer bind or activate C3aR but it is a ligand for C5a-like receptor 2 (C5L2) (Kalant et al., 2003). $C3a_{desArg}$ is more commonly known under the name acylation stimulating protein (ASP) due to its marked stimulating action on triacylglycerol synthesis in human adipocytes and skin (Cianflone et al., 1989). It has therefore been linked to the pathogenesis of obesity, insulin resistance and metabolic syndrome (Zimmet et al., 1999; Sniderman et al., 2000; Wamba et al., 2008; Mamane et al., 2009).

Activation of the complement cascade

Classical pathway

The classical pathway was the first activation pathway of the complement system to be discovered and it involves complement components C1, C2 and C4. It is initiated by

the C1 complex consisting of recognition protein C1q, which binds primarily to antigen–antibody complexes, a pair of C1r and a pair of C1s molecules. C1q is a homohexamer with subunits consisting of C-terminal globular head domain and N-terminal collagenous tail. At least two out of the six globular heads need to be bound to the immune complexes through the Fc region of type G or M immunoglobulin for the classical pathway to be fully activated (Janeway et al., 2005). This binding leads to conformational change in the C1q molecule and subsequent activation of C1r and C1s molecules. Activation of C1r and C1s, with generation of C1s esterase, is followed by cleavage of C4 and C2. This cleavage releases small peptides and allows the assembly of the C3 convertase, C4bC2a complex (Figure 2).

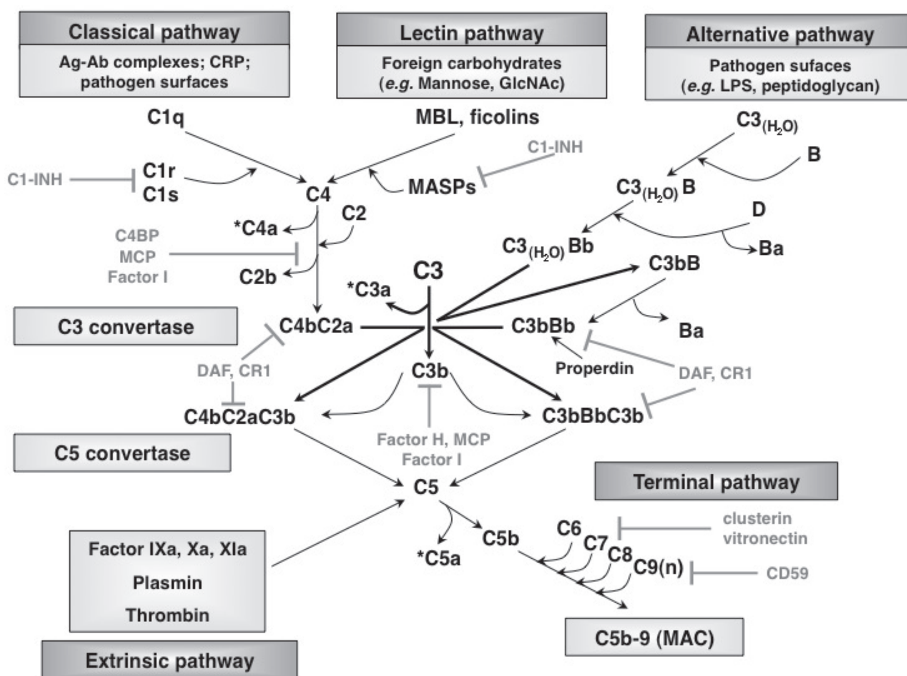

Figure 2. **Activation and regulation of the complement system.** The three activation pathways converge at the level of C3 convertase which together with coagulation factors, activate the terminal pathway. Inhibitors and their interaction points are indicated in gray. Anaphylatoxins are marked with asterisks. Ag-Ab – antigen-antibody complexes; GlcNAc – N-acetyl-glucose, LPS – lipopolysaccharide.

The classical pathway is also activated by antibody-independent mechanisms involving direct binding of C1q to viral envelopes, cell walls of Gram-negative bacteria, C-reactive protein (CRP), intermediate filaments and central nervous system (CNS) myelin (Janeway et al., 2005).

Lectin pathway

The lectin pathway is initiated by the binding of mannose-binding lectin (MBL) and ficolins to carbohydrate groups on the surface of bacterial cells (Fujita et al., 2004). MBL and ficolins are typical pattern-recognition molecules, which serve to attach the MBL-associated serine proteases (MASP) 1, 2, and 3, thus activating MASP esterase activity. Upon activation, MASPs cleave and activate C4 and C2, thus generating the C3 convertase, C4bC2a, similar to the classical pathway (Petersen et al., 2000; Dahl et al., 2001). MBL can also bind to apoptotic cells thus serving as an opsonin (Nauta et al., 2003).

Alternative pathway

The alternative pathway of complement activation is initiated by a constant, low level spontaneous hydrolysis of thioesther bonds in C3 in plasma, leading to the formation of C3(H2O) molecule (Pangburn et al., 1981). C3(H_2O) formation involves conformational "tick-over" of C3, which exposes neo-epitope in a conformation very similar to C3b. Activation of a serine protease, factor D, cleaves factor B into Ba and Bb when factor B is complexed with C3b or C3(H_2O). The C3(H_2O)Bb complexes generate C3b as they cleave circulating C3. However, C3b is short-lived while remaining in the fluid phase. Upon binding to an activating non-self surface, C3b builds a complex with Bb, generating a solid phase C3 convertase of the alternative pathway, C3bBb the stability of which is promoted by a protein called properdin. Binding of additional C3b fragment to the deposited C3-convertase complexes makes up alternative pathway C5-convertase (C3bBbC3b). Spontaneous low-grade activation of the alternative pathway is believed to be a surveillance mechanism which becomes amplified when blood comes in contact with an activating surface. Among the alternative pathway activators are bacterial or microbial fragments, tumor cells, virus envelopes, plastic surfaces, peripheral nerve myelin, and intracellular organelles. These activators act by protecting the C3 convertase from

inactivation by complement regulators that cause C3b cleavage and Bb decay (Xu et al., 2001).

Terminal pathway

As already mentioned, binding of an additional C3b fragment to the C3 convertase brings about C5 convertase properties to the complex and allows for cleaving parental C5 protein to C5a and C5b fragments. In the recent years, an alternative mechanism for complement activation that bypasses the convertase stages has been identified. It involves direct cleavage of C3 and C5 mainly by coagulation cascade proteases (factors IXa, Xa and XIa, thrombin and plasmin) and is part of the so called extrinsic pathway (Huber-Lang et al., 2006; Amara et al., 2010). C5a is the most potent anaphylatoxin in the body while C5b initiates formation of the membrane attack complex (MAC) on the activated surface. C5b remains attached to the convertase complex but allows for the binding of components C6 and C7. Upon attachment of C8 to the C5bC6C7 complex, insertion into the target membrane is possible due to the exposition of hydrophobic surfaces of the complex. The following attachment of multiple C9 molecules leads to formation of MAC, a pore-like structure in the membrane leading to lysis of the target cell (Janeway et al., 2005).

Control of the complement system

Considering the lack of active discrimination between self and non-self particles and its potentially destructive effect also towards host cells, the complement system has to be tightly regulated. Complement regulation is achieved by the presence of soluble and membrane-bound inhibitors on self-cells, short half-life of convertases (unless they are stabilized by binding to relevant target surface) and transient presence of active proteases, such as factor D (Turnberg and Botto, 2003).

Regulation can occur on different levels of the complement cascade (Fig. 2). For example, C1 complex inhibitor (C1-INH) works by causing dissociation of serine protease subunits C1r and C1s, MASP 1 and MASP 2 from the recognition molecules C1q and MBL, respectively, thereby inhibiting the activation of classical and lectin pathways. The main complement regulators are the inhibitors of crucial enzymatic

complement complexes (C3- and C5-convertases) acting either by accelerating the decay of these complexes or by promoting the degradation of C3b and C4b fragments. Although these active complexes have an intrinsically short half-life of a few minutes, their dissociation is further promoted by C4-binding protein (C4BP) and factor H for the classical/lectin and alternative pathway, respectively. C4BP fulfills its function by accelerating the decay of the C4bC2a complex. Factor H has affinity to neutral or anionic polysaccharides and siaylic acid present on the surface of mammalian cells. By binding C3b, factor H prevents its deposition on host cells. and enables C3b cleavage by factor I (Janeway et al., 2005).

C3b and C4b fragments released from the dissociated convertase complexes become inactivated by a step-wise cleavage. Plasma component factor I cleaves them to iC3b and iC4b fragments, but only in the presence of such co-factors as C4BP, factor H, cell-bound membrane co-factor protein (MCP) or complement receptor 1 (CR1). iC3b is further cleaved by factor I to soluble C3c and membrane bound C3dg. Next, plasma proteases cleave off the free C3g fragment leaving C3d on the cell surface (Janeway et al., 2005).

Membrane-bound inhibitors with decay accelerating activity include the decay acceleration factor (DAF, CD55), membrane co-factor protein (MCP, CD46) and CR1 (CD35). Both DAF and CR1 promote the dissociation of both classical and alternative pathway C3-convertases, while MCP is a co-factor for factor I-mediated cleavage of C3b and C4b but does not dissociate the C3-convertase (Liszewski et al., 2000).

Also the terminal pathway is tightly controlled to prevent damaging the host cells. MAC formation is controlled mainly by glycosylphosphatidilinositol (GPI)-anchored membrane protein CD59 (protectin), which blocks the accumulation of C9 molecules in the proximity of C5b-C8 complexes. Also soluble factors such as vitronectin (S-protein) and clusterin are involved in the control of undesirable MAC formation (Turnberg and Botto, 2003).

Complement receptors

There are three groups of receptors for complement components: receptors for cleavage products of C3, receptors for C1q molecules, and receptors for the anaphylatoxic peptides. Stimulation of these receptors evokes a variety of biological responses involving for example the removal of immune complexes and apoptotic cells, stimulation of phagocytosis and complement inhibition. The C3d-binding CR2 receptor bridges innate and adaptive immune responses by increasing the synthesis of antibodies by B lymphocytes. Moreover complement receptors 1-4 (CR1-CR4) with affinity for C3-derived opsonins are crucial for phagocytosis and thus contribute to efficient antigen presentation (reviewed in Carroll, 2004). Receptor for the collagenous tail of C1q (cC1qR, calreticulin) is expressed on phagocytes and together with CR1 mediates clearance of C1q-coated pathogens, apoptotic cells and immune complexes (Peterson et al., 1997). There are three more C1q receptors (gC1qR, C1qRp and CD91), which are believed to stimulate phagocytosis and interact with integrins to promote cell adhesion (Nayak et al., 2012).

Three 7-transmembrane domain G-coupled receptors, C3aR, C5aR and C5L2 have differential affinity for the anaphylatoxins C3a, C4a, C5a and their derivatives. C3aR binds C3a but not $C3a_{desArg}$, while C5aR binds C5a with high- and $C5a_{desArg}$ with somewhat lower affinity (Klos et al., 2009). The third anaphylatoxin, C4a seems to be structurally related to C3a but evokes markedly weaker responses and its functions seem to be species-specific. C4a is a potent agonist of guinea pig but not human C3aR and no other definite human receptor for C4a has been identified (Lienenklaus et al., 1998). The G-protein-coupled receptors (GPCRs) that detect C3a or C5a are linked with several well-defined intracellular signaling pathways (Figure 3).

Upon C3a binding to C3aR, intracellular signal transduction is promoted via heterotrimeric G-proteins that mobilize calcium fluxes from the extracellular compartment. Downstream signaling events include activation of protein kinase C by phospholipase C, and mitogen activated protein kinases (MAPKs) Erk1 and Erk2. In some cell types, signaling pathways require activation of phosphatidylinositol-

bisphosphate-3-kinase (PI3Kγ) and subsequent phosphorylation of Akt as well as Erk1 and Erk2 (Klos et al., 2009).

C5a binding to C5aR causes calcium fluxes from intracellular stores as well as the extracellular compartment, and receptor internalization via clathrin coated pits. C5aR activation leads to downstream activation of several components of different signaling pathways such as PI3Kγ kinase, phospholipase C and D, Raf-1/B-Raf mediated activation of MEK-1, and Wiskott-Aldrich syndrome protein (WASP). WASP is a multifunctional protein that regulates actin dynamics and therefore may play an important role in the C5a dependent chemotaxis (Klos et al., 2009).

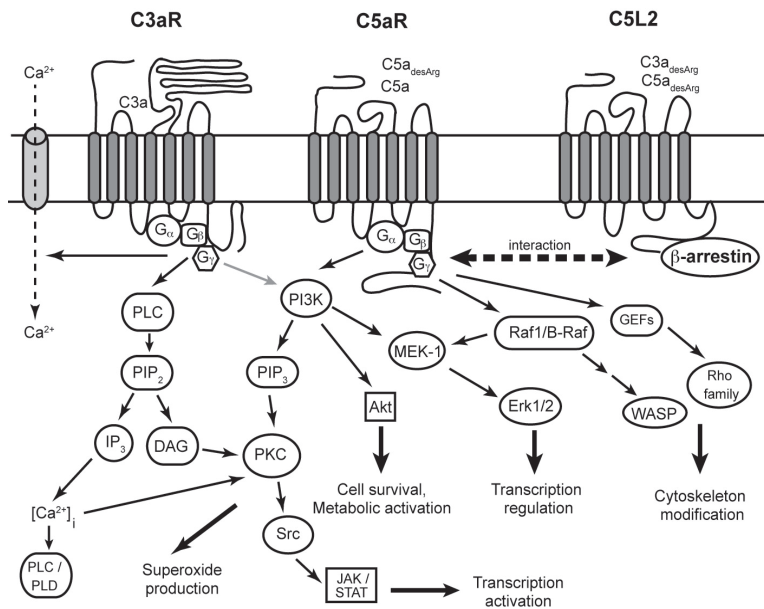

Figure 3. **Intracellular signaling of the anaphylatoxin receptors.** Schematic structure of the receptors is given together with an overview of the major downstream signaling pathways and their cellular effects. In contrast to C3aR and C5aR, C5L2 does not bind G proteins but acts via binding β-arrestin and interaction with C5aR (see text for details). Gα, β, γ – subunits of G protein; PLC/PLD- phospholipase C/D; DAG – diacylglycerol; PIP_3 - phosphatidylinositol triphosphate; IP_3 – inositol triphosphate; PKC - protein kinase C; GEFs – guanine exchange factors; protein; JAK – Janus kinase; STAT - Signal Transducer and Activator of Transcription; $[Ca^{2+}]_i$ – intracellular calcium storage.

Activation of C5aR leads to chemoattraction and activation of neutrophils, basophils, eosinophils and macrophages as well as endothelial cells activation and smooth muscle contraction. Upon activation with C5a, eosinophils release major basic protein, monocytes/macrophages produce inflammatory cytokines and mast cells release the content of their granules including histamine, which in turn causes vasodilation and increases vascular permeability. C3aR stimulation also attracts eosinophils and mast cells but not neutrophils and macrophages, and in addition promotes the secretion of lysosomal enzymes from leukocytes and histamine from mast cells, and smooth muscle contraction (Klos et al., 2009).

The function of the third and most recently discovered anaphylatoxin receptor, C5L2 (also known as $C5a_2R$ or GPR77) is much less defined. This molecule can be found on a similar repertoire of cell types as the canonical C5aR and the expression of these two receptors predominantly occurs in tandem, suggesting a functional link between them (Li et al., 2013). Unlike C5aR and the majority of GPCRs, C5L2 is expressed predominantly intracellularly. Interestingly, it is capable of binding C5a, $C5a_{desArg}$ (with higher affinity than C5aR does) and according to some studies, it also interacts with C3a and $C3a_{desArg}$ (Cain and Monk, 2002; Cui et al., 2009). Due to deviations from the conserved sequence and structure in regions required for G-protein coupling, C5L2 has been proposed to be a non-signaling decoy receptor for C5aR, although this hypothesis has been challenged by subsequent studies pointing to a much more complex role of this molecule.

Although the above described functions support the traditional view of anaphylatoxin receptors as pro-inflammatory immune regulators, in recent years, a large body of evidence has emerged that points to their role in limiting the inflammatory responses in a context-dependent manner. Specifically, the function of C3aR seems to be more selective and anti-inflammatory than C5aR as C3a has been shown to reduce LPS-induced pro-inflammatory cytokine release from peripheral blood mononuclear cells and lymphocytes (Takabayashi et al., 1998; Fischer et al., 1999). Studies of C3aR-deficient mice in endotoxic shock and gut ischemia/reperfusion injury models support the *in vitro* findings (Kildsgaard et al., 2000; Boos et al., 2005; Wu et al., 2013). Furthermore, the biological role of C5L2 appears to be to decrease the pro-inflammatory response conveyed by C5aR, at least in studies of non-disease models. However, based on the observations of

C5L2 function in septic animals, an alternative pro-inflammatory role for this receptor has been suggested, rendering it the most enigmatic complement receptor (Li et al., 2013). Also, owing to their expression on a wide variety of cell types, several non-immunological functions have been assigned to the anaphylatoxin receptors.

Non-immunological functions of the complement system

In the last two decades, novel functions of the complement system have been identified which suggest it has roles far beyond immunity. Among them are tissue regeneration, stem cell chemoattraction and regulation of their migration well as various non-immune functions in CNS.

Tissue regeneration

First evidence for a non-immune function of the complement system came from a study that found C3 mRNA in blastema cell layer of regenerating limbs of salamander (Del Rio-Tsonis et al., 1998). Next, both C3 and C5 were detected in regenerating but not intact limbs and lens of newt (Kimura et al., 2003). Soon it became apparent that complement involvement in tissue regeneration is not restricted to lower vertebrates, known for their remarkable regeneration capabilities. Mice deficient in C3 or C5 showed reduced liver regeneration in response to toxic liver injury or hepatectomy (Mastellos et al., 2001; Strey et al., 2003; Daveau et al., 2004; Markiewski et al., 2004). The liver regeneration in these studies has been shown to be dependent on C3a and C5a signaling through their canonical receptors (Mastellos et al., 2001; Markiewski et al., 2004), by priming and stimulation of hepatocyte proliferation. Further, C3-deficient mice showed delayed removal of damaged and apoptotic liver cells following toxic liver injury.

Regulation of stem cell translocation

Human mesenchymal stem cells (MSC), which are involved in the repair of various tissues, have also been found to express complement peptide receptors and to be chemoattracted by C3a and C5a (Schraufstatter et al., 2009). Furthermore, C5aR has been shown to control osteoblast migration during fracture healing and efficient osteoclast

differentiation required local complement activation (Ignatius et al., 2011b; Ignatius et al., 2011a).

The complement cascade has in recent years also emerged as an important and up to now underappreciated modulator of trafficking of hematopoietic stem/progenitor cells (HSPCs) (reviewed in Ratajczak et al., 2008). It has been reported that the complement becomes activated in bone marrow during mobilization of hematopoietic stem cells to the periphery. C3a increases the responsiveness of HSPCs to stromal derived factor-1 (SDF-1) gradient, which is the primary factor regulating trafficking of these cells between marrow stroma and peripheral blood (Reca et al., 2003). Also C5a and soluble MACs regulate the homing of stem cells back to bone marrow (Lee et al., 2009; Kim et al., 2011).

Complement in the central nervous system

For decades, the brain has been considered an immune-privileged organ owing to the existence of the BBB, formed by endothelial cells, pericytes and astrocytes. This tight barrier prevents plasma proteins, including complement components, from entering the intact brain. However, complement proteins are produced locally in the CNS and complement is considered to provide local defense mechanisms against invading pathogens (Morgan and Gasque, 1996). Both murine and human neurons and glia collectively produce nearly all of the complement proteins *in vitro*, especially upon stimulation with pro-inflammatory cytokines (Barnum, 1995; Thomas et al., 2000).

Complement in unchallenged CNS

The presence of C3aR, C5aR or C5L2 on glia was somewhat expected owing to the monocytic origin of microglia and the fact that astrocytes also are the immunocompetent cells that participate in antigen presentation, and can produce a number of cytokines, chemokines and complement components (Weber et al., 1994; Morgan and Gasque, 1996; Gavrilyuk et al., 2005). However, the discovery of these receptors on neurons was surprising and led to speculations that complement may play a role in CNS homeostasis and modulation of neuronal functions. (Davoust et al., 1999; O'Barr et al., 2001; Gavrilyuk et al., 2005). Hippocampal and cortical neurons express C3aR and C5aR most

abundantly of all cell types in the unchallenged CNS. Additionally, spinal cord motor neurons express relatively high levels of C5aR and C5L2 (Nataf et al., 1998; Woodruff et al., 2008). In the recent years, C3aR and C5aR have been found to be expressed on neural stem/progenitor cells (NSPCs) in murine neurogenic brain regions (Rahpeymai et al., 2006). In line with the surprisingly widespread role of anaphylatoxic peptides and their receptors in regulating the migration of stem cells, C3a has been identified as an organizer of the migration of neural crest cells and (together with C5a) of cerebellar neuronal precursors during development (Benard et al., 2008). Furthermore, C5a has been found to be mitogenic for human undifferentiated neuroblastoma cells and neuroprotective for mature neurons (O'Barr et al., 2001).

A group of proteins related to C1q, namely cerebellins (Cbln) and C1q-like molecules (C1ql) have been found to be expressed in the cerebellum, as well as other regions of the developing and mature brain (Yuzaki, 2008; Bolliger et al., 2011). Furthermore, half of the more than 50 genes encoding putative complement regulators predicted in the mouse genome are expressed in the CNS. These molecules are predicted to be involved in synapse organization and plasticity (Gendrel et al., 2009; Yuzaki, 2010).

Complement during ischemic brain injury

The diverse roles played by the complement system in acute brain disorders are not fully elucidated, however a growing body of evidence suggests a role for the complement system in secondary brain damage (Stahel et al., 1998). During stroke, BBB integrity is disrupted allowing for influx of plasma proteins, including complement components. Investigation of brain tissue of patients with ischemic stroke revealed deposition of C1q, C3c and C4d in all ischemic lesions, suggesting activation of the classical pathway. In necrotic zones of the brains from the same patients, C9, C-reactive protein and IgM were found (Pedersen et al., 2009). The possibility of harmful uncontrolled complement activation following ischemic insults was underlined by the findings of virtual absence of CD59 and CD55 in ischemic lesions. Moreover, systemic activation of the complement cascade is evident in stroke patients as judged by elevated plasma levels of C3, C3a, C4d and soluble terminal complexes C5b-9 (Pedersen et al., 2004; Tamam et al., 2005; Mocco

et al., 2006a; Szeplaki et al., 2009). Consistent with the above, a number of complement inhibition strategies were found to be beneficial in terms of limiting the infarct volume and improving behavioral outcome following experimental cerebral ischemia with reperfusion (Huang et al., 1999; De Simoni et al., 2003; Costa et al., 2006; Mocco et al., 2006b).

However, in the animal model of permanent ischemia, genetic C3 deficiency led to increased infarct volume suggesting potentially beneficial effects of complement activation in the course of stroke (Rahpeymai et al., 2006). Moreover, both C3a and C5a were found to be neuroprotective against excitotoxicity, although through two distinct mechanisms (Mukherjee and Pasinetti, 2001; van Beek et al., 2001). This function of C3a appears to be mediated by astrocytes, whereas C5a acts through the regulation of the caspase cascade and glutamate receptor subunit 2 (GluR2) expression in neurons (Mukherjee et al., 2008). These two protective mechanisms could be relevant for the ischemic brain injury, glia and neurons highly upregulate their C3aR and C5aR expression in response to permanent focal cerebral ischemia (Van Beek et al., 2000). In support of that, a recent report revealed marked neuroprotective properties both of C3a overexpression in the brain and of administration of exogenous C3a peptide following neonatal ischemic brain injury (Järlestedt et al., 2013).

Taken together, these findings indicate that the role of complement activation in the ischemic brain is complex and can be viewed as a double-edge sword (similar to inflammation in general) which is detrimental or beneficial depending on the context.

NEURAL PLASTICITY

It was long thought that the brain only changed during development and that the adult brain was fixed in its functional organization, with specific areas allocated to and hard-wired for specific functions. Today, there is no doubt that the brain is reorganizing itself constantly, for example every time new knowledge is stored or a new motor skill learned. This ability of the brain to change and adapt due to experience is called “neural plasticity”. The term was introduced by the Polish neuroscientist Jerzy Konorski in 1948 with the formal hypothesis embodying these ideas advanced shortly thereafter by the Canadian neuroscientist Donald Hebb (Konorski, 1948; Hebb, 1949). Neural plasticity refers to a variety of structural and functional changes in neural pathways and synapses (connections between neurons, through which information is transmitted from one neuron to another), which occur in response to changes in behavior, environment and neural processes, as well as changes resulting from injury. The study of mechanisms underlying brain plasticity during development and learning provides a good basis for the understanding of functional reorganization of the brain after injury such as stroke. Therefore, physiology of plastic changes is most often studied using model systems such as organotypic slice cultures of hippocampus, a brain structure indispensable for memory formation and learning.

Mechanisms of plasticity

Plastic changes of the brain can be either short-lived or long-lasting and depend on several mechanisms, which constitute the basis for functional and structural reorganization associated with both learning and recovery after CNS injury.

Functional synaptic plasticity

Neurons communicate with each other primarily through fast chemical synapses. At such synapses, an action potential generated near the cell body of the presynaptic cell propagates down the axon where it opens voltage-gated Ca^{2+} channels. Ca^{2+} ions entering nerve terminals trigger the rapid release of vesicles containing a neurotransmitter, which is ultimately detected by receptors on the postsynaptic cell. Virtually all types of synapses are regulated by a variety of short-lived or long-lasting processes.

Short-term synaptic plasticity

Short-term synaptic plasticity is the modulation of synaptic strength following repetitive synaptic activity that occurs within milliseconds (ms) to a few minutes (reviewed in Zucker and Regehr, 2002). Synaptic strength, or in other words efficacy, is determined by the number of functional neurotransmitter release sites (n), the release probability of these sites (p), and the quantal size *i.e.* the magnitude of post-synaptic response to the release of single presynaptic vesicle (q). Therefore, the synaptic efficacy equals n*p*q and it is represented by the mean amplitude of the evoked synaptic response (Korn and Faber, 1991). The change of synaptic efficacy follows Hebbian rules (discussed in the next section) and is one of the fundamental principles of cortical plasticity (Hebb, 1949). Synaptic efficacy and the threshold for activation can be influenced by the temporal structure and synchronization of impulse arrival and neuronal firing.

Short-term synaptic plasticity has a pre-synaptic nature and may involve both enhancement and decrease of synaptic transmission. Synaptic enhancement lasting ~100 ms is referred to as facilitation and it can be observed with pairs of brief stimuli. Due to residual Ca^{2+} in the pre-synaptic terminal following the first discharge, the release probability of the second discharge increases up to several times the size of the first one. The reverse phenomenon is called depression. In synapses with a high initial release probability the large initial discharge causes a depletion of neurotransmitter vesicles available for release from the pre-synaptic terminal, rendering the second post-synaptic response smaller. A third phenomenon, short-term potentiation, occurs with tetanic stimulation which is a series of high-frequency stimuli, and results in postsynaptic potentials of increasing size. Repeated high-frequency stimulation causes calcium ions to accumulate in the pre-synaptic terminal which increases the release probability (p) and/or, via fusion of vesicles, the quantal size (q) (Figure 4). The degree of enhancement of each potential is proportional to the number of preceding high-frequency stimuli. Likewise, when stimulation returns to baseline frequency the time taken for the post-synaptic potential to return to the original magnitude is proportional to the number of rapid stimuli in the tetanic phase and can be up to a few minutes in length (Zucker and Regehr, 2002).

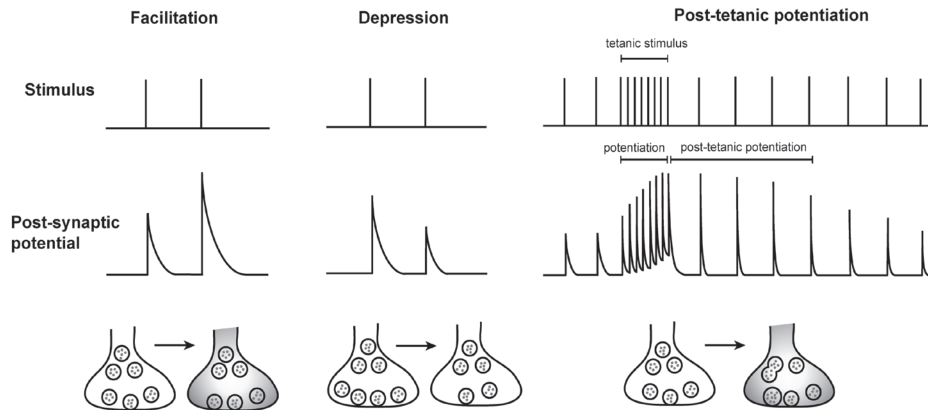

Figure 4. **Short-term synaptic plasticity and the related presynaptic mechanisms**. Postsynaptic facilitation in response to the second stimulus occurs due to residual calcium ions in the presynaptic terminal (gray shading) that increase neurotransmitter release probability. Postsynaptic depression results from depletion of neurotransmitter-containing vesicles in the active zone following previous large discharge. Repeated high frequency stimulation (tetanic stimulus) causes extended potentiation of the postsynaptic response due to accumulation of calcium ions in the presynaptic terminal (gray shading) leading to increased release probability and/or increased quantal size due to fusion of vesicles.

Long term synaptic plasticity

The second and extensively studied mechanism of synaptic plasticity is long-term potentiation (LTP). This process is assumed to be the basis for the acquisition of knowledge and memory formation. It relies on specific patterns of synaptic activity which lead to the strengthening of connections between the synapses of neighboring neurons which fire together, thus providing physiological substrate for a lasting association. This concept was proposed in 1949 by Donald Hebb and was confirmed experimentally twenty years later using hippocampal circuits as a model system (Bliss and Lomo, 1973). LTP requires activation of NMDAR, which acts as a coincidence detector, and an increased intracellular calcium concentration (Wigstrom and Gustafsson, 1986).

NMDAR is a voltage- and ligand-gated ion channel, which opens only when the glutamate release from a presynaptic vesicle to the synaptic cleft coincides with postsynaptic membrane depolarization, *i.e.* when both pre- and postsynaptic neurons are activated (Figure 5). Post-synaptic depolarization is evoked by initial activation of glutamate-gated α-amino-3-hydroxyl-5-methyl-4-izoxazolepropionic receptors (AMPARs), an event that is required for expelling the Mg^{2+} ion that blocks the NMDAR ion channel (Nicoll et al., 1988). Opened NMDAR allows for the influx of calcium, an intracellular second messenger that is bound by calcium-dependent kinases and induces a cascade of intracellular signaling. This activation initiates several pathways which lead to the induction of transcription factors and thus protein synthesis. The latter is the basis for lasting changes in properties of activated neurons (Kauer et al., 1988). These effector events might differ between different types of synapses but in the model hippocampal CA1-CA3 synapse, LTP causes potentiation of AMPAR conductance as well as recruitment of additional AMPARs from the peri-synaptic pool to the postsynaptic membrane (Malinow, 2003). As mentioned above, LTP has been described originally in the hippocampus but it is observed in many brain structures such as cerebral cortex, cerebellum and amygdala (Clugnet and LeDoux, 1990; Hess and Donoghue, 1994).

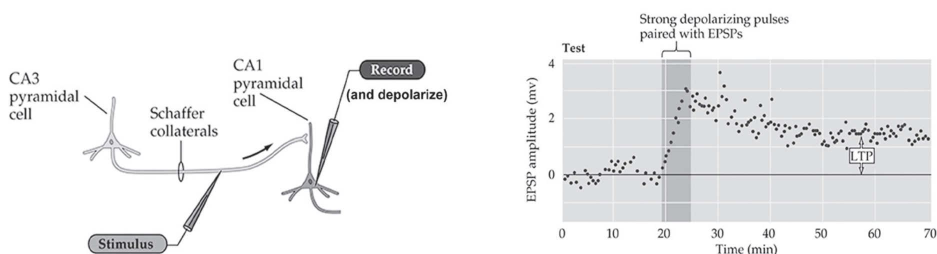

Figure 5. **LTP in hippocampal circuitry caused by synchronized pre- and postsynaptic activity**. Single stimulus applied to Schaffer collateral synaptic input evokes normal excitatory postsynaptic potentials (EPSPs) in the postsynaptic CA1 neuron. However, if the CA1 neuronal membrane is briefly depolarized (by applying current pulses through the recording electrode) in conjunction with Schaffer collateral stimulation, a persistent increase in the EPSP amplitude occurs (synapse undergoes LTP). Modified from Purves et al. (2008).

Synapses can also undergo a long-lasting weakening termed long-term depression (LTD). Similar to LTP, this phenomenon seems to be dependent on the action of NMDARs although its mechanisms are far less understood. LTD was observed for the first time in the CA1 region of the hippocampus after a low-frequency stimulation protocol and is therefore believed to be evoked by synchronized weak neuronal activation (Mulkey and Malenka, 1992).

Metaplasticity and homeostatic plasticity

There are two additional forms of synaptic plasticity, namely metaplasticity and homeostatic plasticity. The term metaplasticity refers to the "plasticity of synaptic plasticity" and assumes that previous history of activity of the synapse determines its current plasticity (Abraham and Bear, 1996). It consists of changes in induction thresholds for synaptic plasticity, which are caused by prior activity that itself did not change the synaptic efficacy. For example, if a given high frequency stimulation is not strong enough to evoke an increase in synaptic strength, it can inhibit subsequent induction of LTP (Huang et al., 1992). On the other hand, trains of low-frequency current pulses often do not produce LTD, however such stimulation protocol given after previously established LTP can result a depression of the responses, *i.e.* cause depotentiation (Bashir and Collingridge, 1994).

When the general activity of the synaptic network is altered for a prolonged time, homeostatic mechanisms are activated and upregulate the synaptic strength if activity level was low, or downregulate it if activity was increased (Turrigiano and Nelson, 2000). This phenomenon is called homeostatic plasticity (also referred to as synaptic scaling) and is believed to occur in order to maintain functional stability of neuronal networks.

Structural plasticity

Another aspect of plasticity has a structural character and involves changes in the number and complexity of dendrites, density of receptors, formation of new dendritic spines and the growth of new axon terminal, which lead to the increase in density and size of synapses., In some brain regions, structural plasticity involves also increase in number of neurons (as discussed further on). Consequently, existing pathways are

strengthened or new connections are developed, either within one neural network or between different neural networks. This may be a slow process taking place over weeks or months, since the growth of new connections takes time. These structural constituents of neural plasticity determine the complexity of neuronal networks and their activity, and can be demonstrated especially well during the process of motor learning. For example, rodents trained to traverse a demanding obstacle course had more synapses in the motor cortex and cerebellum than rats performing a simple walking task (Warraich and Kleim, 2010). Furthermore, animals trained to perform a skilled reaching task show dendritic growth, synaptogenesis, and enhanced synaptic responses which are connected to the expansion of wrist and digit movement representations within the forelimb motor cortex. Similar functional changes have been observed in primate and human motor cortex in connection with skill training (Pascual-Leone et al., 1995; Nudo et al., 1996).

Forms of neural plasticity in recovery of function after ischemic stroke

In cerebral ischemia, the initial pathological events associated with direct neuronal cell death are followed by the activation of regenerative processes for days, weeks or even months after stroke. After the ischemic attack, a majority of stroke patients exhibit certain levels of motor weakness and sensory disturbances, however, over time, most will show a certain degree of functional recovery (Donnan et al., 2008), which may be explained by brain reorganization and plasticity. Recovery of function can be viewed as a relearning process, the basic principles of which do not differ from the ones in the intact CNS. Unlike the previously described mechanisms, which occur mainly on the level of single neurons and synapses, provision of a structural basis for new connections (including long projections) allows for more widespread changes in the form of functional brain remapping that is critical in the recovery of function after stroke and other CNS injuries. It allows the neurons in the spared regions to take over the sensory or motor functions that had previously been performed by the damaged areas.

<u>Ipsilateral axonal sprouting and synaptogenesis</u>

Following cerebral ischemia, extensive remodeling of dendritic spines occurs in the peri-infarct cortex. In rodent models of stroke, an increase in dendritic spine number and spine turnover rates is observed in somatosensory areas of the limbs during the first two

to three weeks (Brown et al., 2009). These changes represent synaptogenesis and circuit plasticity of the areas that exhibit remapping after stroke (Winship and Murphy, 2008).

On the other arm of this structural plasticity is the axonal sprouting, as cortical dendritic spines are known to be postsynaptic partners for axonal boutons. Such a neuronal growth response after stroke has been demonstrated by anatomical tracing studies in rodents (Carmichael et al., 2001) and it is associated with increased expression of growth-promoting proteins and decrease of the growth-inhibitory ones (Carmichael et al., 2005). Massive axonal rearrangements are also seen in primates following brain injury (Dancause et al., 2005). For example, lesions to the primary motor area eliminate major inputs to premotor and somatosensory areas. Over time, new connections develop from the premotor area to the primary sensory cortex, replacing inputs from the primary motor area that had been lost.

Tonic inhibition

Some "global" changes can occur even before the new projections have sprouted, due to changes in the balance of excitatory and inhibitory connections between the whole neural networks. This process depends on neurons and neuronal pathways having much larger region of anatomical connectivity than their usual territory of functional influence as well as a surprisingly high degree of overlap and redundancy of the connections across the cerebral cortex. Some zones may be kept relatively inactive by a phenomenon termed tonic inhibition. Tonic inhibition is mediated by a major inhibitory neurotransmitter of the brain, γ-aminobutyric acid (GABA) and its extrasynaptic $GABA_A$ receptor, the activation of which decreases neuronal excitability (Walker and Semyanov, 2008). If this physiological inhibition is removed, the region of influence can be quickly increased or unmasked, which is associated with fundamental changes in cellular excitability including long-term potentiation (Jacobs and Donoghue, 1991; Hess et al., 1996).

Increased tonic inhibition plays a role in the pathological context of ischemic stroke and it is considered to be a protective mechanism against possible overactivation of the cortical circuits after injury, which could be epileptogenic. It is caused for example by a decreased expression of astrocytic GABA transporters (GATs) in the peri-infarct cortex, resulting in the surplus of GABA in the neuronal surrounding that would normally be

rapidly cleaned up and recycled by astrocytes (Neumann-Haefelin et al., 1998; Frahm et al., 2004). The GABAergic mechanisms mediate changes in neuronal excitability that have a central role in functional recovery of peri-infarct cortex, however, increased and prolonged tonic inhibition after stroke may hamper recovery of function offered by cortical reorganization (Clarkson et al., 2010).

Involvement of the contralesional hemisphere

Mammalian brains are endowed with rich intracortical network that enables reciprocal communication among various sensory and motor areas. In contrast to the uninjured brain, the contralesional hemisphere can contribute to movement controlled from the ipsilesional cortex and after ischemic stroke, contralesional motor cortex increases its activity during the movement of the affected limb (Chollet et al., 1991; Enzinger et al., 2008). Moreover, animal studies show increased synaptogenesis in the non-lesioned motor and somatosensory cortical regions as well as transcallosal projections from the uninjured to the injured hemisphere following ischemic stroke (Stroemer et al., 1995; Carmichael and Chesselet, 2002; Takatsuru et al., 2009). Contralesional activation after stroke usually diminishes at the later stages of recovery. Persistent activation of contralateral cortical regions predicts slower and less complete recovery and is often associated with larger infarcts (Murphy and Corbett, 2009).

Spontaneous behavioral recovery strongly correlates with remodeling of the contralateral corticospinal tracts (CST) at the level of cervical or lumbar spinal cord as well as with the accompanying reorganization of connections of pyramidal neurons in the cerebral cortex in rodents (Liu et al., 2009). CST consists of long projections descending from motor cortex that are organized in a topographic fashion and mediate voluntary movements of the limb musculature. Deafferentation due to stroke causes sprouting of collateral branches from both ipsi- and contralesional CST axons. These projections form new synapses on motor neurons that innervate the stroke-affected side of the body. Such a remodeling of CSTs has been linked to the recovery of fine motor function also in human subjects, so that the bilateral engagement of CST correlated with better recovery than the involvement of the ipsilesional tract alone (Schaechter et al., 2009).

The temporal overview of plasticity-related changes occurring after stroke is presented in the Figure 6.

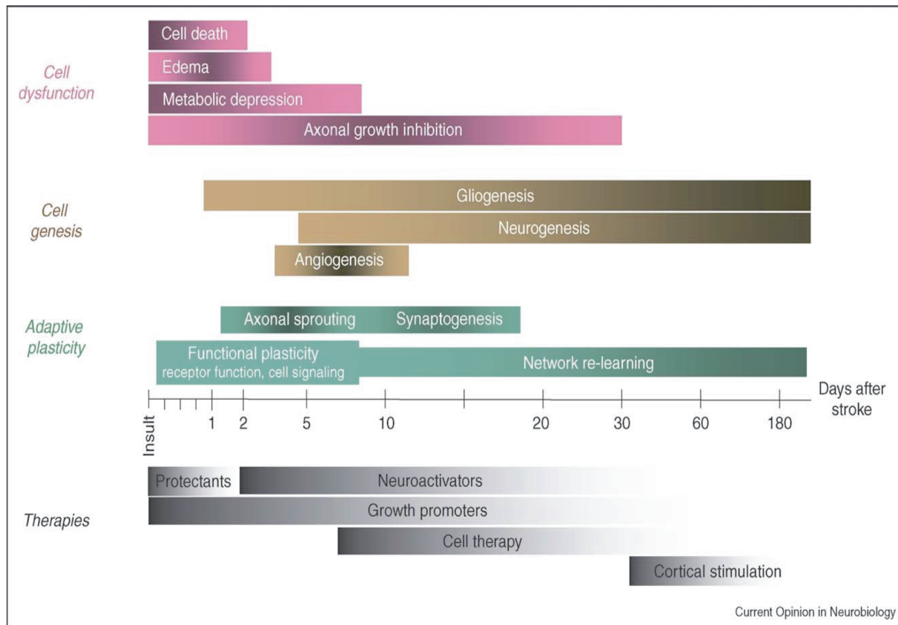

Figure 6. **Processes activated following ischemic stroke and the related therapeutic approaches**. The temporal sequence of events is shown along a schematic timeline. Darker shading highlights the maximum intensity of the specific mechanism. Processes that are detrimental for recovery are shown in pink. Regenerative responses in the form of cell genesis are shown in brown, whereas those that underlie adaptive plasticity are shown in green. Prospective therapies aimed at improving functional recovery are shown in gray. From Wieloch and Nikolich (2006).

The role of neurogenesis in neural plasticity

In the adult rodent and human brains, neural stem cells continue producing new neurons, astrocytes and oligodendrocytes in two neurogenic niches (Figure 7), namely the subgranular zone (SGZ) of hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles (Alvarez-Buylla and Lim, 2004). In rodents, SVZ-derived neuronal progenitors migrate through the rostral migratory stream towards the olfactory

bulb, whereas SGZ-born cells move only short distances to the granular cell layer. The physiological role of adult mammalian neurogenesis has not been fully elucidated owing to the lack of highly specific and isolated ablation models. However, several lines of evidence indicate that neurogenesis is important for plasticity-dependent function of the target brain structure of the neurogenic niches. Correlative observations suggest that adult neurogenesis might contribute to the learning and memory functions subserved by the hippocampus, and to the perceptual and memory functions performed by the olfactory bulb. It is therefore plausible that newborn cells instruct changes in the networks that they join or, through a general alterable role, they simply permit changes to occur in those circuits.

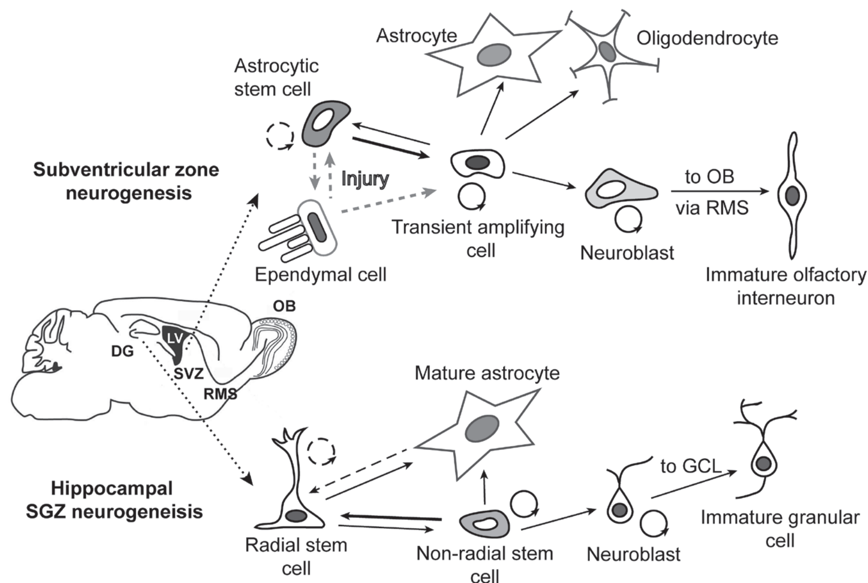

Figure 7. **Adult neurogenesis in the rodent brain**. Model of lineage relationships in the two neurogenic niches: SVZ and SGZ of the hippocampal DG. Dashed arrows represent infrequent events. DG - dentate gyrus, LV – lateral ventricle, RMS – rostral migratory stream, OB – olfactory bulb, GCL – granular cell layer. Adapted from Duan et al. (2008).

At the systems level, physical activity and environmental enrichment increase both neurogenesis and performance on hippocampus-dependent learning tasks, whereas age and stress are associated with deficits in both phenomena. In addition, acquisition of spatial memory in the water maze correlates with the proliferation and survival of newborn dentate gyrus neuron, whereas in the olfactory bulb, olfactory enrichment links increased newborn cell survival with improvements in olfactory memory (Lledo et al., 2006). The mechanism by which neurogenesis is thought to be well suited to mediate formation of memory is that young granule cells in the adult dentate gyrus show a greater propensity for synaptic plasticity compared to older granule cells, whereas newborn granule and periglomerular cells in the olfactory bulb show markedly different active membrane properties compared to the existing neurons around them and show greater plasticity in response to sensory deprivation. In addition, for immature neurons, before glutamatergic synapses are developed, GABA is the major excitatory neurotransmitter which further adds to a repertoire of distinct properties of newly-generated neurons that contribute to their increased plasticity (Ge et al., 2006).

Ischemic stroke in rodents stimulates proliferation of stem cells in SVZ and a fraction of NSPCs cells, which normally migrate through the rostral migratory stream towards olfactory bulb, divert form that route and migrate towards ischemic penumbra. There is evidence that neural stem cells may reside in the cortex or a subpopulation of astrocytes may acquire stem cells properties after injury (Magavi et al., 2000; Jiang et al., 2001; Shimada et al., 2010). Regardless of the origin, some of these cells will transiently persist in the vicinity of injury in an undifferentiated neuroblast state and a small fraction will turn into neurons and astrocytes. Manipulations that are directed at increasing neurogenesis have been shown to improve functional outcome, while the depletion of neuroblasts results in impaired performance after cerebral ischemia (Wang et al., 2004; Ohab et al., 2006; Leker et al., 2007; Wang et al., 2012). However the mechanism behind the positive effects of injury-induced neurogenesis seems unlikely to be mediated by direct neuronal replacement due to very few mature and functionally integrated neurons being generated. Instead, stem cells and progenitors, which are transiently present in penumbral tissue, may produce a variety of growth factors such as brain-derived neurotrophic factor (BDNF), neutrophin-3 (NT-3), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor bFGF, and vascular

endothelial growth factor (VEGF), all of which provide a supportive milieu for recovery-related plasticity (Chopp and Li, 2002; Lu et al., 2003). Remarkably, neurogenesis after stroke seems to be coupled to angiogenesis and revascularization of the ischemic brain tissue, possibly through production of VEGF by stem and progenitor cells thus neurogenesis appears to contribute to tissue remodeling through enhanced perfusion as well as blood flow-independent mechanisms (Hermann and Chopp, 2012). Apart from plasticity-promoting trophic effects, newly-born, partially differentiated neural stem cells may protect the ischemic penumbra via direct cell-cell transfer of molecules (Jaderstad et al., 2010).

The immune system and brain plasticity

As already mentioned, the limited access of peripheral immune cells to CNS due to the existence of BBB, made it necessary for the brain and spinal cord cells to generate inflammatory mediators such as cytokines and complement proteins locally in order to maintain protection from invading microorganisms. However, in the recent years it became clear that the immune system is actively involved in brain plasticity and therefore its activation in CNS is important both for physiological functions and recovery after injury.

Microglia

One of the most prominent immune cell types that influence brain plasticity is microglia. Non-activated microglia in the healthy brain are highly active cells, extending and retracting their processes as they survey the microenvironment in CNS (Nimmerjahn et al., 2005). The most interesting mechanism by which this cell type influences neural plasticity under physiological conditions seems to be their interaction with synapses. Microglia have been recently identified as “the sculptors” of synaptic networks during development by performing elimination of weak synapses (Paolicelli et al., 2011). Moreover, microglial processes were observed to be in association with synapses during visual experience, including contacting axon terminals, dendritic spines, perisynaptic astrocytic processes and synaptic clefts in adult animals (Tremblay et al., 2010). Hence, developmental and experience-dependent plasticity may involve microglial interactions with synapses and physical remodeling of this component of neural circuits.

Microglia also play an important physiological role in controlling adult neurogenesis. Mouse-derived microglia release soluble factors, which contribute to migration and differentiation of neural progenitors *in vitro*, and the number of activated microglia displaying ramified morphology correlates with increased neurogenesis and number of nestin-positive cells in SGZ of hippocampus (Aarum et al., 2003; Battista et al., 2006; Walton et al., 2006). Normal function of microglia has been also shown to be required for spatial learning and memory (Ziv et al., 2006).

The effect of activated microglia in CNS seems to be dependent on the nature of the stimulating factor. Microglial activation phenotype has been defined as either the classical, M1 or the alternative, M2 state, adopted from descriptions used for peripheral macrophage activation (Ransohoff and Perry, 2009). For example, LPS and irradiation induce pro-inflammatory and cytotoxic profile of microglia activation, which has detrimental consequences e.g. by inhibition of neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). Therefore microglia inhibition has been shown to ameliorate these negative effects (Ekdahl et al., 2003; Hoehn et al., 2005). On the other hand, microglia stimulated by IFNγ or anti-inflammatory cytokine interleukin 4 (IL-4), are neuroprotective for cultured hippocampal slices and stimulate neurogenesis and oligodendrogenesis from adult neural stem cells (NSCs). The putative mechanism for these effects appears to be microglial secretion of insulin growth factor 1 (IGF-1), suppression of TNFα and upregulation of glutamate transporter 1 (GLT-1), which are important for clean-up of excitotoxic glutamate from the extracellular space (Butovsky et al., 2005; Shaked et al., 2005; Butovsky et al., 2006).

Microglia can also support neuronal functions and promote neural plasticity by secretion of the main neurotrophic growth factors, BDNF and NGF (Elkabes et al., 1996; Madinier et al., 2009). Noteworthy, physical activity which increases both BDNF and NGF levels in the brain and stimulates neurogenesis, leads also to microglial proliferation in mouse cerebral cortex (Ehninger and Kempermann, 2003).

Macrophages

Similar to microglia, blood-derived macrophages seem to play an important role in CNS plasticity. These cells are assumed to be one of the major contributors to successful

regeneration of damaged peripheral nerves as opposed to the limited direct regenerative properties of CNS pathways. Macrophages recruited to the damaged CNS are less effective in removal of growth-inhibiting myelin debris (Lazarov-Spiegler et al., 1996). However, increased numbers of macrophages at the injury site due to infiltration or transplantation, improve axonal regeneration of damaged optic nerve and spinal cord, effect of which is mediated by efficient myelin debris clean-up and secretion of factors such as oncomodulin and IL-10 (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998; Yin et al., 2006; Shechter et al., 2009).

Astrocytes

Astrocytes are the important components of neurogenic niches and have been implicated in shaping their microenvironment by secreted factors as well as direct cell-cell contact and thus regulating neurogenesis (Song et al., 2002). They can also function as multipotent neural stem cells in both the developing and adult brain (Figure 7). Furthermore astrocyte-derived factors regulate synaptogenesis and synapse maturation (Nagler et al., 2001; Christopherson et al., 2005; Eroglu et al., 2009). Moreover, astrocytes regulate and control the number of neurotransmitter molecules present in the synaptic cleft and the function of NMDARs, thus they influence synaptic transmission by being part of an entity termed the tri-partite synapse (Araque et al., 1998; Panatier et al., 2006; Jourdain et al., 2007).

Upon CNS injury, astrocytes become active, which is hallmarked by cellular hypertrophy and upregulation of intermediate filaments proteins, collectively known as reactive astrogliosis. Reactive astrogliosis is neuroprotective in the acute stages after injury by restricting the secondary tissue damage though the formation of a glial scar (Li et al., 2008). However during the later stages of the recovery, this astrocyte barrier poses a serious obstacle for effective regeneration by secretion of powerful inhibitors of CNS regeneration (Pekny and Pekna, 2004). Astrocytes are also an essential component of the neurovascular unit and directly regulate the properties of BBB. Thus, they can also regulate neurogenesis and synaptogenesis indirectly, by determining the accessibility of blood-derived factors modulating neural plasticity (Barres, 2008).

T lymphocytes

A growing body of evidence suggests that the adaptive immune system and T lymphocytes in particular interact with CNS providing neuroprotection and regulating of neurogenesis. Especially T-cells directed against myelin binding protein (T_{MBP}) reduce the spreading of damage following optic nerve and spinal cord injury (Moalem et al., 1999; Hauben et al., 2000). These autoreactive T-cells become activated and produce NGF and BDNF supporting neuronal growth and survival (Kerschensteiner et al., 1999; Moalem et al., 2000).

T cells also play a role in the maintenance of the hippocampal neurogenic niche by the stimulation of progenitor cells proliferation. As a consequence, immunodeficiency leads to decreased neurogenesis, decreased BDNF expression in the brain and deficits in spatial learning in Morris Water Maze. Conversely, replenishing the $CD4^+$ lymphocytes rescues precursor cell proliferation (Wolf et al., 2009). It seems that CNS-specific T_{MBP} cells specific for the myelin basic protein are mainly responsible for these effects as transgenic T_{MBP} mice show increased hippocampal neurogenesis and perform better in learning and memory tasks. T lymphocytes exert their beneficial properties partially through their interaction with microglia by production of M2-type cytokine, IL-4 and without the need for infiltration of the brain parenchyma (Ziv et al., 2006; Derecki et al., 2010).

Pro-inflammatory cytokines

A number of immune proteins have been found to be associated with developing and mature synapses and some of them have been found to regulate synaptic transmission (Boulanger et al., 2001). A prominent example is TNFα released from microglia which promotes cell surface accumulation of AMPARs and concomitant decrease in $GABA_A$ receptors due to increased endocytosis in hippocampal neurons (Pascual et al., 2012). This way, TNFα increases the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) in hippocampal neurons *in vitro* (Beattie et al., 2002). TNFα is thought to be one of the important regulators of homeostatic plasticity by scaling-up the excitatory synapses as it may be released in response to drop in synaptic activity (Stellwagen and Malenka, 2006). Further, TNFα alters excitability throughout

CNS via its effects on glutamate transporters. It decreases the expression of the GLT-1 and decreases glutamate uptake by other glial transporters such as GLAST (Schafers and Sorkin, 2008). *In vitro* exposure of adult SVZ-derived NSCs to TNFα produces a dose-related increase in proliferation with no effect on differentiation (Widera et al., 2006).

Another pro-inflammatory cytokine, IL-1β, is thought to reduce neurotransmitter release, impair LTP in the hippocampus and modulate synaptic transmission in the neocortex (Schafers and Sorkin, 2008) by rapid inhibition of voltage–dependent calcium channels in CA1 and cortical neurons. On the other hand, IL-1β may have excitatory effect especially on nociceptive fibers as the IL-1 receptor is expressed in dorsal root ganglions, thus IL-1β can act directly on sensory neurons (Marchand et al., 2005). Evidence from several *in vitro* and *in vivo* experiments suggests the existence of a functional interaction between IL-1β and glutamate receptors: IL-1β reduces the frequency of AMPA dependent spontaneous excitatory postsynaptic currents (sEPSCs) and mEPSCs. Physiological levels of IL-1β in the hippocampus take part in memory consolidation and facilitation, while IL-1 receptor blockage impairs memory formation (Goshen et al., 2007). Interestingly, endogenous IL-1β may be required for peripheral nerve regeneration (Guenard et al., 1991).

IL-6 has been shown to induce proliferation of brain microvessels *in vitro*, while IL-6-deficient mice show delayed tissue repair associated with increased neuronal death and slower revascularization in the injury site (Fee et al., 2000; Swartz et al., 2001). Furthermore, IL-6 levels are dramatically upregulated by physiological LTP *in vivo*, however, exogenous IL-6 inhibits LTP in hippocampal slices (Jankowsky et al., 2000; Balschun et al., 2004).

It cannot be forgotten that an ample amount of studies show negative influence of CNS inflammation on learning, memory and neurogenesis in disease. However since the basal levels of cytokines in quiescent conditions are required for all forms of neural plasticity, it means that cytokines effects are dose-, time- and context-dependent.

Emerging roles of complement system in neural plasticity

C3 seems to be the central complement-derived factor that regulates different forms of neural plasticity. As mentioned above, the discovery of anaphylatoxin receptors expression on the surface of NSPCs pointed to the role of complement in regulation of basal neurogenesis. While C3aR signaling was indeed found to stimulate basal neurogenesis in olfactory bulb and dentate gyrus as well as migration and differentiation of NSPCs towards neurons *in vitro*, the role of C5aR in these processes has not been confirmed (Rahpeymai et al., 2006; Bogestål et al., 2007; Shinjyo et al., 2009). Additionally, in a model of permanent cerebral ischemia, genetic deficiency of C3 resulted in greater brain tissue damage and decreased neurogenesis in the peri-infarct cortex (Rahpeymai et al., 2006). The latter finding suggests the importance of C3 and/or its activation fragments in neuroprotection or healing of ischemic brain tissue and extends the neurogenic role of the complement system to injury-related context. It is plausible that the positive complement-related effects are conveyed indirectly by other cells as *in vitro* studies revealed the stimulatory effect of C3a on the release of NGF by microglia and astrocytes (Heese et al., 1998; Jauneau et al., 2006).

Both C3 and C1 have been found to mediate microglia-dependent elimination of synapses during early postnatal maturation of neuronal circuits (Stevens et al., 2007; Schafer et al., 2012). C1q, expressed by developing neurons, localizes to synapses that are thus tagged for elimination through the activation of the complement cascade and deposition of C3b fragments which are recognized by CR3 on microglia. Therefore, mice deficient either in C1q or C3 showed significant and sustained defects in synapse elimination in the thalamus. Additionally, C1q-deficient mice showed enhanced neocortical excitatory synaptic connectivity and epileptiform activity (Chu et al., 2010).

Finally, significant upregulation of C3 mRNA in the growth-associated transcriptome of post-ischemic brain tissue points to the prominent role of the C3 protein or its derivatives in neural plasticity and recovery processes, although the mechanisms thereof are yet to be determined (Li et al., 2010).

AIM OF THE THESIS

The overall aim of this thesis was to study the role of the complement system in brain plasticity and recovery after ischemic stroke as well as to test whether complement activation could be a predictive biomarker of functional outcome after stroke.

Specific aims were:

- To investigate the extent of complement activation in blood plasma of patients suffering from ischemic stroke of various etiologies and to test the hypothesis that these measurements can be helpful in predicting the functional outcome after ischemic stroke. (Paper I and II)

- To investigate whether genetic variation in the C3 gene is associated with ischemic stroke. (Paper III)

- To test the hypothesis that signaling through C3aR stimulates stroke induced neurogenesis and axonal sprouting/regeneration and leads to better functional outcome after experimental stroke. (Paper IV)

- To investigate the effect of C3 deficiency on hippocampal synaptic plasticity and cognitive performance. (Paper V)

METHODS

In this chapter, a summary of the methods used in the current studies will be given together with some methodological considerations. For further details of the experimental procedures, please refer to the papers.

Human subjects (I, II, III)

The study population for the clinical component of the thesis consisted of a large case-control cohort, the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), which constitutes a well characterized sample of ischemic stroke patients and healthy controls form Western Sweden (Jood et al., 2005). Briefly, patients (n=844) younger than 70 years of age and presenting with first ever or recurrent stroke at four Stroke Units in Western Sweden have been prospectively recruited between 1998 and 2008. During the acute phase of stroke, all patients were examined by a physician trained in stroke medicine and all patients underwent electrocardiography and neuroimaging with computer tomography and/or magnetic resonance imaging. Additional diagnostic work-up was performed when clinically indicated. Based on clinical presentation and results of the diagnostics, cases were classified into etiological subtypes according to the TOAST criteria (Adams et al., 1993). Stroke severity at inclusion was scored using the Scandinavian Stroke Scale (SSS) (Scandinavian Stroke Study Group, 1985). At three-month follow-up, functional outcome was assessed with the modified Rankin Scale (mRS) (van Swieten et al., 1988) through examination by a physician trained in stroke medicine. At two-year follow-up, surviving patients were contacted by a research nurse trained in stroke medicine for a structured telephone interview.

Healthy population controls (n=668), free from coronary or periphery artery disease, were randomly selected to match cases with regard to age, sex and geographical residence through the community-based health survey or from the Swedish Population Registry (Wilhelmsen et al., 1997). The control subjects were examined once by a research nurse trained in stroke medicine using the same questionnaire and protocol as for patients. All participants provided their written informed consent. Next-of-kin consented for those participants who were unable to communicate. The distribution of

etiological subtypes and baseline characteristics of the entire study population including vascular risk factors have been published elsewhere (Jood et al., 2005).

In the studies addressing the associations between peripheral blood complement levels and outcome (papers I and II), a subset of individuals from the entire SAHLSIS population was selected in order to avoid sample size bias related to the uneven distribution of etiological subtypes among patients. To this end, all patients classified as having stroke due to LVD (n =73) were included along with a similar number of patients with cryptogenic, SVD and CE stroke (n=79 for each type). Because the cryptogenic and SVD groups have a relatively favorable functional outcome, to minimize the effect of uneven distribution of outcome categories in this subgroup and thus improve the statistical power in the outcome regression analysis, all patients form these two groups with mRS score >2 were selected. The remaining patients in these two groups were selected so that they represented an even distribution of the mRS scores 0, 1 and 2. Regarding the selection of control subjects, for each of the four patient groups, half the number of controls was analyzed (selected so as to have the same mean age and sex distribution as the cases). In contrast, in paper III, data for all 844 patients and all 668 controls were analyzed as genetic association studies require large sample size to allow detection of relationships.

Comment: SAHLSIS includes only patients younger than 70 years, which constitute the vast majority of all of the admissions to stroke units in the region. The upper age limit for inclusion was chosen as the SAHLSIS project was designed primarily to study genetic associations and genetic components show greater influence in younger individuals (Flossmann, 2006). Generally, due to the younger age, unfavorable outcome including mortality was relatively rare. This fact together with the additional selection of patients that was required for reliable statistical analysis renders the presented results not necessarily representative of the general stroke population. Furthermore, as the SHALSIS project was not intended initially to study inflammatory markers, a detailed history of recent infections or inflammatory diseases was not obtained. This could have potentially influenced the levels of complement components in the blood.

Mice (IV, V)

To study the role of the complement system in synapse elimination in the hippocampus (paper V), homozygous mice deficient in C3 (C3 KO) were used (Pekna et al. 1998). They were backcrossed onto the C57BL/6 background for 13 generations to obtain mice congenic with the control wild type (WT) strain (Jackson Laboratories, Bar Harbor, ME, USA). Male mice of various ages were used for these experiments. For electrophysiology, 17 to 30 days old mice were used to cover the postnatal circuit refinement period, whereas learning behavior and epileptiform brain activity were assessed in 2.5 to 3 months old mice to study post-developmental consequences of altered synaptic plasticity as well as for practical reasons. Microglial activation was assessed at 6 months of age, to assess the potential occurrence of delayed or chronic differences between the strains.

To study the role of C3aR in experimental stroke (paper IV), two sets of adult (7 to 9 months old) male mice were used to approximate the clinical situation, while avoiding age-related complications. In the first cohort, mice deficient in C3aR ($C3aR^{KO}$) and $C3aR^{WT}$ mice were used (Kildsgaard et al., 2000). The second cohort consisted of transgenic mice expressing C3a under the control of glial fibrilary acidic protein (GFAP) promoter (C3a-GFAP) and their WT littermates which served as controls (Boos et al., 2004).

All experiments on animals were performed in accordance with the guidelines of the local ethical committee for animal research at the University of Gothenburg or the Malmö-Lund Ethical Committee for the use of laboratory animals, according to the location of the experiment was carried out. All experiments were conducted in accordance with European Union directives on animal rights.

ELISA (I, II)

The enzyme-linked immunosorbent assay (ELISA) is an antibody-based assay commonly used to quantitatively detect a specific antigen in plasma or serum. In the current thesis, sandwich ELISAs were used. The principle of the assay consist in applying diluted plasma sample onto microtitter plate previously coated with capture

antibodies directed against target protein and blocked to prevent unspecific binding of the analyte to the plate surface. Then the plate is washed to remove the unbound antigen followed by incubation with enzyme-linked detection antibody. After subsequent washing, the substrate is added, which is converted by the enzyme to the detectable form. Quantification is performed by absorbance measurements which are proportional to the antigen concentration in the sample and comparison against the standard values, *i.e.* values obtained from a serially diluted sample of known concentration of the target protein.

Plasma C3 levels were measured by an in-house developed assay using polyclonal rabbit anti-C3c immunoglobulins (Dako, Danemark) as capture antibodies and horseradish peroxidase-conjugated sheep (Biogenesis, UK) or horseradish peroxidase-conjugated rabbit (Dako) anti-C3c antibodies for detection. As a color substrate, 1,2-phenylendiamine-dihydrochloride (Sigma, MO, USA) was used and reaction product measured at 490 nm and compared against standard which consisted of pooled human serum. Plasma C3a levels were measured by a commercial assay kit according to the manufacturer's recommendations (Quidel Corporation, CA, USA).

To minimize the occurrence of errors in both pre-analytical as well as analytical phase, venous blood samples were taken into EDTA-coated tubes, according to the standardized protocol (Jood et al., 2005). For patients, sampling was performed within 10 days after admission to the stroke unit (median 4.25 days) and at three months, while for control blood was collected once. Obtained EDTA-plasma samples were aliquoted and stored at -80°C until the analysis. During the analytical procedures, the quality control measures included accurate pipetting preceded by vortexing of samples and inclusion of samples with known antigen concentration.

Comment: Plasma samples were chosen over serum as obtaining serum requires blood clotting, which can result in the activation of complement cascade. Conversely, EDTA used in plasma preparation prevents activation of coagulation- as well as complement cascade enzymes by chelating their essential co-factors Ca^{2+} *and* Mg^{2+} *ions. Plasma C3 levels reflect the net effect of C3 synthesis in the liver and its consumption due to complement cascade activation. Anti-C3 antibodies used in our studies recognize the*

internal fragment of C3 α chain but do not discriminate between intact C3 and its activation and degradation products (C3b, iC3b or C3c), thus they detect total C3 content. In contrast, the extent of activation of complement cascade is estimated by measurements of C3a levels. Of note, as C3a half-life in plasma is extremely short rendering it nearly impossible to correctly measure its content, the assay is designed to detect the more stable $C3a_{desArg}$ *form. The latter peptide is devoid of the classical anaphylatoxic properties but its levels faithfully reflect the extent of C3 cleavage due to cascade activation.*

Genotyping (III)

DNA for genotyping of the single nucleotide polymorphisms (SNPs) in the *C3* locus, was extracted from venous whole blood using commercially available kits. In the C3 gene, 16 tagSNPs (HapMap project) selected to cover a large amount of the variations in the gene using as few SNPs as possible, were analyzed, including rs344555, which has previously been shown to associate with C3 plasma levels. Genotyping was performed as a part of the analysis of a larger panel of SNPs using the Golden Gate assay (Illumina Inc., San Diego, CA, USA). The SNP assay for rs3745565 failed, so this SNP was genotyped using the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). Genotyping was performed blinded to case/control status by professional genotyping facilities at the Uppsala University and University of Gothenburg.

Comment: The tagSNP strategy takes advantage of the phenomenon of linkage disequilibrium in the human genome and allows for informative analysis of putative associations for many SNPs without the need for genotyping all of them (Barrett and Cardon, 2006). However, certain SNPs, which are indeed associated with the disease in question, may not be chosen as the tagSNP, although this information may be indirectly deduced. Additionally, high correlation of the remaining SNPs in the region with the tagSNP, allows for determination of haplotype associations, which might be more biologically informative than the association of a single marker.

Experimental stroke model (IV)

Cortical phothrombotic stroke was induced using the Rose Bengal method (Watson et al., 1985; Lee et al., 2007). The procedure requires anaesthetizing the animal with isoflurane and securing its head in a stereotaxic frame. Midline incision in the scalp is made to expose the scull surface. Photosensitive dye Rose Bengal is injected intraperitoneally (i.p.) and the target cortical area (in this study: left forelimb sensorimotor region) is illuminated through the intact skull for 12 min. As a consequence, photosensitive dye in the blood becomes focally activated by a cold light beam, which leads to production of singlet oxygen (a form of ROS). This leads to endothelial activation and formation of platelet-rich thrombi in the vessels within the illuminated area. Following illumination, scalp is sutured and animal is placed in a heated chamber until full recovery from anesthesia.

Upon returning to their home cage, animals were provided with moist mashed food in a dish placed on the floor of the cage to encourage eating. Animals received 1 ml of sterile saline i.p. to replenish lost fluids 24 h after stroke if they lost more than 2 g of weight overnight.

Comment: This stroke model is characterized by high reproducibility in the size and location of the infarct. Small cortical infarcts, producing deficits in the fine functions of the limbs represent a good model of clinical post-stroke rehabilitation. As opposed to ischemic stroke induced by classical middle cerebral artery occlusion (MCAo), the photothrombotic stroke model is minimally invasive and leads to an infarct with a clearly defined border. For these reasons, photothrombotic stroke model seems to be well suited for studies on post-stroke plasticity and regeneration without evoking an extensive inflammatory response, which may interfere with assessment of regenerative processes. However this form of cerebral injury does not allow significant collateral blood flow around the infarct and results in a relatively narrow penumbra region. Further, it does not permit recanalization, which occurs commonly in humans either spontaneously or as a result of tPA treatment.

BrdU administration (IV)

To be able to detect newly-born neurons, mice were injected with thymidine analogue bromo-deoxyuridine (BrdU). Systemic BrdU administration is a common way of labeling proliferating cells *in vivo.* The method relies on the incorporation of BrdU into DNA of dividing cells and its subsequent immunohistochemical detection in the nuclei of their daughter cells. Animals were injected with 50 mg/kg BrdU in saline i.p. twice a day, 8 h apart. First injection was given 24 h - and the last one on day 7 after stroke induction, as neurogenesis has been shown to peak during the first week after ischemic stroke in rodents.

Comment: A potential problem with BrdU labeling is that this analogue can be taken-up by the apoptotic cells during the aberrant cell cycle re-entry to attempt the DNA repair. However, the extent of this type of labeling is very low in comparison with the amount of BrdU incorporated in the process of DNA replication during normal cell division. Further, the labeled damaged cells do not last as long as the survival period for the animals used in our studies (Kuan et al., 2004). Additionally, BrdU is available in the body for around 2 h after administration and high doses of this drug have been shown to be toxic to the neural stem cells (Ross et al., 2008). Therefore, two smaller doses were chosen instead of a single large dose to minimize the potential toxicity and maximize the effectiveness of BrdU uptake.

Immunohistochemistry and fluorescent-dye neuron loading (IV, V)

Tissue preparation

Animals were deeply anaesthetized with sodium pentobarbital and transcardially perfused with saline followed by ice-cold 4% paraformaldehyde solution in phosphate buffer saline (PBS) 21 days after stroke induction. Brains were removed and immersed in the same fixative at 4ºC for 16 to 24 h. In paper IV, brains were dehydrated in graded series of ethanol washes, cleared with xylene, embedded in paraffin and cut coronally in 6µm thick slices. In paper V, fixed brains were cryoprotected by infusion with 30% sucrose in 0.1 M phosphate buffer (PB) for 3 days at 4ºC, frozen and cut into 30 µm thick floating sections.

Live hippocampal slices after patch-clamp recordings and concomitant neuron loading with neurobiotin (paper V) were fixed in 4% paraformaldehyde in PBS at 4°C overnight.

Comment: The advantage of using free-floating sections is that it allows deep penetration of antibody into the tissue and thus enables subsequent three-dimensional visualization of structures in thicker slices. On the other hand, paraffin embedding enables better preservation of the tissue morphology. This was important in the experimental stroke study as the fragile infarcted tissue is otherwise easily broken during sectioning or staining. Paraffin sections do not require special storage conditions and remain suitable for staining for many years. However, some antigens do not tolerate paraformaldehyde fixation and paraffin infiltration and may not be detectable on paraffin sections even after antigen retrieval.

Immunofluorescent staining

For the detection of post-stroke born neurons as well as synapses and axonal growth cones in the peri-infarct cortex (paper IV), brain sections were deparaffinized and underwent heat-induced antigen retrieval by intermittent gentle boiling in citrate buffer for total of 15 min. This step breaks the protein cross-links generated during fixation and is required for the unmasking of some antigens to be efficiently detected with antibodies. After cooling down and washing, unspecific protein binding was blocked by 30 min incubation at room temperature (RT) in PBS containing 0.05% Tween and 1 % bovine serum albumin (BSA) or 4% normal donkey serum (for BrdU/NeuN staining). For the quantification of microglia in the hippocampus (paper V), floating sections were washed and blocked with 5 % normal goat serum and 0.25 % Triton X-100 in PBS for 1 h at RT. Next, sections were incubated overnight at 4ºC with primary antibodies diluted in the respective blocking buffer. Following washing, sections were incubated with appropriate biotinylated or fluorescently-tagged secondary antibodies for 2 h at RT. Additional 1 h incubation with fluorescent dye-conjugated streptavidin was performed if the previous step involved biotin. Using the three-stage staining, less abundant antigens or antigens present in very small structures can be readily detected owing to the signal amplification

conveyed by each additional deposition step. After final washing, sections were mounted and coverslipped.

For the dendritic spine quantification, fixed hippocampal slices were washed with PBS and permeabilized with 0.01% Triton-X in PBS for 1 h at RT. After washing in PBS, slices were incubated with dye-conjugate in PBS for 3 h, followed by additional washing, mounting and coverslipping. For detailed information about antibodies and conjugates used for staining, see Table 2.

Target structure	Primary antibody (company)	Dilution	Secondary antibody (company)	Dilution	Fluorescent conjugate (company)	Dilution
post-stroke-born mature neurons	rat anti-BrdU (AbD Serotec)	1:150	donkey anti-rat-Alexa Fluor 488 (Invitrogen)	1:500		
	mouse anti-NeuN-biotin (Millipore)	1:100			streptavidin-Cy3 (Sigma)	1:100
pre-synaptic terminals	goat anti-synapsin I a+b (Santa Cruz)	1:150	donkey anti-goat-biotin (Jackson Research Lab)	1:200	streptavidin-Cy3 (Sigma)	1:100
axonal growth cones	mouse anti-GAP-43 (Millipore)	1:1000	rabbit anti-mouse-biotin (Dako)	1:200	streptavidin-Cy3 (Sigma)	1:100
microglia	rabbit anti-Iba1 (Wako)	1:1000	goat anti-rabbit-Alexa Fluor 488 (Invitrogen)	1:200		
dendritic spines on neurobiotin-filled neurons					streptavidin-Alexa Fluor 488 (Invitrogen)	1:1000

Table 2. Antibodies and dye-conjugates used for tissue stainings

Comment: Immunofluorescence is a sensitive method and an invaluable tool in determination of cell morphology and the localization of cells or subcellular structures. It is based on specific binding of the primary antibody to the antigen in the tissue and its subsequent visualization with fluorescent dye-conjugated secondary or tertiary reagent. As opposed to bright-field immunohistochemistry, it allows for the detection of more than one type of antigen in the same tissue section using different fluorophores thus extending the possibilities of the characterization of cells and other structures in the tissues. It also allows for clear visualization of very fine structures even in thick sections due to

adjustable high signal/background ratio. The disadvantage of using fluorescence as a detection system in comparison to light-stable chromogen, is the fact that it fades with time especially upon prolonged imaging. The quality of the staining depends on many factors and staining protocol needs to be optimized for each antigen and tissue. A negative control, most commonly consisting in the omission of the primary antibody, needs to be included in every assay to assess specificity.

Evaluation of the infarct volume (IV)

Infarct volume at 21 days post-stroke was evaluated on every 20th section containing the infarct (12-16 sections per mouse). After deparaffinization, sections were rehydrated and incubated in hematoxylin (which stains basophilic cell nuclei violet/blue) followed by eosin solution (which stains cytoplasm and extracellular deposits) for 2 min each with washing in water in between (HE stain). Next, sections were dehydrated in ethanol and isopropanol, cleared in xylene and coverslipped. Infarct size was evaluated morphometrically on digital bright-field microscopic images (Eclipse 80i, Nikon, Japan) with ImageJ software (NIH, USA) by manual delineation of the infarct and hemisphere areas by an investigator blinded to the experimental group. Volume of the injury was derived by multiplying area of total tissue loss that includes shrinkage due to scarring [(contralateral hemisphere - ipsilateral hemisphere) + infarcted tissue] on each section by the total inter-section distance.

Comment: HE staining is a simple and widely-used method of evaluating morphology on tissue sections. Clear infarct border created by a pronounced glial scar after photothrombotic stoke and negligible cytoplasmatic staining of the necrotic core enables straightforward and reliable delineation of the infarcted brain tissue. Measuring the area of the infarct on a few sections but within fixed coordinates with regard to bregma is generally used in experimental stroke studies (especially in MCAo models). However, in the present injury model with highly controlled location and clean border, analyzing sections spanning the entire infarct was considered a more accurate method.

Quantitative analysis of immunostainings (VI, V)

All image acquisitions and quantifications were performed by an investigator blinded to the genetic background of the animals.

Cell counting (IV, V)

$BrdU^+/NeuN^+$ cells (paper IV) were counted on images obtained by a confocal microscope (LSM TCS SP2, Leica Microsystems, Germany), using 40x objective, on 12-16 sections (separated by 120 µm intervals) per mouse. The results were expressed as cell density, obtained by dividing cell numbers by volume of tissue analyzed.

The $Iba1^+$ cell counting (paper V) was performed bilateral in 6 to 8 hippocampal sections per animal. Cells were counted in the dentate hilus and the granule cell layer (GCL) and the subgranular zone (SGZ), the latter defined as two cell body diameters below the GCL, using an epifluorescence microscope.

Peri-infarct synaptogenesis and axonal sprouting (IV)

Highest signal intensity single plane images of sections stained for synapsin I and GAP-43 were obtained by laser scanning confocal microscope (LSM TCS SP2) with 63x objective. Images were taken from two adjacent but not overlapping optical fields (referred to as proximal and distal) in several regions of the peri-infarct cortex and dorsal striatum. Corresponding images were taken in the contralateral hemisphere. Three standard sections per animal spaced by 160 µm were analyzed. All sections were scanned with the same acquisition parameters and images were analyzed with MetaMorph® software (Molecular Devices, USA) to obtain average number, size and intensity of positive punctate structures per image.

Comment: Because of the small size and high numbers of axonal terminals and synaptic puncta, automated high content image analysis (HCIM) was used for unbiased quantifications. Such an approach is highly reliable as opposed to measuring the total intensity of pixels per image. Artefacts from staining and microfolds of the tissue usually have very high intensity, seriously confounding the whole-image measurements. Conversely, by assessing the average value of parameters of the individual round-shaped

structures, this problem is largely eliminated due to these unwanted objects being automatically excluded as not fulfilling the set criteria. Moreover, such analysis is more informative as size of the puncta is related to number of pre-synaptic vesicles in terminals or number of boutons in multiple-synapses. The key point of this approach is to ensure homogenous conditions of staining and image acquisition, however even somewhat uneven intensity level of the staining would affect the other parameters only negligibly.

Dendritc spines (V)

Images of neurobiotin-filled neurons were obtained by a confocal microscope (LSM 700, Zeiss, Germany) at 63x magnification. Dendritic spines on apical secondary and tertiary dendrites of hippocampal CA1 pyramidal neurons were counted manually with the help of ImageJ software (NIH, USA). Spine densities were calculated as the number of spines divided by the length of the segment to approximate the density of excitatory synapses, which preferentially localize to the spines.

Electrophysiology (V)

Acute slice preparation

Mice were anesthetized with isoflurane and decapitated. Brains were rapidly removed and placed in ice-cold preparation solution to lower the tissue metabolism and thus increase neuronal survival. Transverse hippocampal slices (400 µm thick) were cut with a vibratome in the same ice-cold solution. Slices were subsequently stored in artificial cerebrospinal fluid (ACSF) at 25 ºC for a minimum of 1 h. For the recordings, a single slice was transferred to a recording chamber where it was kept submerged in a constant flow of perfusion solution at 25-28 ºC. Calcium and magnesium chloride content in the perfusion solution varied between the different experimental paradigms depending on the purpose: in the single and paired pulse experiments, the solution contained physiological (2 mM) concentration of $CaCl_2$ and $MgCl_2$ each, while in the MK-801 experiments, the solution contained 3 mM $CaCl_2$ and no $MgCl_2$ to enable NMDA receptor opening. In the whole-cell patch-clamp recordings and the burst experiments, the solution contained higher dicationic salt concentration, 4 mM each of $CaCl_2$ and $MgCl_2$, to suppress spontaneous network activity. Picrotoxin was always present in the perfusion

solution to block GABA receptor-mediated activity. All solutions were continuously bubbled with 95% O_2 and 5% CO_2 (pH 7.4).

Comment: Hippocampal slices provide distinct experimental advantages over other in vitro and in vivo preparations of the hippocampus. The most significant advantages are that, except for the absence of afferent input, hippocampal slice preparations retain the cytoarchitecture and synaptic circuits of the intact hippocampus, yet are readily accessible for optical imaging or electrophysiological studies that can also include pharmacological manipulation.

Extracellular field recordings

For field excitatory postsynaptic potential (fEPSP) recordings, electrical stimulation and recordings of synaptic responses were carried out in the CA1 region of the hippocampus with the stimulation and recording electrode positioned in the *stratum radiatum*. Biphasic constant current stimuli (at varying frequency dependent on the experiment type) were delivered via tungsten wires to the axons of the pyramidal cells of the CA3 area, called the Shaffer collaterals, while a glass pipette, placed in the layer of apical dendrites of the pyramidal CA1 neurons, was used for field recordings. The stimulation intensity was set such that it would not evoke firing in the postsynaptic neurons, as evidenced by the absence of a population spike distorting the fEPSP. Evoked responses were analyzed off-line in a blinded manner using custom-made IGOR-Pro software (WaveMetrics, Lake Oswego, OR, USA).

Input/output measurements (synaptic efficacy)

Synaptic efficacy evaluation in C3 KO and WT mice was performed by input/output measurements after pulse stimulation of 0.2 Hz. In this type of experiment, fEPSP, that is the response of the population of activated synapses, is preceded by a pre-synaptic fiber volley, the amplitude of which is proportional to the number of stimulated axons. Plotting the magnitude of fEPSP against size of the fiber volley at the different stimulation intensities allows for calculation of total synaptic efficacy per axon.

Release probability measurements

The differences in release probability (p), were first assessed by measuring the response ratio to paired pulses (50 ms apart) to obtain a straightforward measure of facilitation or depression. Next, recordings with an irreversible NMDAR open channel blocker, MK-801, were performed (Tocris Cookson, UK). In these experiments, in response to a 0.5 Hz-stimulation, the release of the synaptic vesicle results in the opening of the opposing NMDARs. The resulting EPSP is recorded but due to the presence of MK-801, channels become immediately blocked, preventing further flow of ions *i.e.* they are being turned-off in a release-dependent way. The probability of the neurotransmitter release is estimated in a nearly direct manner by evaluating the propensity of EPSP magnitude to decay. In this experiment, AMPARs were blocked by the presence of NBQX compound (Tocris Cookson).

To mimic the *in vivo* situation where the isolated stimuli, used in the experiments described above, happen very rarely, fEPSP were measured also as a response to a burst stimulation. In each recording, 30 trains of 10 high-frequency (20 Hz) impulses were delivered at 30 s intervals (total of 300 pulses).

Comment: The extracellular signal from a single neuron is extremely small and thus hard to record in the brain. However, in the hippocampus, neurons are arranged in such a way that they all receive synaptic inputs in the same area. Because these neurons are in the same orientation, the extracellular signals from synaptic excitation do not cancel out, but add up to giving a robust signal that can easily be recorded with a field electrode. The advantages of extracellular recordings are experimental simplicity, unchanged intracellular environment and generation of stable recordings over long period of time. A common obstacle, however is that the fEPSP often becomes distorted by back propagating action potentials. To overcome this problem, the initial slope of the EPSP was measured instead of the amplitude of the burst response.

Intracellular recordings

Whole cell patch-clamp recordings

Patch-clamp technique is based on establishing a high resistance seal between the tip of glass pipette containing an electrode, and a cell, thus enabling control of the cell membrane voltage or current (Neher and Sakmann, 1976). In this thesis, by setting voltage over the membrane (”clamping the membrane potential”), miniature currents flowing over the cell membrane required to maintain that potential were measured and analysed in a blinded manner using Mini-analysis program (Synaptosoft, Decatur, GA, USA). Recordings were performed on visually identified neurons under the infrared differential-interference contrast microscope (Olympus BX51WI, Japan). The recording pipette solution contained 0.5% of neurobiotin, which loaded the patched-clamped neuron (for subsequent visualization) during the recording.

Comment: The major advantage of whole cell recordings is that they enable control the cellular environment and the assessment of the quantal (q) parameters of the neuron. However, patch-clamp recordings are technically demanding and more invasive (due to disruption of the intracellular milieu) than extracellular recordings, and can sometimes induce LTP (Malinow and Tsien, 1990).

***In vivo* electroencephalography (V)**

In order to detect possible spontaneous epileptiform activity, C3 KO and WT mice were unilateraly implanted with a recording electrode in the hippocampal CA3-CA1 region. EEG activity was recorded 10 days later, twice for 12 hours and evaluated for seizures and focal interictal spike activity by an investigator blind to the animal’s genotype.

Behavioral testing

All tests were performed and scored by an investigator blinded to the genotype of the animals tested.

Evaluation of sensorimotor deficits (IV)

Spontaneous forelimb asymmetry task: Mouse was placed in a glass cylinder allowing it to stand on the base and small enough to encourage rearing. Mice were videotaped for 2 min with a mirror behind the cylinder to ensure good visibility from all directions. The number of times the right and the left forepaw were used to make first contact with the cylinder was recorded. Due to stroke-related deficits mice are expected to avoid using the right paw to support the body while rearing. Forelimb asymmetry index was calculated as the ratio of right (affected) first paw touches to total first paw touches [R/(R+L)]. Testing was performed at 48 h as well as at 7, 14 and 21 days after stroke.

Adhesive removal: Circular adhesive stickers, cut in half, were used as bilateral tactile stimuli on the palmar surface of each forepaw of the mouse, and the time to remove each sticker was recorded. Mice were trained to readily remove both stickers within 15 seconds before stroke induction. Sensorimotor deficit due to stroke is expected to cause impaired perception and delayed removal of the sticker on the affected paw. Forelimb asymmetry was calculated as a ratio of time to remove the sticker from right (affected) paw to time to remove the sticker from the left paw [R/L]. Animals were tested 24 and 48 h as well as post-stroke day 7, 14 and 21.

Comment: Behavioral tests are the invaluable tool for studying the effects of different factors on animals' recovery from the experimental stroke. However, high variability of the individual performance within the group may easily preclude finding a between-group difference. Both of the tests used in this study depend on animal's eagerness to explore and repeated testing may result in the decline in exploratory behavior. Additionally, stress related to the handling procedures (restraining prior adhesive removal test) may result in anxiety-like behavior, further increasing measurement variability.

Evaluation of hippocampal-dependent cognitive performance (V)

The IntelliCage® platform (New Behavior, Zurich, Switzerland) enabling unbiased analysis of learning over time in a social context, in home cage environment was used to evaluate hippocampal function. First, mice were tested for baseline exploratory behavior

for 5 days. In the second experiment using different cohort of animals, mice had an introduction period of 5 days in the IntelliCages, during which they were acclimatized to performing nose pokes to gain access to the water bottles. The introduction period was followed by a place learning period, for which each animal was randomized to one corner. This allocated corner was programmed as the correct corner while the other three corners were programmed as incorrect. The mice were only allowed to drink from the water bottles in the correct corner, where a nose poke would open a door and give them access to the water bottles. In the incorrect corners, the doors to the water bottles could not be opened by performing nose pokes. After 5 days, the animals were randomized to a new corner for the reversal learning. Food was provided *ad libitum* during the experiments and mice were provided with shelters within the cage. Only the active (dark) period was analyzed.

Comment: The great advantage of using the IntelliCage® platform is that it eliminates stress resulting from handling and changing animals' environment as e.g. in Morris water maze test. The system is fully automated, as mice are carrying a subcutaneous microtransponders to allow identification of individual animals and tracking of their behavior. However, drawing conclusions from the wealth of data generated by this system requires advanced statistical analysis. Besides, a number of considerations prior to the experiment as well as during data analysis are needed to avoid confounding the measures. For example, one should avoid testing too young animals to prevent two animals from being able to use the corner at the same time; exclude the long-lasting visits, which might indicate unintentional touching the sensor with the back while e.g. sleeping in the corner; or exclude animals with very low frequency of visits and water licks which might indicate the animal's failure to understand how the corners work or transponder registration failure.

Statistics

Different statistical approaches were employed throughout the studies depending on the nature of the data and the type of scientific question asked. Due to predominantly non-Gaussian distributions of the clinical data (especially C3 and C3a measurement), almost exclusively non-parametric statistics were used in the studies described in paper I

and II. Associations between complement levels and ischemic stroke or functional outcome after stroke (paper I and II) as well as between genetic variants of C3 SNPs and ischemic stroke or its subtypes (paper III) were analyzed by binary logistic regression (R statistical computing package v2.6, R DC Team, Vienna, Austria - http://www.R-project.org/ or SPSS v17.0, SPSS Inc. Chicago USA). To be able to practically determine the importance of the detected significant association, a measure of effect size is reported. To this end, odds ratios (ORs) and their confidence intervals (CIs) were estimated. All the association analyses were additionally adjusted for the effect of traditional vascular or outcome-related risk factors. Furthermore, the values of complement protein levels were divided into tertiles to obtain biologically informative unit of effect measures in the regression analyses. Associations between the genetic variants and C3 or C3a levels were estimated by linear regression with genotypes coded as ordinal categorical variables (R statistical computing package).. Values for complement measurements were first logarithmically transformed to obtain approximately normal distribution of the residuals, therefore back-calculated values of protein levels are reported instead of a difficult to interpret β coefficients of log-data.

For the statistical analyses of all electrophysiology experiments (paper V), brain slices were treated as replicates, *i.e.*, reported n equals number of slices analyzed. Input/output data were modeled by linear regression and the resulting functional coefficients were compared by Student's *t*-test. Glutamate release probability in the MK-801 experiment was modeled by non-linear regression (exponential decay function). Resulting best-fit curves for C3 KO and WT strains were compared globally with the F-test, *i.e.,* comparing the sum of squares of fits of two separate curves for each data set versus fitting one common curve for both strains. Additionally, the time constant of the decay was compared with *t*-test after adjusting for the correct number of degrees of freedom (GraphPad Prism, v5.0a, San Diego, CA, USA).

Behavioral stroke data (paper IV) were analyzed by two-way ANOVA (effect of time and strain) with repeated measurements, followed by Dunnett post-hoc tests (GraphPad Prism). For the analysis of different parameters measured in the IntelliCage (paper V), generalized estimating equations (GEE) were used to estimate the average response of the populations. These analyses were performed using SigmaStat 2.0 (SPSS Inc).

The remaining data were analyzed by unpaired t-tests between groups or paired t-tests within group (GraphPad Prism). For data sets with non-Gaussian distribution, non-parametric tests - Mann-Whitney *U* test and Wilcoxon signed rank test, respectively - were used. To avoid false positive results, Bonferroni correction was employed for testing with multiple pair-wise comparisons. Reported *P*-values are the corrected ones. In the genetic association study (paper III), correction for multiple testing was performed by multiplying the *P*-values by the number of haplotype blocks as Bonferroni correction would be an excessively conservative method due to genetic variants being dependent on each other. Data are presented as mean ± SEM, or median ± interquartile ranges (IQRs), when data followed a non-Gaussian, unless stated otherwise.

RESULTS AND DISCUSSION

Systemic complement response differs between ischemic stroke subtypes (Papers I and II).

To date only a few and relatively small studies addressed the question of systemic complement activation during ischemic stroke while the reports on complement influence on functional outcome are even scarcer. Therefore, to evaluate the extent of complement response in ischemic stroke, we measured plasma C3 and C3a levels at the acute and delayed stage after stroke in patients classified to the four major ischemic stroke subtypes and compared them to the levels of the control subjects.

We observed marked differences in the temporal pattern of plasma complement levels among patients suffering from ischemic stroke due to different etiologies. Plasma C3 levels were increased above the control level in all stroke groups in the acute phase while they remained elevated at three-month follow-up in LVD, SVD and cryptogenic but not CE stroke. Interestingly, only for the cryptogenic stroke patients the follow-up C3 levels did not decline as compared to acute stage, although this state was not associated with systemic elevation of C3a levels. In the remaining subtypes, C3a levels were still increased at three months after stroke. High (upper third) acute phase levels of both C3 and C3a were associated with CE and cryptogenic stroke independently of vascular risk factors, while high levels of C3 and C3a in both acute and convalescent phase showed independent association with the LVD and SVD stroke. Moreover, in the cryptogenic and CE stroke group, C3 levels at both time points were correlated with CRP levels. Levels of C3-activation-derived C3a peptide were correlated with CRP levels at both time points only among the LVD patients.

The finding that three-month levels (assumed to approximate the pre-stroke levels) of C3 and C3a were associated with patient status in LVD and SVD independently of traditional risk factors points to similarities between these subtypes, the chronic nature of the underlying pathology and the potential role of complement in this process. Indeed, atherosclerosis is often associated not only with LVD but also SVD stroke (Jung et al., 2012) and the complement system, and C3 in particular, is involved in the regulation of atherogenesis (Persson et al., 2004; Oksjoki et al., 2007; Leung et al., 2009). However,

with respect to the patterns of complement activation and the role of CRP, SVD and LVD subtypes appear to be two distinct entities as C3a and CRP levels correlate only in the LVD group. On the other hand, with regard to the correlation between systemic hsCRP and C3 levels, the cryptogenic stroke subtype appears to resemble CE subtype, and not the milder form of LVD as previously suggested (Bang et al., 2003). This may also indicate that a substantial fraction of cryptogenic strokes are indeed caused by a form of cardioembolism, at least in our study population. Such a possibility has been actually proposed based on gene expression profiling (Jickling et al., 2012). However, as that study did not distinguish between the two subcategories of undetermined etiology stroke, data presented there and our results cannot be directly compared.

Plasma C3 and C3a levels show etiology-dependent associations with functional outcome after ischemic stroke (Papers I and II).

We also investigated the correlation of plasma C3 and C3a levels with the functional outcome after stroke. Three-month follow-up C3 levels in the LVD patients as well as C3 levels at both time points in the CE group showed a correlation with the extent of initial impairment measured by SSS at admission. Moreover, the high three-month C3 levels were associated with unfavorable outcome (mRS: 3-6) both at 3 months and 2 years after stroke in LVD and CE patients and this association was independent of age and sex. Such an association was not found in the remaining groups. Interestingly, moderate levels (middle third) of C3a/C3 ratios at 3-month follow-up in CE patients were associated with favorable outcome (mRS: 0-2) at 2 years post stroke. All the associations in the CE group withstood additional correction for SSS score and CRP levels.

It cannot be ruled out that infarct size affects C3 levels in blood and the association with functional outcome among different subgroups. However, unlike high acute phase CRP levels, that are associated with long–term outcome and correlate with brain infarct volume (as shown here as well as in other studies), only C3 levels in the delayed phase after stroke showed significant and independent association with outcome in our study. The associations with outcome in CE stroke remained significant even after adjustment for CRP levels and SSS score, which is an approximated measure of the

extent of the injury, supporting the notion that the complement system plays a role in stroke and that for CE stroke patients, three-month plasma complement measurements may be better predictors of functional outcome than CRP. It is noteworthy, however, that low number of patients suffering from cryptogenic and SVD stroke displayed mRS score above 3 on either time point of outcome evaluation and therefore low statistical power might have precluded finding any significant associations in those groups.

Additional factors, such as *e.g.* infection or subsequent more subtle thromboembolic events that can contribute to high systemic C3 levels and negative outcome in the CE and LVD patients could have also affected the results. The lack of a detailed record of recent infections or chronic inflammatory states in SAHLSIS is a clear limitation. The finding that moderate level of complement activation measured by C3a/C3 ratio (middle third) is associated with favorable outcome in the CE patients is intriguing and warrants the mechanistic studies on the beneficial properties of complement activation, such as the atheroprotective effects of $C3a_{desArg}$ (Lewis et al., 2011).

Taken together, studies described in paper I and II show complex associations of plasma complement levels with ischemic stroke and patient's functional outcome. Future larger studies controlling for infections and stratifying patients both according to stroke severity and etiology are warranted.

Genetic variation in complement component C3 shows association with ischemic stroke (Paper III) as well as C3 and C3a levels (unpublished data).

To evaluate the potential associations of genetic variation in the *C3* locus with ischemic stroke, we genotyped 16 tag SNPs within the *C3* region in entire SAHLIS population and analyzed the results with relation to clinical data.

Two SNPs were found to be associated with ischemic stroke independently of the effect of age and sex. The minor allele (G) of rs2277984 was associated with increased risk of overall ischemic stroke, while the minor allele (C) of rs3745565 showed

association with decreased risk of stroke. These associations remained significant after additional adjustment for risk factors such as hypertension, diabetes, and smoking. Interestingly, when these two SNPs were analyzed in each of the main stroke subtypes individually, a positive association between the minor allele of rs2277984 and the cryptogenic stroke was observed. This association was also independent of all the mentioned risk factors in a multivariate model. Haplotype analysis did not add any further information. After correction for multiple testing, only the association of rs3745565 with overall ischemic stroke remained significant.

Both rs2277984 and rs3745565 are intronic variations and are not linked to any known functional SNP(s). In contrast, no association was detected for rs2230199, a non-synonymous SNP in exon three resulting in a change in the electrophoretic properties of C3 protein, which is in line with other published studies (Kramer et al., 2000). The association of rs2277984 with cryptogenic stroke only may be caused by a possible subtype-specific effect, but also simply the lack of statistical power to detect associations in the remaining stroke subtypes. Indeed, our cryptogenic stroke population includes the largest number of participants out of the four subtypes, being around 1.5 to 2 fold greater than each of the remaining groups.

We also investigated the possible influence of genetic variants of the *C3* gene on the plasma levels of C3 and C3a among the selected individuals (n=469, 310 patients and 159 controls, Table 3) from the SAHLSIS population (characterized in papers I and II). In this analysis, using pooled control and three-month patient measurements, two SNPs were found to be associated with complement protein levels in the univariate as well as in multiple-adjusted model. Inclusion of rs3745565 was found to significantly improve (P=0.0034) the basic prediction model of C3a levels that included age, sex and case/control status through the addition of 2.55% of the explanatory value of C3a variance, which then equaled 4.74% (P=0.0007). Its rare allele (C), associated also with the control status in SAHLSIS population, was associated with the decrease of 12.04 ng/L (13.32% of the geometric mean) in plasma C3a level (P=0.021) in heterozygotes. However, homozygous C allele carriers had on average 40.46 ng/L higher C3a levels (44.77%) than homozygous major allele carriers (P=0.025), most probably due to predominance of patients in this genotype group. The other variant, the rs237554,

was found to significantly improve the basic model of plasma levels of C3 and C3a (*P*=0.0200 and *P*=0.0026, respectively), which then predicted 6.78% and 4.03% of the respective variances. Although the contribution of this variant to the determination of C3 and C3a level was only 1.66% and 1.84% respectively, homozygous minor allele (AA) carriers had 129.00 mg/L (31.22% of geometric mean) higher C3 levels than homozygotes for the dominant allele (GG) (*P*=0.014), while in heterozygotes (AG) the increase of C3a levels was on average 17.10 ng/L (18.92% of geometric mean, *P*=0.005).

SNP	Genotype	Controls	Ischemic stroke	Total
rs3745565	GG, n (%)	106 (66.7)	233 (76)	338 (72.8)
	CG, n (%)	50 (31.4)	64 (21)	114 (24.6)
	CC, n (%)	3 (1.9)	9 (3)	12 (2.6)
rs237554	GG, n (%)	109 (68.6)	232 (75.6)	341 (73.2)
	AG, n (%)	47 (23.6)	70 (22.8)	117 (25.1)
	AA, n (%)	3 (1.9)	5 (1.6)	8 (1.7)

Table 3. Genotype frequencies in controls and patients with the plasma complement levels data available

Both rs237554 and 3745565 are deep-intronic SNPs in the central region of the C3 gene (intron 28 and 26, respectively), therefore it is difficult to speculate about any potential causal relation with plasma C3, and in particular C3a levels. It is unlikely that these SNPs represent any direct link with complement components level, although theoretically introns may contain sequences that could influence regulation of protein expression level (Cooper, 2010). Instead, these associations might be indicative for other, yet unidentified, causative variants remaining in the tight linkage disequilibrium with the analyzed SNPs, and localizing within the coding region of the *C3* gene or even outside its locus. Regardless of the actual type of relationship, the mechanism behind the effects of these variants seems to play only a subtle role in regulation of C3 production and activation. It is intriguing that the rare allele, which showed association with decreased risk of stroke, was also associated with lower levels of C3a independently of the case/control status. It is also possible that these associations are mediated by other factors.

Although potentially interesting, due to a very small sample size for the genetic association study and an extremely low number of homozygous uncommon allele carriers, these results should be treated with caution. The effects and strength of the association are rather small and some of them would not withstand correction for multiple testing. Therefore at least some of the above findings can be false-positive and future larger studies, with detailed fine mapping of these SNPs and controlling for factors such as levels of inflammatory markers, may clarify the issues raised by this study.

Receptor for complement peptide C3a stimulates neural plasticity after experimental brain ischemia (Paper IV).

C3, C3a peptide and its receptor have been implicated in the neuroprotection, regulation of adult neurogenesis and synaptic reorganization during development in rodents. To determine the role of C3a-C3aR signaling in ischemia-induced neural plasticity and post-stroke recovery, we subjected C3aR-deficient ($C3aR^{KO}$) mice, transgenic mice with brain-specific and complement activation-independent C3a peptide expression (C3a-GFAP) and their respective wild type (WT) control mice to photothrombotic stroke.

We found that the infarct size at 21 days after stroke did not differ between the groups but C3a overexpression increased, whereas C3aR deficiency decreased post-stroke neurogenesis as assessed by the number of newly-born neurons in the peri-infarct cortex. We found significantly increased number and size of synpasin I^{+} pre-synaptic puncta in the proximal peri-infarct cortex as compared to the contralateral, uninjured cortical regions in all of the experimental groups. However, $C3aR^{KO}$ mice had markedly fewer synaptic puncta than $C3aR^{WT}$ mice in the contralateral hemisphere, whereas C3a-overexpression did not lead to further increase in the number of putative synapses. Also in the striatum, the number and size of synaptic puncta was reduced in the uninjured hemisphere in $C3aR^{KO}$ as compared with $C3aR^{WT}$ animals. The difference between the strains was present also in the peri-infarct motoric cortex but only in regions located more than 240 μm away from the injury border.

The quantification of GAP-43$^+$ objects revealed a decreased number of activated axonal growth cones in the ipsilesional striatum and in both the ipsi- and contralesional somatosensory cortex of the C3aR^{KO} mice. Interestingly, C3a-GFAP mice had more and larger GAP-43$^+$ structures than WT animals, both in the injured and uninjured cortex as well as more growth cone-like objects in the striatum. Jointly, these findings suggest that C3aR signaling is important in several forms of structural plasticity after ischemic stroke. Two independent behavioral tests measuring fine sensorimotor performance indicated that mice with a deletion of C3aR have greater impairment of the stroke-affected forepaw function than their WT counterparts. However, the C3a-overexpressing mice did not show any difference in synaptogensesis and performance compared to their WT littermates.

Although previous studies showed that C3 deficiency, that precludes production of the C3a peptide, is associated with increased infarct volume after MCAo, and C3a-GFAP mice are protected against neonatal ischemic brain injury, we did not observe any neuroprotection mediated by C3a-C3aR axis in the current stroke model. However, the photothrombotic stroke model is characterized by a limited penumbra region and therefore not likely to show any neuroprotective effects (Porritt et al., 2012). On the other hand, this study corroborates and extends the previous findings of the positive role of C3aR signaling in the regulation of neurogenesis. We showed that the overexpression of C3a in reactive astrocytes was associated with an increased number of newly born neurons in the peri-infarct region, implying that the endogenous neurogenic response to ischemia can be boosted by C3a. However, the comparable functional performance of C3a-GFAP mice and their WT littermates suggests that the contribution of peri-infarct neurogenesis to sensorimotor function recovery in mice, at least during the first three weeks after stroke, is negligible.

As the effect of C3aR deficiency on synaptic plasticity was observed primarily in the infarct-remote areas, it is conceivable that the potential positive effects of C3aR signaling on synaptic plasticity in the direct infarct vicinity are diminished by its other functions, such as glial cell activation or recruitment of blood-borne inflammatory cells in the infarct-proximal cortical region. Alternatively, as the contralateral synaptic plasticity in rats (Luke et al., 2004) has been associated with the engagement of the unaffected limb

and improvement of function, it is possible that employment of compensatory behaviors by the animals in our study contributed to the contralateral hemisphere response.

Our observation that the expression of GAP-43 was reduced in the absence of C3aR, whereas it was increased when C3a was expressed in reactive astrocytes, together with the reports of C3 upregulation in sprouting neurons isolated from the rat cortex after ischemic stroke (Li et al., 2010) and the stimulatory effect of C3a on neurite outgrowth *in vitro* (Shinjyo et al., 2009), supports the conclusion that C3a signaling through C3aR plays a positive role in axonal sprouting after stroke. The unchanged functional performance of C3a-GFAP mice in spite of the marked increase in axonal growth marker expression might be due to the behavior tests used being not sensitive enough to detect subtle changes offered by C3a overexpression during the first three weeks post-stroke. It is also plausible that, although beneficial at a later stage, high C3a levels, resulting from reactive gliosis-driven C3a expression in the acute phase do not provide an optimal milieu for the induction of regeneration and instead hinder post-ischemic neural plasticity.

In conclusion, these results indicate that C3aR signaling stimulates post-stroke cortical neurogenesis as well as synaptogenesis and axonal sprouting and should be considered as a target when designing therapeutic strategies to improve functional recovery.

Altered cognitive performance and synaptic function in the hippocampus of mice lacking C3 (Paper V).

As C3 was found to regulate both the number of synapses and hippocampal neurogenesis, we sought to determine the effects of constitutive genetic ablation of C3 on the functions of an unchallenged adult hippocampus by assessing place and reversal learning ability and synaptic function in C3 KO mice.

Hippocampus-dependent function test using the IntelliCage platform required mice to first learn where to access water by performing a nose poke (place learning,

corner 1) and then, in the subsequent phase (reversal learning, corner 2), to unlearn the previous behavior and learn to find the new allocated water corner. We observed a significant difference in both the number of incorrect visit ratio and absolute number of incorrect nose pokes ratio per day between C3 KO and WT mice over the five days of testing. For both of the measures, C3 KO mice performed better and displayed greater improvement per day in odds ratio in both place learning and reversal learning phase.

Using extracellular field recordings in hippocampal slices, we found that the net efficacy of CA3-CA1 synapses was unchanged in C3 KO mice, whereas paired-pulse and MK-801 experiments revealed decreased glutamate release probability (p) in these mice. Since quantal parameters (q) of synapses of the single CA1 pyramidal neuron did not differ between the strains as judged by AMPA mESPC intracellular recordings, we concluded that C3 KO mice must have increased number of functional synapses in the hippocampus. Based on the electrophysiological data, the increase is estimated to be around 20% in C3 KO mice close to puberty. Despite the putative increase in number of glutamate release sites, we did not observe any spontaneous epileptiform activity in C3 KO mice, which is in contrast to studies investigating the neocortex of C1q KO mice (Chu et al., 2010). As both C1q and C3 are needed for synaptic circuit refinement and mice deficient in either of these components show the same phenotype, at least within the retinogeniculate system (Stevens et al., 2007), the decrease in release transmitter probability can be viewed as a homeostatic compensatory plasticity, the capacity of which might be brain region-dependent.

Additional experiment revealed that in contrast to single pulse, high-frequency burst stimulation evokes significantly larger overall postsynaptic response in C3 KO than in WT mice. This finding points to a limited capacity of these compensatory mechanisms in the hippocampal CA1 region of C3KO mice. Surprisingly, we did not find any significant difference between the strains in the number of dendritic spines, which are the primary excitatory synapse-bearing structures. The discrepancy between morphological and electrophysiological results is intriguing and merits further investigation. Possible explanation might be related to quantification inaccuracy due to limitations of conventional light microscopy and the type of labeling method, or predominant differences in the numbers of shaft, but not spiny synapses.

Although C3-deficincy has been shown to result in reduced hippocampal neurogenesis (Rahpeymai et al., 2006), enhanced place and reversal learning of C3 KO mice implies that neural plasticity at the level of synaptic function is a more important factor in determining hippocampus-dependent learning than the rate of generation of new neurons in the dentate gyrus, at least in the context of C3 deficiency. Therefore, the role of C3 in synapse elimination in the context of neurodegenerative pathology such as Alzheimer's disease and the possible benefit for cognitive performance of synapse sparing by C3 inhibition warrants further studies on the role complement in the aging or diseased hippocampus.

MAIN CONCLUSSIONS

The results of this thesis support the view that complement system is involved in the pathophysiology of ischemic stroke. Further, this work demonstrates that complement system regulates different forms of basal as well as stroke-induced synaptic plasticity.

Specifically we found that:

- Plasma C3 and C3a levels are elevated in ischemic stroke as long as 3 months after stroke but the dynamics of this increase as well as the potential prognostic value of these markers are strongly influenced by ischemic stroke etiology. Importantly, for CE stroke patients, C3 levels measured at follow-up may be a better predictor of outcome than CRP.

- In the *C3* gene, rare allele of rs3745565 SNP is associated with decreased risk of overall ischemic stroke as well as lower C3a levels, while rs2277984 shows association with cryptogenic stroke subtype. Additionally, rs237554 is associated with increased level of both C3 and C3a.

- C3aR signaling plays a positive role in neural plasticity after experimental ischemic stroke and deletion of C3aR leads to disturbance of these processes and greater functional impairment. Brain-targeted overexpression of C3a stimulates post-stroke cortical neurogenesis and axonal sprouting, which may potentially contribute to functional recovery.

- C3 deficiency alters synaptic function and most probably the synapse number in the maturing hippocampus. These changes are associated with improved spatial learning.

CONCLUDNG REMARKS AND FUTURE DIRECTIONS

It needs to be noted that the results from human subjects and animal models cannot be directly compared due to profoundly different aspects of the role of complement in stroke investigated in these studies. Nevertheless, they both are relevant for the development of a holistic approach to treat ischemic stroke. Deciphering inflammatory mechanisms in ischemic stroke, including those involving the complement system, is of a great importance for better understanding of stroke pathophysiology. This in turn is indispensible for designing successful and safe therapies that could help limit the extent of brain damage resulting in smaller initial deficit. Equally or even more important is the investigation of the mechanisms underlying post-stroke plasticity and repair processes and possibilities to boost them in order to promote recovery of lost functions.

Apart from the limitations due the lack of data on possible underlying inflammatory conditions in SAHLSIS, the inclusion criterion of age restricts the generalizability of our results from this relatively young cohort of patients as ischemic stroke incidence increases with age. Therefore, a replication study including older patients would be necessary to confirm our findings. Furthermore, it would be useful to measure plasma levels of complement proteins at several strictly defined time points to obtain a better temporal profile of the complement response. Such information could help in defining a suitable time frame for eventual acute phase therapies aimed at inhibiting complement activation.

Our current data do not allow us to establish the mechanism by which C3a and its receptor regulate synaptogenesis and axonal sprouting. As most or all brain cells express C3aR, it remains to be determined whether the observed effects are neuron-autonomous or are mediated by the responses of glia or other cell type. These issues could be resolved by using WT, C3 and C3aR-deficient primary cultures of for example astrocytes and microglia and their co-cultures with neurons under normal as well as ischemic conditions. We are currently addressing the possibility that C3aR-deficient mice have altered synaptic density in the cortex in the absence of ischemic injury. Such a finding would indicate yet another aspect of the complement-dependent regulation of synapse number during development.

Our findings of the beneficial effects of brain specific C3a expression on post-stroke neurogenesis and axonal plasticity together with a recent report showing that C3a overexpression confers a robust neuroprotection against neonatal hypoxic-ischemic injury and intraventricular administration of C3a peptide ameliorates hypoxia-ischemia induced cognitive deficits (Järlestedt et al., 2013), are very encouraging. Despite the differences in ischemic injury model and the maturation stage, the latter results prove the feasibility and effectiveness of exogenous administration of C3a peptide to the brain. However, determining the precise timing of such a therapy for ischemic stroke would be crucial as it might be suboptimal or even deleterious to administer C3a immediately after stroke. Another challenging aspect of designing such a complement-based therapy for modulation of plasticity response after ischemic stroke would be the development of an efficient, safe and non-invasive administration route for the drug to avoid systemic effects and problems with crossing BBB.

Although previous studies from our laboratory showed that mouse NSPCs express receptors for both C3a and C5a, neither C5aR-deficient nor C5a-overexpressing animals showed an altered basal neurogenesis (Bogestål et al., 2007). However, it is possible that the effects of C5a may not observable in unchallenged animals. Therefore, we are in the process of evaluating the role of C5a-C5aR signaling using the methods described in paper IV. Taking into consideration that the half-life of C3a and C5a is extremely short, it is possible that at least some of the effects of C3a and C5a in CNS are mediated by their more stable desarginated forms. Therefore, due to its affinity for both $C5a_{desArg}$ and $C3a_{desArg,}$ and a possible modulatory effect on C5aR signaling, it would be interesting to evaluate the role of C5L2 receptor in different forms of neural plasticity including basal neurogenesis.

Despite clear results obtained by electrophysiological methods, we could not confirm at a structural level the conclusion of higher number of synapses in the hippocampus of C3KO mice. Therefore, an alternative labeling technique allowing for simultaneous visualization of pre- and post-synaptic components together with a super-resolution confocal microscopy could help clarify this issue. As C3 was shown to mediate synapse elimination from spinal cord motor neurons after peripheral nerve axotomy (Berg et al.,

2012), it remains to be tested whether C3 plays any role in microglia-mediated in synapse elimination after stroke (Wake et al., 2009).

Another important question is the role of the complement system and in particular synapse elimination and altered synaptic plasticity in cognitive decline during aging. The first observations in this regard point, perhaps not surprisingly, to an even larger complexity of this problem. As Stephan and colleagues recently showed, C1q KO mice are protected from age-related cognitive impairment independently of synapse elimination. This study further showed that the absence of C3 and C1q had opposing effects on activity-dependent synaptic potentiation of perforant path to dentate gyrus synapses (Stephan et al., 2013). These results show that C1q contributes to age-related decline of hippocampus-dependent cognitive functions independently of complement activation and without affecting the synapse numbers in the hippocampal dentate gyrus. Better understanding of the complement activation dependent as well as independent functions of the complement proteins, in particular C1q and C3 may therefore pave the way to tackle one of the greatest health threats of old age.

In summary, this thesis contributes to the growing body of evidence for the role of complement in ischemic stroke and stroke recovery. The roles of individual complement proteins and their activation products in stroke may, however, be profoundly different depending on both the temporal and spatial context. The currently established roles of the complement system range from the more classically immune functions such as initiation of inflammation, opsonization, regulation of antibody responses and cell lysis through the elimination of dead cells and cell debris, atherogenesis and stem cell homing to stem cell differentiation, neuroprotection and synapse elimination in CNS. The next decade will undoubtedly unravel even less expected functions for a system that was once regarded as “an antibody helper”.

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