THE NEURONAL CELL ADHESION MOLECULE ICAM-5

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To the memory of my father

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals **I-IV**, and on unpublished results presented in the text.

I Tian, L., **Nyman, H.**, Kilgannon, P., Yoshihara, Y., Mori, K., Andersson, L.C., Kaukinen, S., Rauvala, H., Gallatin, W.M., and Gahmberg, C.G. (2000) Intercellular adhesion molecule-5 induces dendritic outgrowth through homophilic adhesion. *J. Cell Biol.* **150** (1), 243-252.

II **Nyman-Huttunen, H**.*, Tian, L.*, Ning, L., and Gahmberg, C.G. (2006) α-Actinin-dependent cytoskeletal anchorage is important for ICAM-5-mediated neuritic outgrowth. *J. Cell Sci.* **119**, 3057-3066.

III Tian, L., Stefanidakis, M.*, Ning, L.*, Van Lint, P., **Nyman-Huttunen, H**., Libert, C., Itohara, S., Mishina, M., Rauvala, H., and Gahmberg, C.G. (2007) Activation of NMDA receptors promotes dendritic spine development through MMP-mediated ICAM-5 cleavage. *J. Cell Biol.* **178** (4), 687-700.

IV **Nyman-Huttunen, H**.*, Ning, L.*, Aatonen, M., Syrjä, S., Rauvala, H., Tian, L., and Gahmberg, C.G. (2011) ICAM-5 regulates the association of NMDA receptor subunit 1 with α-actinin in central neurons. S*ubmitted.*

* These authors have contributed equally to the work.

ABBREVIATIONS

ABD	agonist-binding domain
AB	actin-binding domain
AD	Alzheimer's disease
ADIP	afadin DIL domain-interacting protein
ALCAM	activated leukocyte cell adhesion molecule
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptor
APC	antigen-presenting cell
AxCAM	axon-associated cell adhesion molecule
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BIG-1	brain-derived Ig molecule
CAM	cell adhesion molecule
CaM	calmodulin
CaMK	CaM kinase
CaMKII	calcium/calmodulin-dependent protein kinase II
CAV-21	coxsackie virus A21
CD	cluster of differentiation
CNS	central nervous system
CRP	cysteine-rich protein
CSF	cerebrospinal fluid
CYFIP-1	cytoplasmic FMRP interacting protein 1
DBF-actin	drebrin-binding F-actin
DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrin
DCX	doublecortin
DenCAM	dendrite-associated cell adhesion molecule
DIV	day in vitro
Dm	double-mutant
DNQX	6,7,-dinitroquinoxaline-2,3(1H,4H)-dione
EAE	experimental autoimmune encephalomyelitis
ECD	extracellular domain
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
E3KARP	NHE_3 kinase A regulatory protein
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ERM	ezrin/radixin/moesin

F-actin	filamentous actin
FAK	focal adhesion kinase
FMRP	fragile X mental retardation protein
Fn	fibronectin-type domain
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell-derived neurotrophic factor
GluR	glutamate receptor
GpIb-IX	platelet glycoprotein Ib-IX
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
HB-GAM	heparin-binding growth-associated molecule
ICAM	intercellular adhesion molecule
ICD	intracellular domain
IFN	interferon
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IgSF	immunoglobulin superfamily
IL	interleukin
iNOS	inducible nittic oxide synthase
kDa	kilodalton
LFA	leukocyte function-associated antigen
LIM	Lin11/Isl-1/Mec -3
LPS	lipopolysaccharide
LTD	long-term depression
LTP	long-term potentiation
LW	Landsteiner-Wiener
mAb	monoclonal antibody
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MAGI-1	membrane associated guanylate kinase-1
MAGUK	membrane-associated guanylate kinase
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C-kinase substrate
MEK	MAPK/ERK kinase
MEKK1	MEK kinase 1
mGlu(5b)	metabotropic glutamate receptor type 5b
MIDAS	metal ion-dependent adhesion site
MMP	matrix metalloproteinase

MS	multiple sclerosis
MT	microtubule
NCAM	neural cell adhesion molecule
NgCAM	neuron-glia cell adhesion molecule
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NHE3	sodium -hydrogen exchanger 3
NrCAM	NgCAM-related cell adhesion molecule
NR1	NMDA receptor subunit 1
NTD	N-terminal domain
pAb	polyclonal antibody
PdBu	phorbol 12,13-dibutyrate
PDGF	platelet-derived growth factor
PDZ	PSD-95/DlgA /zo-1
PECAM-1	platelet endothelial cell adhesion molecule-1
PI3K	phosphoinositide 3-kinase
PIP2	phosphoinositide (4,5)-bisphosphate
PIP3	phosphoinositide (3,4,5)-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKN	protein kinase N
PM	plasma membrane
PMN	polymorphonuclear neutrophil
PSA	polysialic acid
PSA-NCAM	polysialylated NCAM
PSD	postsynaptic density
RIL	reversion-induced LIM-protein
SHP2	Src homology domain 2-containing tyrosine phosphatase 2
sICAM	soluble ICAM
siRNA	small interfering RNA
SPR	surface plasmon resonance analysis
SynCAM	synaptic cell adhesion molecule
SynGAP	synaptic Ras GTPase activating protein
TAG-1	transiently expressed axonal surface glycoprotein-1
TCR	T cell receptor
TMD	transmembrane domain
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VLA	very late antigen

ABSTRACT

Cell adhesion molecules and extracellular matrix (ECM) molecules are very important in providing physical connections and generating cellular signalling events. In brain, the connections between cell surface adhesion proteins, such as members of the immunoglobulin superfamily (IgSF) family, integrins, cadherins and neurotransmitter receptors, with the actin cytoskeleton and cytoskeletal proteins, are important in neuronal cell migration, synapse formation and synaptic plasticity. This thesis focuses mainly on the intercellular adhesion molecules (ICAMs), especially ICAM-5.

ICAM-5 (telencephalin) belongs to the ICAM-family of adhesion proteins which is part of the immunoglobulin superfamily. ICAMs are involved in leukocyte adhesion and adhesion-dependent functions in the central nervous system (CNS) through binding to the leukocyte-specific β2 integrins. ICAM-5 is specifically expressed in the mammalian forebrain, appears at the time of birth, and is located at the cell soma and dendrites of neurons. Recent studies also demonstrate that it is important for the regulation of immune functions in the brain and for the development and maturation of neuronal synapses. The clinical importance of ICAM-5 is still poorly understood; it may have a role in the development of Alzheimer's disease (AD).

In this study, the role of ICAM-5 in neuronal differentiation and its associations with α-actinin and N-methyl-D-aspartic acid (NMDA) receptors were examined. NMDA receptors (NMDARs) are involved in many neuronal functions, including the passage of information from one neuron to another one, and thus it was thought important to study their role related to ICAM-5. The results indicated that ICAM-5 was able to induce dendritic outgrowth through homophilic binding (ICAM-5 monomer binds to another ICAM-5 monomer in the same or neighbouring cell), and the homophilic binding activity appeared to be regulated by monomer/multimer transition. Furthermore, an interaction with α-actinin was shown to be important for neuritic outgrowth. It was examined whether matrix metalloproteinases (MMPs) are the main enzymes involved in ICAM-5 ectodomain cleavage. The results showed that stimulation of NMDARs leads to MMP activation, ICAM-5 is cleaved, accompanied by dendritic spine maturation. These findings also indicated that ICAM-5 and NMDA receptor subunit 1 (NR1) compete for binding to α-actinin, and ICAM-5 may regulate the association of NR1 with the actin cytoskeleton. Thus, it is concluded that ICAM-5 is a crucial cell adhesion molecule involved in the development of neuronal synapses, especially in the regulation of dendritic spine development, and its functions may also be involved with memory formation and learning.

REVIEW OF THE LITERATURE

1. ROLE OF NEURONAL IGSF ADHESION MOLECULES AND NMDA RECEPTORS IN THE CNS

1.1. COMMONLY USED TERMS IN NEUROBIOLOGY

In this review multiple terms special to neurobiology are widely used, and they are explained below.

amygdala	within the brain temporal lobe, involved in emotion and memory processing
cerebellar vermis	a structure between the cerebellar hemispheres
cerebellum	little brain, involved in motor control
cerebral cortex	the outermost neural tissue surrounding the cerebrum (forebrain)
cerebral piriform cortex	in telencephalon, involved in smell perception
contextual fear conditioning	a learning form in which the animal can associate the stimulus and the aversive consequences of the stimulus
corpus callosum	a bridge between the right and left cerebral hemispheres
corticospinal tract	axons that connect the spinal cord and the cerebral cortex
dendritic arborization	dendritic outgrowth and branching
dendritic spines	small protrusions in dendrites of the postsynaptic neurons, receive the information from the axons from presynaptic neurons, involved in mediating information forward
diencephalon	involved in regulation of the autonomic nervous system
excitatory neurons	neurons which release a neurotransmitter (glutamate) which causes activation and/or depolarization of the postsynaptic neurons
excitotoxicity	neuronal death caused by neurotransmitter receptor overactivation
glial cells	astrocytes and oligodendrocytes, protect and support neurons in brain
growth cone	a protrusion at the tip of an axon, forms contacts with the postsynaptic neurons
hippocampus	in the telencephalon, involved in memory formation
hydrocephalus	brain swelling
inhibitory neurons	neurons which release a neurotransmitter (GABA) which causes deactivation and/or hyperpolarization of the postsynaptic neurons
locomotor activity	movement from one place to another
long-term potentiation	a long-lasting increase in synaptic transmission between two neurons, involved in learning and memory processing
long-term depression	a long-lasting decrease in synaptic strength, selective, can weaken specific synapses

mesencephalon	the midbrain, involved in vision, hearing, motor control
metencephalon	contains pons and cerebellum, involved in regulating breathing
myelencephalon	contains medulla oblongata, also involved in regulating breathing
neocortex	part of the cerebral cortex, involved in higher brain functions such as sensory perception and language
neural tube	CNS develops from it in the embryo
neuron depolarization	neuronal stimulation
neurotransmission	neurotransmitter receptors are activated, can lead to long-term or short-term changes in synaptic strength
olfactory bulb	in the forebrain, involved in smell perception
prepulse inhibition in the acoustic startle response	can be used in animals to study human neurological diseases having deficits in sensorimotor gating
radial maze task	measures spatial learning and memory in animals
radial migration	differentiating neural stem cells migrate along radial glial cells to their destinations
reference memory	long-term memory
retinal ganglion cells	neurons in the eye retina
Schwann cells	glial cells of the peripheral nervous system
sensory gating	the brain can adjust its responses depending on the stimulus
septum	inside the forebrain, associated to the basal ganglia system
spatial learning	spatial memory is needed to navigate around, for example in a maze
synaptic plasticity	structural or functional changes in the CNS, basis of learning and memory
synaptogenesis	synapse formation
telencephalon	the forebrain, is involved in higher brain functions, including movement, language, memory, learning, communication
thalamus	between the cerebral cortex and midbrain, is involved in mediating spatial sense signals to the cerebral cortex
ventricular and subventricular zones	the location of proliferating neural progenitor cells in the neural tube

1.2. NEURONAL IGSF ADHESION MOLECULES AND NMDA RECEPTORS IN DEVELOPMENT AND PLASTICITY OF THE CNS

In the central nervous system (CNS), neurons contact other neurons at a site called the synapse. Synaptic transmission is involved in all higher brain functions, including memory formation and learning (Brose, 1999).

The adult brain develops in several stages. In the beginning of the neural development, neural progenitor cells are generated and they migrate to different target areas within the brain. Neural progenitor cells develop into neurons and glia. Glial cells are the supporting cells of the neurons. During their journey to their destination, neural progenitor cells form contacts with other neurons by developing axons. Axons select the proper target cells and a synapse is formed between two neurons (Brose, 1999). Subsequently synapses enable the brain to

receive information via these synaptic connections.

Adhesion molecules are known to participate in target recognition and stabilization during synapse formation. These molecules are required for axonal development, and they are involved in reorganizing synaptic connections in the adult brain. The cell adhesion molecule (CAM) expression level is regulated by changes in synaptic activity and this can reciprocally be modulated by different CAMs. Several studies have revealed that cell adhesion molecules have a role in the synaptic plasticity which underlies higher brain functions such as memory formation and learning (Murase and Schuman, 1999, Gerrow and El-Husseini, 2006). Dysfunction of cell adhesion molecules may contribute to several psychiatric disorders, and to the development of brain pathology in certain neurological diseases, such as multiple sclerosis (MS) and Alzheimer's disease (AD) (Cotman et al, 1998, Gerrow and El-Husseini et al, 2006).

NCAM and L1

Several families of adhesion molecules, including the neural cell adhesion molecule (NCAM), L1, synaptic cell adhesion molecule (SynCAM), cadherins, protocadherins, neuroligins and integrins, are involved in synapse formation (Gerrow and El-Husseini, 2006). Only the members of the immunoglobulin superfamily (IgSF), NCAM and L1, will be discussed here. N-methyl-D-aspartic acid (NMDA)-type glutamate receptors are involved in the rapid regulation of synaptic transmission, and their role in synaptic plasticity and associations with NCAM and L1 will be described in more detail.

NCAM was characterized as a cell surface glycoprotein over 30 years ago (Jorgensen and Bock, 1974, Rutishauser et al, 1976). The extracellular portion of NCAM contains several motifs (**Fig 1A**): five immunoglobulin (Ig) domains and two fibronectin-type III (FnIII) domains. Due to alternative splicing, there are three different isoforms of NCAM (**Fig 1B**). NCAM-180 is a transmembrane protein passing through plasma membrane once; NCAM-140 is also a transmembrane protein with a shorter cytoplasmic domain; NCAM-120 contains a glycosylphosphatidylinositol (GPI) anchor. Soluble forms of NCAM are also found (Ronn et al, 2000, Gascon et al, 2007). A 10 amino acid insertion in the region encoding the extracellular part of the molecule generates NCAM forms whose levels increase during brain development. NCAM can be modified by post-translational mechanisms, such as glycosylation. A polysialic acid (PSA) is linked to NCAM. It is composed of α2,8-linked sialic acids (Finne et al, 1983). Earlier studies have shown that PSA is able to weaken NCAM-NCAM interactions and therefore it regulates cell adhesion events (Kiss and Rougon, 1997, Rutishauser and Landmesser, 1996, Ronn et al, 2000). NCAM knockout mice have presynaptic defects in their neuromuscular junction leading to problems in neurotransmission, furthermore, these mice seem to suffer

defects in synaptic vesicle cycling (Polo-Parada et al, 2001, 2004, 2005).

NCAM belongs to the L1 group of cell surface glycoproteins that show similarities in their domain structure, e.g. Ig domains and FnIII domains are very similar. In vertebrates, this L1 subgroup includes L1, NgCAM (neuron-glia cell adhesion molecule), NrCAM (NgCAM-related cell adhesion molecule), NCAM, BIG-1 (brain-derived Ig molecule), axonin-1/TAG-1 (transiently expressed axonal surface glycoprotein-1) and F11 (Walsh and Doherty, 1997).

The L1 protein is composed of six Ig domains, five FnIII domains, a single transmembrane region, and a cytoplasmic domain (**Fig 2**). Two different forms of L1 have been found. One form containing the RSLE (arginine, serine, leucine, glutamate) sequence can be found in neurons. The form lacking the RSLE sequence and the N-terminal sequence can be detected in non-neuronal cells (Burden-Gulley et al, 1997, Kenwrick et al, 2000, Maness and Schachner, 2007). There are different proteases able to cleave the L1 ectodomain (Maness and Schachner, 2007).

During brain development, both NCAM and L1 are abundantly expressed in the CNS. NCAM and L1 are able to interact in a homophilic manner. IgI-IgIII domains are involved in NCAM-NCAM binding (*cis*- and *trans*-interactions, **Fig 1C**) whereas L1-L1 homophilic binding occurs through its second Ig domains (Soroka et al, 2003, Zhao and Siu, 1995). Both NCAM and L1 interact with a number of molecules in a heterophilic manner. It has been shown that NCAM can bind other members of the Ig family of adhesion molecules such as L1 or TAG-1. In addition, NCAM interacts with different extracellular matrix (ECM) proteins and proteoglycans. Neuropilin-1, integrins, TAG-1, F3/F11/contactin, CD9 and CD24 are known to associate with L1 (Maness and Schachner, 2007) (**Fig 2**). Importantly, NCAM is able to undergo interactions with growth factor receptors.

When NCAM binds in a homophilic manner, the fibroblast growth factor receptor (FGFR) becomes autophosphorylated and dimerized, leading to activation of a phospholipase Cγ-dependent intracellular signalling pathway (**Fig 3**). However, the evidence for L1 signalling through this receptor is not so clear. NCAM also acts as a signalling receptor for members of the glial cell-derived neurotrophic factor (GDNF) family and associates with neurotrophin receptors (Gascon et al, 2007). Furthermore, both L1 and NCAM are known to interact with signalling adaptor molecules such as the non-receptor tyrosine kinases Src and the src-family kinase Fyn through their intracellular domains (Maness and Schachner, 2007, Gascon et al, 2007). The interaction of L1 with the integrins may also lead to signal transduction (Maness and Schachner, 2007). Moreover, the cytoplasmic domains of L1 and NCAM-140/NCAM-180 are linked to the actin cytoskeleton through binding to ankyrin (Bennett and Baines, 2001) and spectrin (Niethammer et al, 2002, Leshchyns'ka et al, 2003, Maness and Schachner, 2007) respectively. The binding may be regulated by tyrosine phosphorylation (Kenwrick et al, 2000, Maness and Schachner, 2007) since it is known that phosphorylated L1 can interact

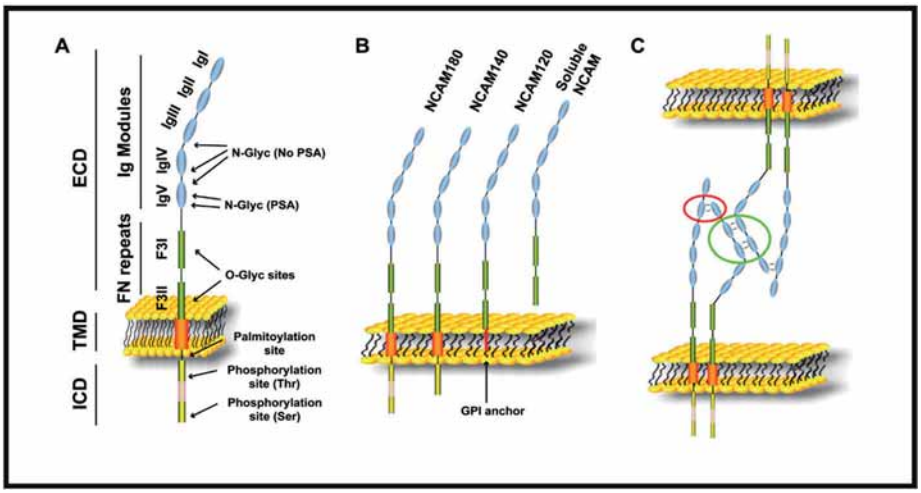

Fig 1. **Molecular features of NCAM. (A) Schema illustrating the identifiable domains (left) and the posttranslational modifications (right) found on the NCAM protein core. (B) Molecular structure of different NCAM isoforms.** As illustrated in the picture, NCAM180 and 140 are transmembrane proteins that only differ in a small portion of the intracellular domains. NCAM120 contains the extracellular domain linked to the membrane through a GPI anchor. Finally, soluble NCAM results either from an alternative splicing or from the enzymatic removal of the extracellular domain of other NCAM isoforms. **(C) The current model for NCAM interactions.** As depicted, NCAM *cis*-dimers involve the interaction between IgI and IgII (red circle). NCAM *trans*-interactions require the initial formation of *cis*-dimers. Then, two kinds of interactions between NCAM molecules on opposing cell membranes are possible (Soroka et al, 2003). The "flat zipper" interaction, illustrated in the picture, involves IgII and IgIII domains (green circle). ECD: extracellular domain; GPI: glycosylphosphatidylinositol; ICD: intracellular domain; TMD: transmembrane domain; IgI-V: Ig-like domain I-V; F3I/II: fibronectin type 3 homology domain I/II. Reprinted from *Brain Research Reviews*, Gascon et al, 2007. Copyright (2007), with permission from Elsevier.

with doublecortin (DCX), which is a microtubule-associated protein involved in the migration of cortical neurons (Kizhatil et al, 2002) (Fig 2). The L1 cytoplasmic tail can also bind other cytoskeletal proteins – the ezrin-radixin-moesin (ERM) proteins (Dickson et al, 2002, Cheng et al, 2005).

NMDA receptors

NMDA receptors (NMDARs) are glutamate-gated ion channels found in the CNS that are involved in excitatory synaptic transmission, plasticity and excitotoxicity. Other ionotropic glutamate receptors (GluRs) include α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. NMDARs exist in many subtypes, and are usually permeable to calcium but the receptors have different biophysical and pharmacological features and there are variations in the subunit composition. After glutamate and glycine binding and depolarization of the neuron, an NMDAR becomes activated, and the magnesium block is removed. Moreover, glutamate receptors have been implicated in several neurological disorders (Waxman and Lynch, 2005, Paoletti and Neyton, 2007).

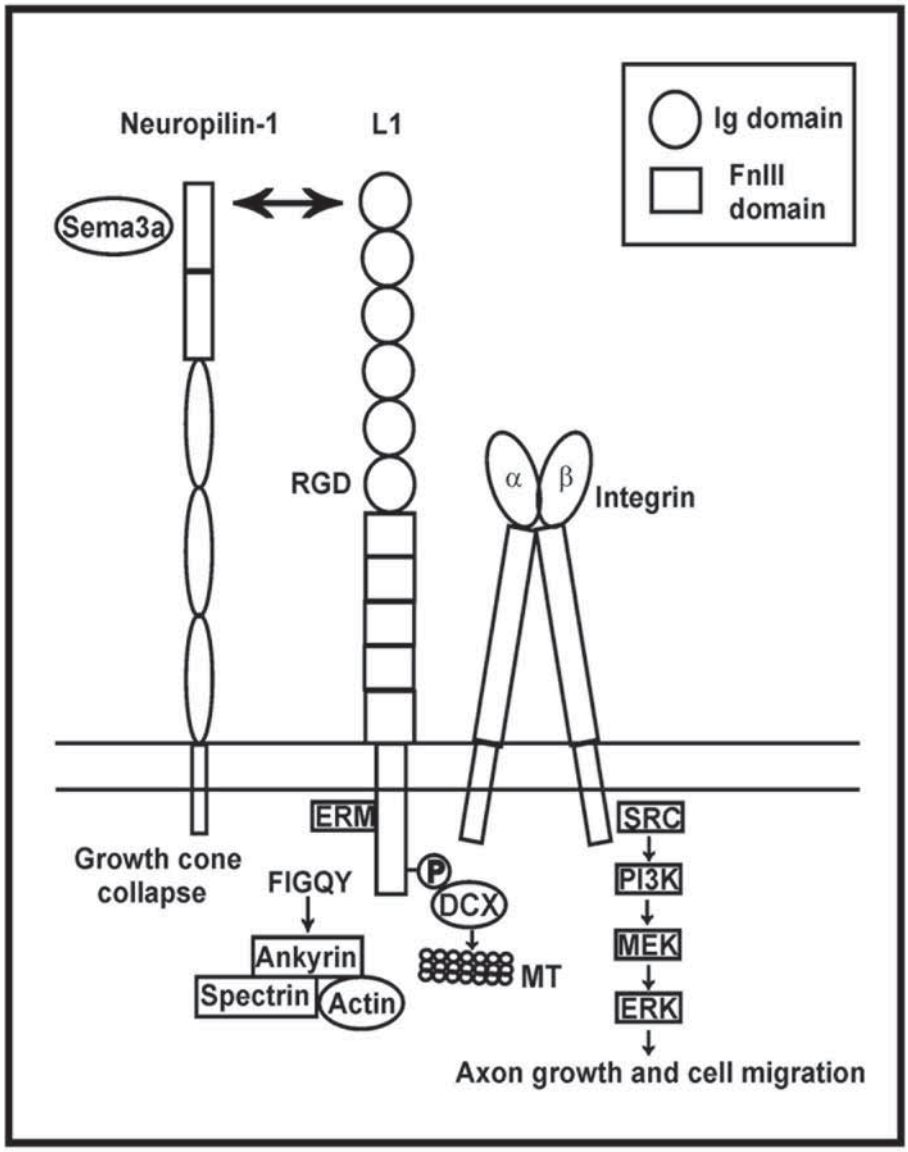

Fig 2. **An illustration of the structure of L1 and downstream signalling pathways.** Different kinases are activated when L1 induces signal transduction through binding to integrins via an RGD (Arginine-Glycine-Aspartic acid) sequence in Ig domain 6. Spectrin links L1 to the actin cytoskeleton through ankyrin and L1 can also associate with microtubules (MT) via binding to doublecortin (DCX). Finally, signalling events lead to axonal outgrowth, growth cone collapse and neuronal cell migration into different areas of the brain. PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase. Modified from Maness and Schachner, 2007.

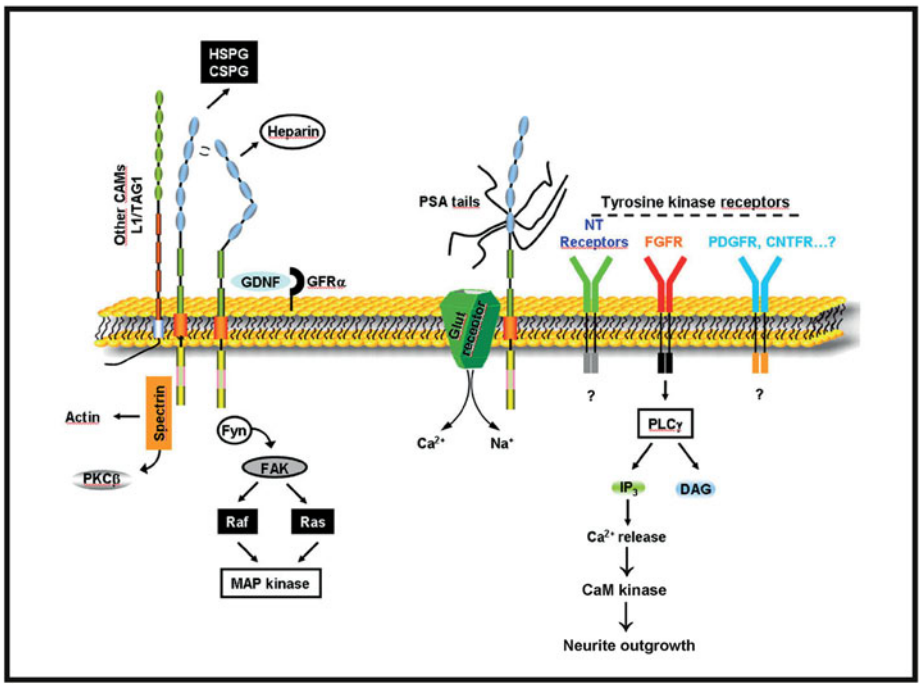

Fig 3. **Molecular interactions and signalling of NCAM/PSA-NCAM.** NCAM intrinsic signalling relies on the heterophilic interactions of the intracellular domain and mainly results in MAPK (mitogen-activated protein kinase) activation. NCAM extracellular domain interacts with a number of other proteins involved in cell adhesion, such as ECM members or other CAMs (left). Importantly, the presence of PSA (right) allows NCAM to interact with diverse signalling molecules (glutamate receptors, tyrosine kinase receptors, and p75 receptors). Since many of these new partners are able to activate intracellular signalling cascades, it has been proposed that PSA might shift NCAM activity from an anchoring to a signalling state. Reprinted from *Brain Research Reviews*, Gascon et al, 2007. Copyright (2007), with permission from Elsevier.

The NMDARs are heteromeric complexes composed of three subunits: NR1, NR2 and NR3. There are eight different NR1 subunits, as well as four different NR2 subunits (A, B, C, D) and two NR3 subunits (A and B). NMDARs are believed to consist of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits. In NR3-expressing cells, this subunit can be detected as a tetrameric complex combined with the NR1 and NR2 subunits (Cull-Candy et al, 2001, Paoletti and Neyton, 2007). NMDA receptor subunits contain a large extracellular N-terminus, three transmembrane segments (TM1-3), and a C-terminal cytoplasmic domain which varies in size and can interact with several intracellular proteins (**Fig 4**) (Waxman and Lynch, 2005, Paoletti and Neyton, 2007).

The NMDA receptor subunit 1 (NR1) is considered to be an obligatory subunit of the NMDAR, whereas NR2 is not. NR1 is expressed in all parts of the brain and during all developmental stages (Moriyoshi et al, 1991). The NR2A-D subunits are

expressed in different developmental stages. For example, before birth, NR2B and NR2D are expressed in high amounts. The expression level becomes diminished in the postnatal brain and subsequently NR2A and NR2C expression levels increase. In adult brain, NR2A can be found in many regions, whereas NR2B is mostly expressed in the forebrain, NR2C is located in the cerebellum, and NR2D in small cell populations in certain brain regions (Monyer et al, 1994, Standaert et al, 1996). The NR1/NR2A/NR2B complex is the major receptor type present in the forebrain (Luo et al, 1997).

The functional roles of NMDARs have been determined by using genetically modified mice. NR1 knockout mice die shortly after birth due to respiratory failure, highlighting the vital nature of the NMDAR. Although the NMDAR may have a role in different developmental stages, the neuroanatomy of NR1 knockout mice appeared rather normal (Forrest et al, 1994, Li et al, 1994). NR2B knockout mice die immediately before birth. NR2A knockout mice are viable, although they display impaired hippocampal plasticity (Kutsuwada et al, 1996, Sakimura et al, 1995). NR2C and NR2D knockout mice are also viable and exhibit no abnormalities in brain morphology but they do show deficiencies in motor functions (Ikeda et al, 1995, Ebralidze et al, 1996, Kadotani et al, 1996). Therefore, the NR1/NR2B subtype is the most important NMDAR type to be found in the CNS. NR2 subunits are more easily replaced by other subunits.

The NR3 subunit is expressed in the developing brain, whereas in the adult rodent brain, it is expressed in the thalamus and spinal cord in minor amounts (Ciabarra et al, 1995). NR3 may function as a modulatory subunit since no specific function has been identified. Studies using NR3 knockout mice have indicated that it may have a role in synapse formation and neurite outgrowth (Sucher et al, 1995, Das et al, 1998).

NMDAR activity is regulated by phosphorylation by different kinases, including protein kinase C (PKC), protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), and Src-family kinases. Phosphorylation of NR1, NR2A and NR2B subunits can change the NMDAR activity and alter its subcellular localization at the synapse (Waxman and Lynch, 2005). Furthermore, both NMDAR subunits bind to several proteins through their intracellular domains: NR2 subunits can bind to postsynaptic density 95 protein (PSD-95) and α-actinin (Kornau et al, 1995, Wyszynski et al, 1997, Husi et al, 2000), and NR1 subunits bind to calmodulin and α-actinin (Wyszynski et al, 1997, Krupp et al, 1999) (**Fig 5**). Previous studies have shown that CaMKII phosphorylation can modulate PSD-95 binding to NMDARs (Gardoni et al, 2006). Moreover, it has been shown that both AMPA and NMDA glutamate receptors are able to bind to polysialylated NCAM (PSA-NCAM) (Vaithianathan et al, 2004, Hammond et al, 2006). The other binding partners for NMDARs will not be discussed here.

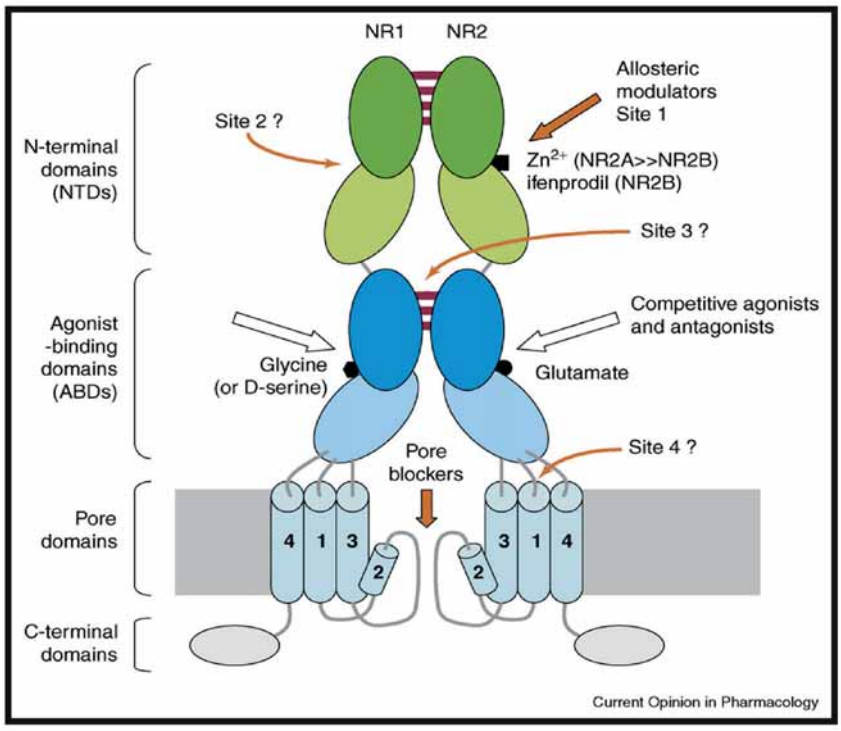

Fig 4. **Potential sites for ligand binding at NMDARs.** Most NMDAR are believed to assemble as tetramers, associating two NR1 and two NR2 subunits in a 'dimer of dimers' quaternary architecture. For clarity, only one of the two NR1/NR2 heterodimers is shown. The extracellular region of each subunit is made up of a tandem of 'Venus-flytrap' domains, the N-terminal domain (NTD) and the agonist-binding domain (ABD). In the extracellular region, the subunits dimerize at the level of the ABDs and probably also at the level of the NTDs. The NR2 ABD binds glutamate, whereas the NR1 ABD binds the coagonist glycine (or D-serine). White arrows indicate binding sites for competitive agonists and antagonists. Thick orange arrows indicate sites known to bind allosteric modulators such as endogenous zinc (NR2A and NR2B NTDs) or ifenprodil-like compounds (NR2B NTDs), both acting as non-competitive antagonists.. The ion-channel domain also forms binding sites for pore blockers such as endogenous Mg2+, MK-801, memantine or ketamine, acting as uncompetitive antagonists. Thin orange arrows indicate putative modulatory sites, which can bind either positive or negative allosteric modulators. The only known NMDAR antagonists that display strong subunit selectivity are the NR2 NTD ligands Zn2+, which selectively inhibits NR2A-containing receptors at nanomolar concentrations, and ifenprodil-like compounds , which selectively inhibit NR2B-containing receptors. Reprinted from *Current Opinion in Pharmacology*, Paoletti and Neyton, 2007. Copyright (2007), with permission from Elsevier.

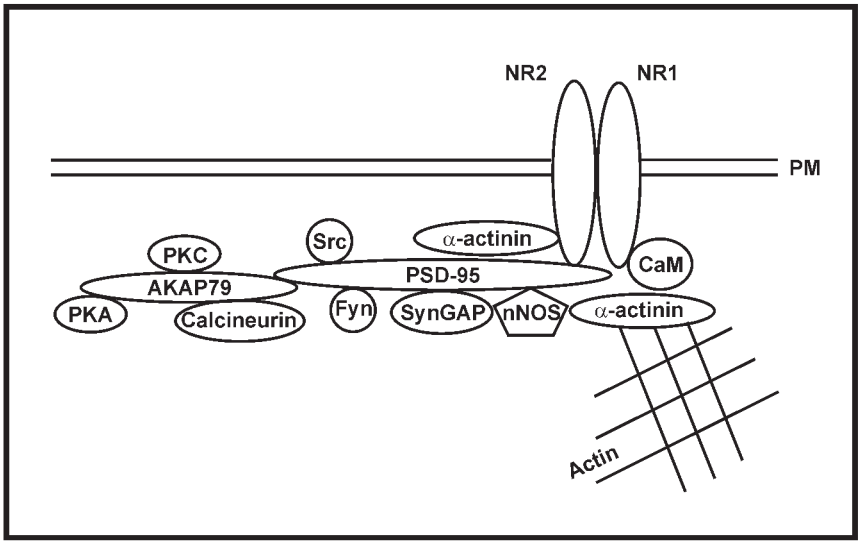

Fig 5. **The NMDA receptor scaffolding complex in the synaptic junction.** NR2 subunit binds to PSD-95 and α-actinin in the cytoplasm. Signalling molecules. such as Src, PKA and the synaptic Ras GTPase-activating protein (SynGAP) localize with the NMDAR. Calmodulin (CaM) and many other proteins, including α-actinin bind to the NR1 subunit. PM, plasma membrane. Modified from Waxman and Lynch, 2005.

Migration of neurons, axonal guidance and neuronal differentiation

Neuronal differentiation begins when the neural tube develops into the spinal cord and brain. Usually the neural progenitor cells vanish after the entire neuronal population has been produced. It is known that neuronal cells do not renew easily. The ventricular and subventricular zones are the regions where the neural progenitor cells are located. Glial cells, the supporting cells of neurons, are derived from these stem cells. The neural progenitor cell usually produces two types of daughter cells. One daughter cell will continue dividing but the other daughter cell will no longer divide. The neural progenitor cell may also produce two postmitotic daughter cells. Those cells that do not divide, instead they start to differentiate into different kinds of neuronal cells. Interactions between cells are the basis for neuronal differentiation, and of the IgSF adhesion molecules, the role of PSA-NCAM in this context has been studied most carefully. The absence of PSA in neuroblasts may affect the differentiation of neurons that have just been produced. The activation of NCAM in hippocampal progenitor cells is known to increase neuronal differentiation (Amoureux et al, 2000). The removal of PSA in adult hippocampal progenitor cells leads to enhanced NCAM interactions which results in neuronal differentiation (Burgess et al, 2008). The differentiated cells migrate to several different regions in the brain, but some cells may remain where they were created.

Neuronal cells use nonradial and radial routes for cell migration. In the nonradial route, neurons migrate in parallel to the surface of the neural tube. In the radial route, neuronal stem cells migrate from the ventricular zone to the surface of the neural tube. The radial cell migration has been studied most carefully, and it was found that migrating neurons are located near to the radial glial cells, which guide them to their final positions in the developing brain (Hatten et al, 1999). Several cell surface adhesion molecules are involved in radial neuronal migration, including members of the IgSF: NCAM and L1, and related molecules (Brummendorf and Rathjen, 1995, Maness and Schachner, 2007). For instance, PSA-NCAM is involved in the neural progenitor cell migration from the subventricular zone to the olfactory bulb. Several studies have indicated that NCAM knockout mice and PSA knockout mice experience a significant reduction in the size of the olfactory bulb, which indicates that neural progenitor cells do not migrate properly to the olfactory bulb (Walsh and Doherty, 1997, Weinhold et al, 2005). L1-ERM interactions may be involved in neuronal migration too, as ezrin is known to colocalize with L1 in migrating cortical neurons, and it is replaced by ankyrin at the time of synaptogenesis (Mintz et al, 2003).

After migrating to the target area in the brain, neurons develop axons and the axon selects its target cells. Axonal outgrowth and the recognition of synaptic targets is mediated by a specialized structure with numerous filopodia at the tip of the growing axons called the growth cone. These growth cones respond to various signalling molecules. These signalling molecules facilitate correct synaptic connections (Suter and Forscher, 2000). These signals include surface-bound and diffusible molecules that either inhibit or attract axonal growth. Moreover, secreted growth factors usually promote and maintain stable synapses. There are a variety of receptors and second messenger molecules which can transduce the signals transmitted through the growth cone and initiate the intracellular events that are involved in directed axonal growth and synapse formation. Various IgSF members, including L1 and NCAM, and related molecules, participate in axonal growth and growth cone guidance during the development of the CNS (Walsh and Doherty, 1997, Maness and Schachner, 2007, Schmid and Maness, 2008). By studying NCAM knockout mice or enzymatic removal of PSA, it has been possible to demonstrate in many different systems that PSA-NCAM expression during different developmental stages and in adult plasticity has a crucial role in regulating axonal outgrowth (Gascon et al, 2007).

Human L1 can stimulate axonal growth in neurons (Williams et al, 1992). There are studies indicating that L1 can promote axonal outgrowth via an L1-L1 homophilic interaction in neurons (Walsh and Doherty, 1997). L1 antibodies are able to block neurite outgrowth in L1-transfected cells or glial cells, and disturb the growth of retinal ganglion cell axons in the rat retina (Williams et al, 1992, Brittis et al, 1996).

Moreover, L1 knockout mice exhibit axon guidance errors in the corticospinal tract, and also abnormal hippocampal and cerebellar development (Cohen et al, 1998, Rolf et al, 2002, Demyanenko et al, 1999, Sakurai et al, 2001). Furthermore, the *cis*-interaction of NgCAM with axonin-1 allows NgCAM to function as an axonal outgrowth promoting receptor (Kunz et al, 1998). Thus, all these results demonstrate that the IgSF adhesion molecules are clearly involved in axonal guidance and growth and if fail to work, this may lead to serious defects in synapse formation, particularly in neurite outgrowth and growth cone guidance.

Two cytoskeletal proteins, spectrin and ankyrin, are binding partners for NCAM-140/NCAM-180 and L1, respectively (Maness and Schachner, 2007).

NCAM and L1 participate in axonal outgrowth by activating intracellular signaling cascades. In NCAM-mediated neurite outgrowth, an FGF tyrosine kinase receptor cascade is activated after *cis*-interaction with NCAM (**Fig 3**). Finally, CaM kinase (CaMK) is activated and there is neurite outgrowth (Walsh and Doherty, 1997, Gascon et al, 2007, Maness and Schachner, 2007).

Synapse formation and synaptic plasticity of the CNS

Synapses are composed of intercellular junctions between neurons. The formation of synapses is tightly controlled in the human brain. Synapse formation is a complicated phenomenon and takes place in several steps. It begins when axons start to contact the dendrites of the postsynaptic cell and the appropriate pre- and postsynaptic proteins have been recruited to the initial contact site. The nascent synapse formed between the axon and dendrite of two neurons is stabilized by different CAMs, which are usually involved in cell adhesion (Gerrow and El-Husseini, 2006). Synapse formation, especially the dendritic spine formation, will be discussed in more detail in chapter 2.

In two neurons, the connections form between axons and dendrites, especially dendritic spines, and this leads to synapse formation. Synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD), occurs at dendritic spines. LTP and LTD both involve changes in synaptic strength. LTP represents a long-lasting increase in synaptic transmission, which results in calcium influx in postsynaptic dendritic spines through NMDARs, and it is believed to be the basis of cognitive functions, such as memory formation and learning (Gascon et al, 2007).

Many studies on synaptic plasticity have focussed on NCAM and L1. For example, LTP was impaired in constitutive and conditional NCAM knockout mice, (Muller et al, 2000, Bukalo et al, 2004). Interestingly, the interaction between NMDARs and polysialylated NCAM (PSA-NCAM) suggests that the high expression levels of PSA-NCAM during neuronal development could be involved in synapse formation by regulating glutamate receptor function (Hammond et al, 2006, Gascon et al, 2007). In the hippocampus, the absence of PSA abolishes LTD and LTP and suppresses spatial learning (Gerrow and El-Husseini, 2006). In an *in vivo* experiment,

antibodies against NCAM or L1 were injected into the hippocampal area of rats, and it was noted that their ability for spatial learning was diminished. L1 knockout mice also display the same kinds of problems. Interestingly, L1 knockout mice seem to exhibit normal LTP in hippocampus, probably because some other adhesion molecules may be replacing L1 adhesion function in these mice (Gerrow and El-Husseini, 2006). The learning problem is probably related to a functional failure in some other part of the brain. The role of L1 in synaptic transmission events is still under investigation. Thus, the neuronal cell adhesion molecules NCAM and L1 are believed to have a role in synaptic plasticity.

1.3. NEURONAL IGSF ADHESION MOLECULES AND NMDA RECEPTORS IN NEUROLOGICAL DISEASES OF THE CNS

Immune diseases: multiple sclerosis

MS is a chronic autoimmune disease which affects the CNS and is characterized by physical and cognitive disabilities. The disease affects women more often than men and young adults can also be affected (Bar-Or et al, 1999).

MS can occur in several forms, but there are two main forms: the relapsing form and the progressive form. In the relapsing form, the patient suffers from several separate attacks and whereas in the slowly progressive form, attacks do not appear frequently. However in both cases, permanent neurological problems usually occur. Nowadays MS patients live almost as long as individuals without the disease even though there is no therapy that can prevent the progression of the disease completely (Pittock and Lucchinetti, 2007).

Multifocal demyelinated plaques, the MS lesions, are found throughout the CNS, and they are the pathological hallmarks typical for MS. The basic features in MS lesions are variable degrees of inflammation, myelin destruction, oligodendrocyte death, glial cell activation, axonal injury, and infiltration of T cells and macrophages (Pittock and Lucchinetti, 2007).

In MS, circulating immune cells pass into the CNS by crossing the blood-brain barrier (BBB), which separates the CNS from blood circulation. The same phenomenon occurs in an MS animal model, experimental autoimmune encephalomyelitis (EAE). The infiltration of inflammatory cells requires adhesion and transmigration of these cells across the BBB (Engelhardt, 2008). Two adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), are upregulated on vascular endothelial cells of MS lesions (Cannella and Raine, 1995). Their ligands, leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), have also been identified on the perivascular inflammatory cells of MS lesions (Bo et al, 1996, Brosnan et al, 1995). ICAM-1/LFA-

1 and VCAM-1/VLA-4 -interactions are also important in the pathogenesis of EAE (Engelhardt et al, 2008).

Therapeutic targeting of immune cell trafficking into the CNS has been regarded as a promising approach for treating human MS diseases. An anti-VLA-4 antibody (natalizumab) is able to block leukocyte entry into the CNS, and it has been used successfully in treating MS patients. Even though there are some side-effects, the clinical benefits outweigh these disadvantages (Engelhardt, 2008).

The expression of adhesion molecules on brain vascular endothelium is increased in other inflammatory neurological disorders as well and inflammatory cells are able to bind to the CNS endothelium. Viral encephalitis is characterised by lymphocyte entry into the CNS. Indeed, when there is viral infection of the CNS, this increases the expression of ICAM-1 and VCAM-1 in the cerebrovascular endothelium (Irani and Griffin, 1996). Increased levels of soluble ICAM-1 and intercellular adhesion molecule-5 (ICAM-5) (telencephalin) have been detected during viral encephalitis (Hartung et al, 1993, Lindsberg et al, 2002).

Neurological diseases related to NCAM, L1 and NMDA receptors

Several cell adhesion molecules, including NCAM and L1 of the IgSF, are known to participate in synapse formation and therefore if the adhesion molecules are not working properly, then this can cause different neurological disorders. Failure in the regulation of synaptic proteins, especially, NCAM, has been noted in some psychiatric disorders, including hereditary schizophrenia (Gerrow and El-Husseini, 2006, Maness and Schachner, 2007). A soluble NCAM form produced by proteolysis (containing the extracellular part) can be found in the post-mortem brain of schizophrenic patients (Poltorak et al, 1995, Vawter et al, 1998). It is also found in the cerebrospinal fluid (CSF) of these patients (Poltorak et al, 1995, Vawter et al, 1998). Soluble NCAM may be associated with schizophrenia by blocking NCAM-NCAM binding. NCAM knockout mice either without NCAM or expressing the soluble NCAM, show several interesting features: the amount of inhibitory and excitatory synapses is reduced, they have higher basal locomotor activity and their response to amphetamine is enhanced (Pillai-Nair et al, 2005). These features are usually similar to the situation in schizophrenia. However, some features in the knockout mice having a total loss of the NCAM molecule are not typical characteristics of schizophrenia: they have normal sensory gating (Plappert et al, 2005). Schizophrenic patients exhibit impaired sensory gating, i.e. they suffer from auditory hallucinations and delusions (Gerrow and El-Husseini, 2006). NCAM has also been linked to other human brain disorders, such as bipolar disorder and AD. Some of the other behavioural abnormalities observed in NCAM knockout mice also resemble some human disease states; the mice show several features

resembling serotonin 1A-related aggressive behaviour and anxiety (Maness and Schachner, 2007).

L1 mutations are linked to many diseases in humans, including X-linked mental retardation and schizophrenia (Kenwrick et al, 2000, Maness and Schachner, 2007). The L1-related mental retardation form (spastic paraplegia type 1) displays many typical features, including hydrocephalus and lower limb spasticity (unusual muscle tightness). In L1 knockout mice, many malformations can be found in the corticospinal tract, cerebellar vermis, corpus callosum and hippocampus. L1 knockout mice also show defects in cognitive behaviour that resemble the features seen in some forms of human mental retardation. Moreover, L1 knockout mice show impaired sensory function. This is believed to take place because Schwann cell interaction with the unmyelinated sensory nerve axons is reduced (Kenwrick et al, 2000), thereby indicating a role for L1 in axonal myelination.

NMDARs have been regarded as potential therapeutic drug targets since they are involved in many brain disorders, such as epilepsy, stroke, AD, and Parkinson's and Huntington's diseases (Paoletti and Neyton, 2007). NMDARs are also involved in excitotoxicity. They are able to evoke excitotoxic neuronal death in response to overactivation of the receptor. In many neurodegenerative diseases, including Parkinson's and Huntington's diseases, and in ischemia caused by stroke or brain trauma, neuronal death is the main feature observed (Paoletti and Neyton, 2007). NR1 and NR2A knockout mice models have been used to study schizophrenia (Mohn et al, 1999, Miyamoto et al, 2001, Paoletti and Neyton, 2007). There are some studies indicating that failure in NMDAR function may be linked to the pathological features seen in human psychoses (Paoletti and Neyton, 2007). It has been proposed that polymorphisms in the NR2B gene may be associated with AD (Slutsky et al, 2004).

Blockade of the function of NMDARs is reported to protect neurons in animals in models of both seizure and stroke (Cull-Candy et al, 2001). There is growing body of evidence implying that subunit-selective NMDA receptor antagonists may prove to be more effective than the broad-spectrum antagonists. For example, in the future, NR2B-selective antagonists may be useful for the treatment of Parkinson's disease in humans, because some studies have revealed that they are useful in animal models of this debilitating disease. Additionally, NMDA receptor activity increase could be useful for treating cognitive behavioral disorders, including schizophrenia and psychoses (Paoletti and Neyton, 2007).

2. DENDRITIC SPINE MORPHOGENESIS

2.1. STRUCTURE OF DENDRITIC SPINES

Dendritic spines are derived from filopodia, the immature form of dendritic spine. They are very small protrusions (0.5-8 μm) which are expressed on neuronal dendrites in the brain. They contain the postsynaptic component, which is important for excitatory synapses in the brain (Harris and Kater, 1994). However postsynaptic machinery is lacking from the immature dendritic filopodia. The spines are known to be involved in synaptic formation and plasticity. Dendritic spines contain complex structures with different transmembrane, cytoskeletal and scaffolding molecules. The amount of filopodia and spines varies, depending on how much information is passing through the synapses. Usually spines dominate over filopodia when synaptic transmission takes place. Dendritic spine abnormalities are found in many neurological and psychiatric diseases (Calabrese et al, 2006).

Dendritic spines can be divided into three different types: the immature thin type, the stubby type and the mature mushroom type (**Fig 6A**). The thin type is characterized by a small head and a slender neck, whereas the mushroom type has a large head and a short neck, with the stubby type being an intermediate form lacking the neck (Sekino et al, 2007). Depending on the situation, any type of these spines can dominate over the others. Intermediate filaments and microtubules are not found in spines but they can be found in large numbers in the dendritic shaft (Husi et al, 2000).

The actin cytoskeleton and a structure called postsynaptic density (PSD) are two major structural components of dendritic spines (**Fig 6B**) and in neurons, actin is expressed abundantly. Filamentous actin (F-actin) is able to form different networks that are the main components of the actin cytoskeleton (Calabrese et al, 2006, Sekino et al, 2007). Morphological changes in spine structures are modulated by activated glutamate receptors (Matus, 1999).

PSD can be found under the postsynaptic membrane of dendritic spines (Kennedy, 1997). Various scaffold proteins and signalling molecules can be found in the PSD (Sheng, 2001), such as several PSD -95/DlgA/zo-1 (PDZ) proteins and many membrane-associated guanylate kinases (MAGUKs) (Kim et al, 1995). PSD-95, which is a typical PDZ protein, is abundantly expressed in the PSD (Cho et al, 1992, Kistner et al, 1993).

After spine morphogenesis has begun, there is synaptic assembly of PSD-95 (Okabe et al, 2001). Studies on PSD-95 knockout mice indicate that they exhibit no abnormalities in spine structures (Migaud et al, 1998). In contrast, the actin cytoskeleton is involved in controlling spine morphology as the disruption of the actin

cytoskeleton can interfere with spine morphogenesis and disassembly of synaptic elements (Allison et al, 2000, Zhang and Benson, 2001). Therefore, PSD may have a role in spine morphogenesis, but the actin cytoskeleton has a more dominant role.

2.2. DENDRITIC SPINE DEVELOPMENT

At the beginning of synaptogenesis, dendritic shafts contain numerous filopodia. They are long and thin protrusions, and transient in their nature and they contain less F-actin than spines. Filopodia present in growth cones are usually involved in axonal and dendritic outgrowth. Filopodia in dendritic shafts participate in synaptogenesis (Portera-Cailliau et al, 2003).

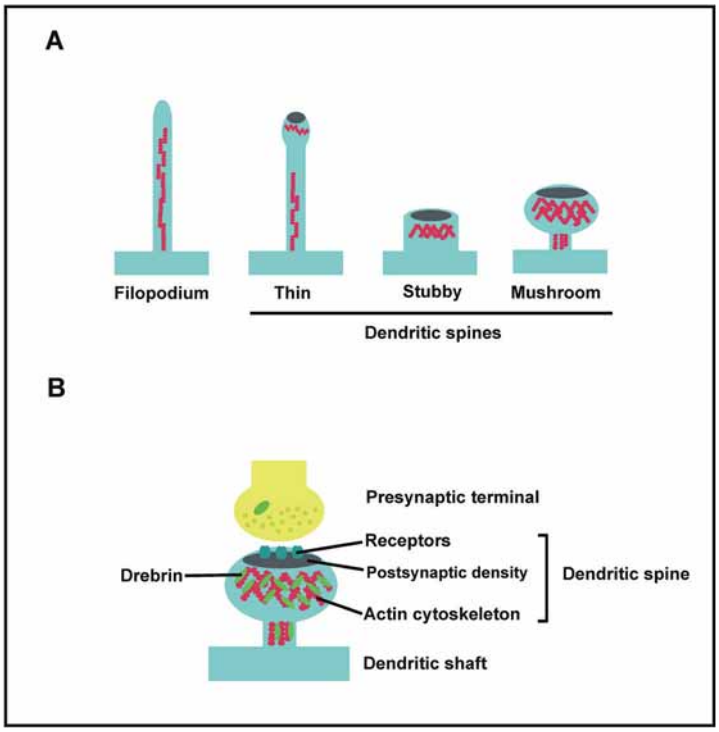

Fig 6. **Structure of dendritic filopodia and spines, and a model of an asymmetric synapse.** (**A**) Filopodia are the precursors of dendritic spines, and they develop into three types of dendritic spines: thin type, stubby type and mushroom type spines. Gray disks: PSD structure and red circles: F-actin. (**B**) The synapse is formed between the presynaptic terminal and the dendritic spine. Synaptic vesicles are found in the presynaptic terminal. Two structural elements: actin cytoskeleton and PSD are important for the function of the dendritic spine. PSD contains the neurotransmitter receptors. Actin-binding proteins, including drebrin, are stabilizing the dendritic spine architecture. Modified from Sekino et al, 2007.

During synapse formation, the spines start to dominate over filopodia (Matus, 2005). Usually when filopodia are converted to stable spines, there may be a transition stage involved (Dailey and Smith, 1996, Dunaevsky et al, 1999). Two types of filopodia can be found: cluster type and diffuse type (Takahashi et al, 2003). The classification depends on how drebrin-binding F-actin (DBF-actin) is clustered. Drebrin is usually highly expressed in the brain (Hayashi et al, 1996), and it controls spine morphogenesis (Mizui et al, 2005, Takahashi et al, 2006).

Diffuse-type filopodia can be found in the early postnatal brain and the amount is high at this stage. Drebrin E is expressed first this being followed by drebrin A (Aoki et al, 2005). When drebrin forms clusters with F-actin, the cluster-type filopodia develop. PSD-95 clusters form next with concomitant formation of spines. Drebrin is also translocated from spines to dendrites, when NMDARs become activated (Sekino et al, 2006).

At the beginning of synaptogenesis, filopodia are expressed on nonspiny neurons (Wong and Wong, 2001). As the neuronal maturation continues, the number of cluster-type filopodia increases, and they have more presynaptic contact sites (Takahashi et al, 2003). As the cluster-type filopodia lack PSD-95 clusters, they are believed to be the transitional filopodia type before turning into spines (Sekino et al, 2007). Nonetheless, only some portion of filopodia are turned into spines (Ziv and Smith, 1996). Therefore, many factors, such as CAMs and signalling molecules, are thought to be involved in controlling synaptogenesis at different developmental stages.

The amount of spines is highest in the later developmental stages when synaptic plasticity is also high. Dendritic spine formation continues at its maximum level until the third postnatal week in rodent hippocampus. The spine density remains at a stable level in adulthood (Zhang and Benson, 2000). However, multiphoton microscopic studies have shown that spine and synapse formation continues throughout life (Trachtenberg et al, 2002). Therefore, the adult brain is able to form new synaptic connections all the time.

2.3. REGULATION OF DENDRITIC SPINE DEVELOPMENT

The formation of contacts between cells at synapses resembles the contact formation between nonneuronal cells. However, the synaptic contacts are different because both the pre- and postsynaptic elements have polarized, asymmetric features. Several adhesion molecules, such as NCAMs, N-cadherins, and protocadherins, are involved in controlling synapse formation (Takeichi and Abe, 2005, Washbourne et al, 2004, Calabrese et al, 2006). Other molecules are also involved, such as neurexins, neuroligins, Eph receptors and their ephrin ligands (Calabrese et al, 2006).

Cadherins belong to the family of calcium-dependent homotypic CAMs and they are expressed by almost all cells. For example, N-cadherin is found in neurons (Takeichi and Abe, 2005). When synapse formation begins, N-cadherins are located on pre- and postsynaptic sides in the newly formed synapse making stable connections. Actin-binding proteins, such as α-actinin and cofilin, link the cadherin/β-catenin complex indirectly to the actin cytoskeleton (Neuhoff et al, 2005). It has been shown that β-catenin has an important role in N-cadherin-mediated adhesion during synapse formation (Murase et al, 2002). δ–Catenins are also binding partners for cadherins and they are involved in spine regulation (Kosik et al, 2005).

The protocadherins belong to a subgroup of the cadherin superfamily, and α- and γ-protocadherins are abundantly expressed in neurons and they can be found in synapses (Phillips et al, 2003). Studies on γ-protocadherin knockout mice have revealed that the amount of synapses is diminished in the spinal cords of these animals, implying that cadherins are required for synaptic development (Weiner et al, 2005).

Several CAMs of the IgSF work together to join cells near to each other through their extracellular domains. CAMs, including NCAM, SynCAM, and nectin, are involved in regulating adhesion in the synapse together with integrins, thus being able to have an effect on dendritic spine development and morphology (Benson et al, 2000, Chavis and Westbrook, 2001, Washbourne et al, 2004).

Neurexins and neuroligins are involved in spine and synapse formation as well (Scheiffele, 2003). Neurexins are usually located on the presynaptic side and they promote postsynaptic differentiation whereas neuroligins reside on the postsynaptic side and are involved in presynaptic differentiation. They function at both γ-aminobutyric acid (GABA)-ergic (inhibitory) and glutamatergic (excitatory) synapses (Graf et al, 2004, Calabrese et al, 2006). The PSD-95 association with neuroligins may regulate neuroligin distribution and functional activity (Calabrese et al, 2006).

Several extracellularly secreted molecules, including brain-derived neurotrophic factor (BDNF) and thrombospondins participate in synaptogenesis too (Ji et al, 2005, Christopherson et al, 2005, Calabrese et al, 2006). A membrane protein, syndecan-2, is also involved (Ethell and Yamaguchi, 1999). Structures resembling spines can also be found in neurons which normally lack spines and these are thought to be made up of intracellular elements. Finally, binding of ephrins to their tyrosine kinase receptors, Ephs, also modulates spine morphology by activating various signalling pathways, involving the Rho-family GTPases (Calabrese et al, 2006).

2.4. REGULATION OF DENDRITIC SPINE MOTILITY AND STABILIZATION

The actin cytoskeleton is involved in regulating spine motility, stability and shape (Cingolani and Goda, 2008). Cadherins, neuroligins and Eph receptors indirectly link the actin cytoskeleton to the neurotransmitter receptors. Drebrin, cofilin, profiling and gelsolin are involved in controlling actin polymerization. Glutamate receptors, including NMDARs, participate in spine outgrowth regulation (Matus, 2000). The CaMK signalling pathway can be activated by NMDARs, and this leads to cytoskeletal changes but it may also lead to the formation of dendritic spines and synapses. Thus, the CaMK pathway is involved in controlling spine morphogenesis (Ciani and Salinas, 2008).

Rho-family GTPases belong to the Ras superfamily of small GTPases. They regulate the dynamics of the actin cytoskeleton (Hall and Nobes, 2000). The Rho GTPases Rac, RhoA and Cdc42 are known to influence dendritic spine formation and shape (Newey et al, 2005). Studies on neurons expressing constitutively active Rac1 show that these neurons form small spines (Luo et al, 1996), whereas in neurons expressing constitutively active RhoA, the spine density and length is diminished (Tashiro et al, 2000). The function of Cdc42 in spine morphogenesis is not known. Studies have shown that constitutively active or dominant negative forms of Cdc42 do not change spine density or length (Tashiro et al, 2000). On the other hand, another study demonstrated that Cdc42 may be involved in dendritic spine development (Irie and Yamaguchi, 2004).

The Rho GTPases may control the dynamics of the actin cytoskeleton by regulating signalling phospholipids, including phosphatidylinositol 4,5-bisphosphate (PIP2) which is able to induce actin polymerization (Weernink et al, 2004). The protein myristoylated alanine-rich C-kinase substrate (MARCKS) binds PIP2 in a phosphorylation-dependent way and thus it has an important role in maintaining the dendritic spine morphology (Calabrese and Halpain, 2005). MARCKS is also able to bind to actin filaments and Ca^{2+}/calmodulin. Therefore it is considered to mediate PKC and calmodulin-dependent spine functions. Moreover, two other GTPases, Ras and Rap, participate in regulating the dendritic spine morphology (Calabrese et al, 2006).

2.5. DENDRITIC SPINE PATHOLOGY IN NEUROLOGICAL DISEASES

Spine disruption is a typical feature found in many psychiatric and neurological disorders (Blanpied and Ehlers, 2004, Calabrese et al, 2006), including stroke, epilepsy, trauma, dementia, major depression, normal aging, schizophrenia, and

addiction (Fiala et al, 2002, Glantz and Lewis, 2001, Nimchinsky et al, 2002, Swann et al, 2000). Spine malformations usually affect synaptic functions.

Changes in spine structure and synaptic plasticity may be related to the cognitive deficits observed in different neurological diseases. In schizophrenia, the amount of spines is significantly reduced in prefrontal cortical pyramidal neurons (Glantz and Lewis, 2001). The same phenomenon is seen in human epilepsy (Swann et al, 2000) and is mimicked in animal models of epilepsy (Isokawa, 2000, Jiang et al, 1998). In AD and Parkinson's disease, which are chronic neurodegenerative diseases, the number of spines is reduced or other changes can be found in dendrites (Selkoe, 2002). Data using an AD animal model showed that when postsynaptic actin-regulatory machinery is disrupted, synaptic functions are disturbed, this being reflected in cognitive deficits.

The major hereditary mental retardation forms, fragile X syndrome and Down syndrome, are characterized by changes in spine morphology. The amount of mature spines is decreased and there is a greater number of filopodia (Irwin et al, 2000, Kaufmann and Moser, 2000). Usually proteins that are involved in regulating spine morphology and development, are not expressed correctly and this causes spine abnormalities (Kojima and Shirao, 2007). In fragile X syndrome, the expression of the fragile X mental retardation protein (FMRP) is disturbed because there is an insertion in the FMR1 gene (Dolen and Bear, 2005). The spine amount is reduced in human patients with fragile X syndrome and in FMRP knockout mice. The spine morphology may be altered in both cases. The FMRP protein has an important function in synapses since it regulates protein translation (Bagni and Greenough, 2005, Reeve et al, 2005). The FMRP protein has cytoskeletal binding partners: e.g. the cytoplasmic FMRP interacting protein 1 (CYFIP-1) is one of them and the interactions with these ligands affect dendritic spine structures (Castets et al, 2005, Schenck et al, 2001).

3. ALPHA-ACTININ

3.1. INTRODUCTION

α-Actinin is an abundantly expressed cytoskeletal protein that belongs to a superfamily of actin-binding proteins including spectrin, dystrophin, and utrophin (Hitt and Luna, 1994, Winder, 1997). Inside the cell α-actinin binds to F-actin. The nonmuscle isoform binds calcium whereas the isoform expressed in muscle is not able to bind calcium (Otey and Carpén, 2004). α-Actinin regulates the activity of several receptors, and it acts as a linker molecule between the cytoskeleton, cell surface receptors and the different intracellular signalling molecules. It is located at the leading edge of a migrating cell, cell adhesion sites and focal contacts (Knight et al, 2000). It is the main actin-binding protein located in postsynaptic dendritic spines in human brain (Shirao and Sekino, 2001).

3.2. STRUCTURE AND PHYSIOLOGICAL FUNCTION

α-Actinin is a rod-shaped antiparallel molecule with a molecular weight of about 100 kilodalton (kDa) which can form homodimers. The monomer can be divided into seven domains: the two N-terminal calponin-homology domains which form the actin-binding domain (AB), four spectrin repeats in the central rod domain region, which show similarity to the actin-binding protein spectrin, and the C-terminal calmodulin-like (CaM) domain consisting of four Ca^{2+}-binding EF-hand repeats (Sjoblom et al, 2008) (**Fig 7**). The α-actinin homodimer formation appears to be mediated by the presence of spectrin repeats (Ylanne et al, 2001). The rod domain region serves as a potential interaction site for many cell surface receptors, and different signalling and adaptor proteins (Djinovic-Carugo et al, 2002, Otey and Carpén, 2004). Crystallographic studies have provided evidence that this central rod domain region is both curved axially and twisted, and other proteins may bind to this region with high affinities (Tang et al, 2001, Ylanne et al, 2001). The twisted rod-shaped structure may affect the stability of the α-actinin dimer. All α-actinin isoforms share this twist in their structure (Ylanne et al, 2001). The crystal structures of ABs have been determined for human α-actinin-1, -2 and -3 (Franzot et al, 2005, Borrego-Diaz et al, 2006, Lee et al, 2008). Electron microscopic studies also have indicated that the actin-binding region can exist in two different conformations. An open domain-swapped conformation and a closed conformation have been found (Liu et al, 2004). α-Actinin may be involved in tension sensing in the actin cytoskeleton (Hampton et al, 2007).

Four different α-actinin genes are expressed in humans. Two striated muscle forms (2 and 3), and two nonmuscle forms (1 and 4) have been found (Otey and Carpén, 2004). α-Actinin-2 is also expressed in nonmuscle cells, especially in neurons (Wyszynski et al, 1998, Walikonis et al, 2001). α-Actinin-4 shows ~86% similarity to α-actinin-1 (Honda et al, 1998). α-Actinin-1 is more abundant in nonmuscle cells than α-actinin-4 but both isoforms can be found in focal adhesions and stress fibers (Tsuruta et al, 2002, Edlund et al, 2001).

Interestingly, α-actinin-4 knockout mice suffer severe glomerular disease possible because podocytes, the visceral epithelial cells in kidneys, display abnormal morphology (Kos et al, 2003). α-Actinin-1 is also found in kidneys but it cannot substitute for α-actinin-4 in its functions. Thus, the high sequence similarity does not necessarily mean that they have redundant functions. More recent studies have indicated that transgenic mice with differences in α-actinin-4 expression exhibit some of the distinct morphologic features of glomerular disease (Henderson et al, 2008). It also appears that under normal conditions in the kidney, α-actinin-4 is able to bind to integrins and it is required for normal podocyte adhesion (Dandapani et al, 2007). In addition, α-actinin-4 gene point mutations have been found in humans displaying a familial kidney disease, and actin-binding affinity is affected by these mutations (Kaplan et al, 2000, Michaud et al, 2003).

The expression of α-actinin-4 is higher in migrating cells and it may have a role in macropinocytosis and phagocytosis (Honda et al, 1998, Araki et al, 2000). In addition, α-actinin-4 binds to inducible nitric oxide synthase (iNOS), which probably plays a role in hypoxia (Daniliuc et al, 2003).

Enhanced movement of cancer cells by the actin cytoskeleton reorganization seems important for cancer invasion and metastasis. Interestingly, there are studies indicating that α-actinin may be involved in metastasis. Metastasis is a pathological procedure in which cell movement and adhesion become disturbed and malignant cells invade other tissues. Thus, α-actinin-4 expression is decreased in malignant human neuroblastoma cell lines and it is known that α-actinin-4 can also act as a tumor suppressor in these cancer cells (Nikolopoulos et al, 2000). Activation of phosphoinositide 3-kinase (PI3K) and the insulin-like growth factor 1 (IGF-1) receptor affects α-actinin activity and disassembly of connections between cells (Guvakova et al, 2002). There are also studies revealing that α-actinin-4 can increase cell movement and promote lymph node metastasis in colorectal cancer (Honda et al, 2005), and ovarian cancer (Barbolina et al, 2008). However, the role of α-actinin-4 in the process of cancer invasion and metastasis still remains to be determined.

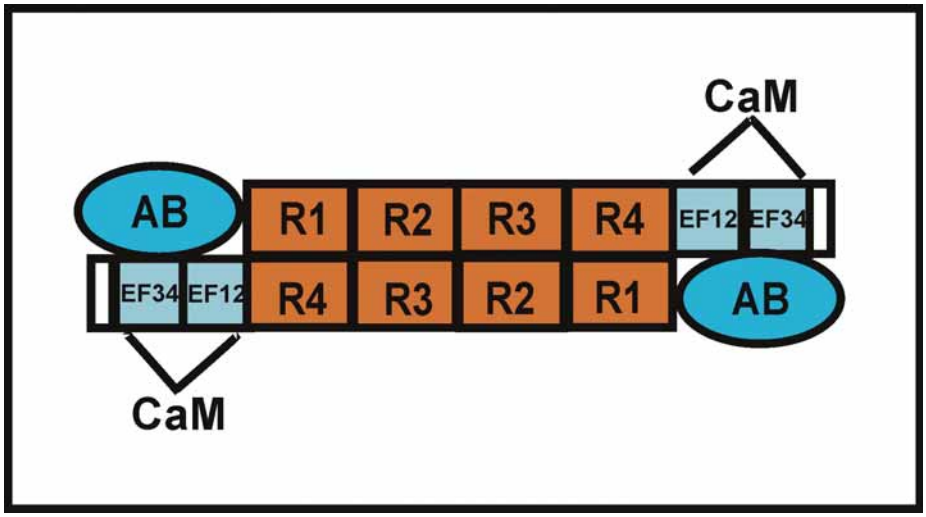

Fig 7. **A schematic illustration of α-actinin in its native form.** Each monomer contains an N-terminal actin-binding domain (AB) consisting of two calponin-homology domains, a central rod domain region with four α-helical spectrin-like repeats (R1-R4), and a C-terminal CaM domain consisting of four Ca^{2+}-binding EF-hand repeats. Modified from Sjoblom et al, 2008.

3.3. INTERACTIONS

In striated muscle, α-actinin and other Z-disc proteins are involved in organizing the actin filaments. In smooth muscle and nonmuscle cell stress fibers, dense bodies (dense regions) function similarly as Z disc proteins. Dense bodies are located regularly along stress fibers. α-Actinin has several binding partners in the dense region, including Lin11 /Isl-1/Mec-3 (LIM) and PDZ domain proteins (Otey and Carpén, 2004). The cytoskeletal proteins zyxin and the cysteine-rich protein (CRP) contain a LIM domain, and they bind to α-actinin (Beckerle, 1997, Crawford et al, 1992, Pomies et al, 1997, Reinhard et al, 1999). Zyxin is known to have a role in cell differentiation and cell movement (Otey and Carpén, 2004).

Enigma/Cypher proteins have N-terminal PDZ domains and C-terminal LIM domains. PDZ domains bind to various cytoskeletal proteins and LIM domains bind to different kinases. One of these proteins, CLP36, is found in nonmuscle cells and it is able to interact with α-actinin through its PDZ domain (Bauer et al, 2000, Vallenius et al, 2000). In contrast, Clik1 kinase binds to its LIM domain (Vallenius and Mäkelä, 2002). Thus, these results show that α-actinin is involved in multiprotein complex formation. This may help to deliver signals between the cytoskeleton and the nucleus.

Myotilin, myopalladin and palladin belong to a newly discovered family of α-actinin binding proteins, and are very similar in structure. They have more than one copy of the Ig-like domain (Salmikangas et al, 1999, Parast and Otey, 2000, Bang et al, 2001, Mykkanen et al, 2001). Myotilin and myopalladin are expressed in striated muscle Z discs (Salmikangas et al, 1999, Bang et al, 2001). In developing mammalian tissues Palladin is the most abundantly expressed protein of this family and it can bind to the C-terminal CaM domain of α-actinin (Parast and Otey, 2000, Ronty et al, 2004). It is involved in the maintenance of the cytoskeletal structure and cell morphology (Parast and Otey, 2000, Mykkanen et al, 2001, Boukhelifa et al, 2001, 2003). Interestingly, the amount of stress fibers and focal adhesions is diminished in fibroblasts where there is decreased expression of palladin (Parast and Otey, 2000). When a recombinant α-actinin construct lacking the CaM domain is expressed, this also leads to loss of stress fibers (Pavalko and Burridge, 1991, Schultheiss et al, 1992). It seems that palladin and α-actinin are needed for maintaining the stress fiber structure.

α-Actinin appears at different adhesion sites: adherens junctions, focal adhesion sites, lymphocyte contact regions and hemidesmosomes (Otey and Carpén, 2004). It is also found in glomerular podocyte foot processes and neuronal synapses (Otey and Carpén, 2004). Several types of adhesion molecules, including ICAM-1 (Carpén et al, 1992), ICAM-2 (Heiska et al, 1996), and NCAM (Buttner et al, 2003), bind to α-actinin. β1, β2 and β3 integrins have been shown to bind to α–actinin (Otey et al, 1990, 1993, Pavalko and LaRoche, 1993). α-Actinin can also bind to platelet glycoprotein Ib-IX (GpIb-IX) and syndecan-4 (Feng et al, 2002, Greene et al, 2003), which are also adhesion molecules. Nonmuscle α-actinin can take several transmembrane proteins as binding partners most of which are adhesion molecules (**Table 1**). The acidic spectrin repeat region of α-actinin usually binds to the cationic residues present in different transmembrane proteins.

α-Actinin has been postulated to have several functions as a linker between the cytoskeleton and plasma membrane. Through binding to the actin cytoskeleton, α-actinin helps to maintain the structural integrity of the cell adhesion site (Rajfur et al, 2002). α-Actinin may regulate the activity of cell surface receptors, such as L-selectin (Dwir et al, 2001), since this is a binding partner for α-actinin (Pavalko et al, 1995). Interactions of α-actinin with different regulatory molecules link signalling molecules to different adhesion sites. Clustering of cell adhesion molecules, including ICAM-2, also involves interactions with α-actinin and in this way cell adhesion may be enhanced (Otey and Carpén, 2004).

3.4. REGULATION

Several mechanisms are involved in regulating the activities of α-actinin: binding to phosphatidylinositol intermediates, protease processing, calcium binding, and tyrosine kinase phosphorylation (Otey and Carpén, 2004, Sjoblom et al, 2008). These signalling pathways can be activated by integrin engagement. α-Actinin may have a vital role in cell movement as it is known to be involved in cell migration (Gluck and Ben-Ze'ev, 1994). However, the mechanism has remained unclear. Phosphatidylinositol intermediates are involved in regulating the focal adhesion disassembly in non-muscle cells. Platelet-derived growth factor (PDGF) activates PI3K inducing the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). As a consequence, focal adhesions become reorganized. Usually, the binding of PIP2 or PIP3 reduces the affinity of α-actinin to bind to β integrins and the actin-binding properties of α-actinin are also reduced (Greenwood et al, 2000, Fraley et al, 2003, Corgan et al, 2004, Full et al, 2007). It has been shown that the binding of phosphoinositides is involved in regulating the association-dissociation rate of α-actinin with actin filaments and integrins (Fraley et al, 2005). PI3K is a binding partner for α-actinin (Shibasaki et al, 1994) and this enzyme has been found to regulate chemotactic cell movement (Merlot and Firtel, 2003).
Calpain is a protease that cleaves α-actinin. At focal adhesion sites, it cleaves different cytoskeletal proteins during cell migration (Franco et al, 2004). MEK kinase 1 (MEKK1), is a mitogen-activated protein kinase (MAPK) kinase kinase, and is found at focal adhesions and stress fiber regions and it cal also interact with α-actinin (Christerson et al, 1999, Cuevas et al, 2003). It has been demonstrated that phosphoinositide binding to α-actinin regulates calpain proteolysis (Sprague et al, 2008) and calpain may also affect the recruiting of α-actinin to focal adhesions (Bhatt et al, 2002, Cuevas et al, 2003). It is possible that α-actinin is able to recruit MEKK1 to focal adhesion sites, where MEKK1 can act as a regulator of calpain activity.

Two calcium-insensitive isoforms: α-actinin-2 and -3 can be found in skeletal muscle. α-Actinin-1 and -4, which are nonmuscle isoforms, are calcium-sensitive and if calcium is present, their actin-binding affinity becomes reduced (Otey and Carpén, 2004, Sjoblom et al, 2008). Linkages between stress fibers and focal adhesion sites may be affected by these affinity changes.

The AB of nonmuscle α–actinin is phosphorylated by the focal adhesion kinase (FAK). Actin-binding affinity decreases, and this can cause cytoskeletal changes perhaps with cell movement being enhanced. Cell adhesion events may also be affected through different signalling pathways (Izaguirre et al, 2001).

Table 1. Proteins interacting with nonmuscle α-actinin. Some ion channel receptors, such as L-type calcium channel (Sadeghi et al, 2002), polycystin-2 (Li et al, 2005), and Kv1.4 and Kv1.5. potassium channels (Cukovic et al, 2001), are not shown. a-Actinin also binds to NCAM (Buttner et al, 2003), the AMPA glutamate receptor subunit 4 (GluR4) (Nuriya et al, 2005) and the metabotropic glutamate receptor type 5b [mGlu(5b)] (Cabello et al, 2007). ADIP, afadin DIL-domain interacting protein; MAGI-1, membrane associated guanylate kinase 1; E3KARP, NHE3 (sodium -hydrogen exchanger 3) kinase A regulatory protein; MEKK1, MEK (MAPK /ERK kinase) kinase 1; PKN, protein kinase N. Reprinted from *Cell Motility and the Cytoskeleton,* Otey and Carpén, 2004. Copyright (2004), with permission from Wiley & Sons, Inc.

Binding partners for nonmuscle α-actinin

Binding partner	Category	Key reference
CLP-36	Stress fiber	Bauer et al, 2000 Vallenius et al, 2000
Palladin	Stress fiber Focal adhesion	Parast and Otey, 2000 Mykkänen et al, 2001
Zyxin	Stress fiber Focal adhesion	Crawford et al, 1992
CRP	Focal adhesion	Pomies et al, 1997
Vinculin	Focal adhesion	Belkin and Koteliansky, 1987 Wachsstock et al, 1987
β1, β3 integrins	Focal adhesion	Otey et al, 1990
β2 integrins	Lymphocyte targeting	Pavalko and LaRoche, 1993
ICAM-1	Lymphocyte targeting	Carpén et al, 1992
ICAM-2	Lymphocyte targeting	Heiska et al, 1996
L-Selectin	Lymphocyte targeting	Pavalko et al, 1995
GpIb-IX	Platelet adhesion	Feng et al, 2002
ADIP	Adherens junction	Asada et al, 2003
α-catenin	Adherens junction	Knudsen et al, 1995
BP180	Hemidesmosome	Gonzalez et al, 2001
MAGI-1	Tight junction	Patrie et al, 2002
Syndecan-4	Cell-matrix adhesion	Greene et al, 2003
ADAM12	Cell-cell adhesion	Cao et al, 2001
NMDA receptor	Synapse	Wyszynski et al, 1997
Densin	Synapse	Walikonis et al, 2001
A2A receptor	Synapse	Burgueno et al, 2003
Rabphilin 3A	Synapse	Kato et al, 1996
E3KARP	Regulatory	Kim et al, 2002
MEKK1	Regulatory	Christerson et al, 1999
PIP3	Regulatory	Greenwood et al, 2000
PIP2	Regulatory	Rezendiz et al, 2004
iNOS	Regulatory	Daniliuc et al, 2003
PI 3-kinase	Regulatory	Shibasaki et al, 1994
PKN	Regulatory	Mukai et al, 1997
CaMKII	Regulatory	Walikonis et al, 2001

3.5. FUNCTION IN THE CNS

α-Actinin-1, -2, and -4 are expressed in the brain, and α-actinin-1 and -2 isoforms are mainly concentrated in the dendritic spines (Wyszynski et al, 1998, Shirao and Sekino, 2001, Nakagawa et al, 2004, Peng et al, 2004).

α-Actinin is involved in the localization of NMDARs which are activated by glutamate. NR1 and NR2B subunits bind to the α-actinin rod domain region (Wyszynski et al, 1997, Husi et al, 2000). In excitatory synapses, colocalization of NMDARs and α-actinin-2 can be observed in the PSD region, and α-actinin-2 is known to be able to bind to the NMDAR (Wyszynski et al, 1998). Modulation of the NMDAR/α-actinin-2 interaction could be a molecular mechanism accounting for inactivation of NMDARs by calcium (Rosenmund et al, 1995, Krupp et al, 1999). When calcium fluxes into the cell through activated NMDARs, calmodulin binds to the NR1 cytoplasmic tail and subsequently, α-actinin dissociates from NMDARs and receptor-channel activity is inhibited because the receptor dissociates from the actin cytoskeleton (Ehlers et al, 1996, Wyszynski et al, 1997, Zhang et al, 1998). Furthermore, α-actinin may link NMDARs to the PSD.

Previous studies have shown that by overexpressing α-actinin-2, the length and density of dendritic extensions can be increased in cultured hippocampal neurons. This requires the expression of the actin-binding domain and the spectrin repeats of α-actinin. Both these domains are also necessary for dendritic spine targeting (Nakagawa et al, 2004). These results suggest that in neurons, α-actinin is able to regulate spine morphology and density. In addition, α-actinin-1 has been shown to interact with other glutamate receptors, including the AMPA glutamate receptor subunit 4 (GluR4) and the metabotropic glutamate receptor type 5b [mGlu(5b)] (Nuriya et al, 2005, Cabello et al, 2007). Interestingly, GluR4 phosphorylation is one way to regulate its interactions with α-actinin-1 (Nuriya et al, 2005). Moreover, α-actinin has been shown to be involved in the trafficking of GluR1 AMPA receptors in dendritic spines and the reversion-induced LIM-protein (RIL) is also involved (Schulz et al, 2004). α-Actinin has been shown to participate in internalization and aggregation of the adenosine A2A receptor (Burgueno et al, 2003).

In the synapse, α-actinin-4 has one further binding partner, densin (Walikonis et al, 2001). Densin is a transmembrane glycoprotein which has a PDZ domain in its cytoplasmic domain, and it is expressed in the PSDs (Apperson et al, 1996). Densin is thought to function as an adhesion molecule in the CNS, and it is involved in stabilizing the synaptic cell connections (Apperson et al, 1996). (Robison et al, 2005a, Walikonis et al, 2001). Densin, α-actinin-4 and CaMKII form a protein complex in the PSD. It is known that intracellular calcium levels may affect the phosphorylation state of CaMKII and the autophosphorylated CaMKII binds to densin more efficiently (Walikonis et al, 2001). CaMKII also binds to the NMDA receptor subunit NR2B (Robison et al, 2005a, 2005b). Studies have also revealed that calcium/calmodulin replaces α-actinin in the NR1 C0 domain and this promotes CaMKII binding (Merrill et al, 2007). Therefore, by binding to all these postsynaptic molecules, α-actinin may be involved in advanced neuronal functions, such as memory formation and learning.

4. INTERCELLULAR ADHESION MOLECULES (ICAMS) 1–5

4.1. INTRODUCTION TO ICAMS

ICAMs are type I transmembrane glycoproteins that belong to the IgSF. These molecules have a large extracellular region which contains Ig-like domains and a short cytoplasmic domain (Gahmberg et al, 1997b, Cavallaro and Christofori, 2004) (**Fig 8**). The cytoplasmic domains of the different ICAMs are not very similar, indicating that they have quite distinct functions and cell signalling capacities (Hayflick et al, 1998). ICAMs are involved in immune and inflammatory responses and in embryonic development. They also have a role in the development of the CNS (Springer, 1990, Tessier-Lavigne et al, 1996). At present, five ICAM molecules have been discovered (Gahmberg, 1997a, Gahmberg et al, 1997b, 1998, Hayflick et al, 1998) (Fig 8). The IgSF members are usually expressed on the cell surface where they are involved in many different functions, including endocytosis, cell migration and adhesion, and antigen recognition. They also have functions related to organization of the cytoskeleton, regulation of growth and tumor progression. Several IgSF members are involved in leukocyte adhesion, including ICAMs, VCAM-1, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), cluster of differentiation 2 (CD2), leukocyte function associated antigen-3 (LFA-3), activated leukocyte cell adhesion molecule (ALCAM), and the neuronal cell adhesion molecule L1 (**Table 2**). One of the major functions of the ICAMs is to mediate leukocyte adhesion through binding to leukocyte-specific β2 integrins, especially LFA-1 (Larson and Springer, 1990, Gahmberg, 1997a, Gahmberg et al, 2009). The binding is regulated by phosphorylation of the β2 integrins (Fagerholm et al, 2006). Leukocyte transendothelial migration is a process in which circulating leukocytes adhere to endothelium and pass through it to the inflammatory tissue. In inflammatory tissues, leukocytes are involved in regulating the immune response (Gahmberg et al, 1998, Ransohoff et al, 2003).

ICAM-1 is mainly found on leukocytes and endothelial cells but also in many other tissues, and it may be upregulated by cytokines (Wawryk et al, 1989, Hubbard and Rothlein, 2000). ICAM-2 is usually found on leukocytes and endothelial cells though platelets also express ICAM-2 (Staunton et al, 1989, Nortamo et al, 1991a). ICAM-3 is a leukocyte protein, and a ligand for dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al, 2000). ICAM-4 is expressed by the red blood cells (Bailly et al, 1994), and it may have an important role in the turnover of senescent red cells (Ihanus et al, 2007, Toivanen et al, 2008). ICAM-5 is only

found in neurons in the mammalian brain, and it is important for the development of neuronal synapses as well as for controlling lymphocyte activation (Mori et al, 1987, Gahmberg et al, 2008, Tian et al, 2008).

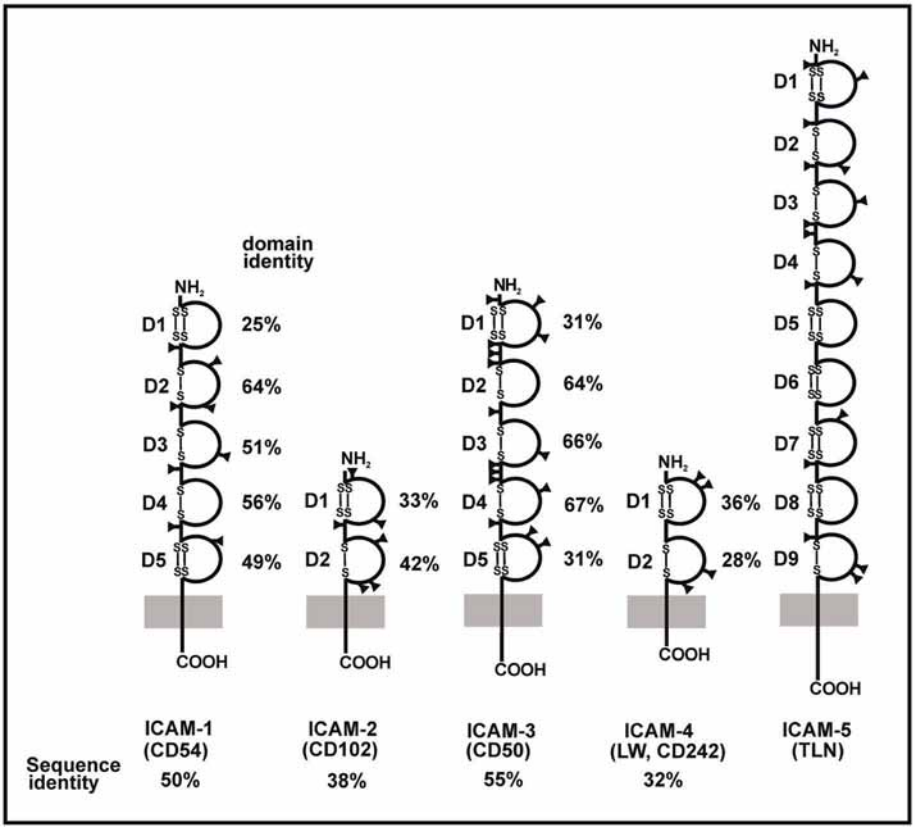

Fig 8. **A schematic representation of the human ICAMs.** The overall or domain-specific sequence identities of ICAM-5 compared to other ICAM family members are displayed as percentages. The domains are numbered. The potential N-glycosylation sites are indicated. Modified from Li Tian's thesis: ICAM-5 (Telencephalin) – a Novel Cell Adhesion Molecule, 2001.

4.2. ICAM-5 (TELENCEPHALIN)

Discovery

When embryonic development begins, five segmental enlargements are formed from the neural tube: diencephalon, telencephalon, mesencephalon (or midbrain), metencephalon (pons and cerebellum), and myelencephalon (or medulla oblongata). The spinal cord is also formed from the neural tube. From these segments,

telencephalon begins to develop and it contains several structures, including the cerebral piriform cortex, neocortex, hippocampus, striatum, amygdala, and olfactory bulb (Mori et al, 1987). It is recognized that telencephalon is involved in several crucial functions of the brain including voluntary movements, sensory perception, memory, learning, language, and communication.

Table 2. IgSF cell adhesion receptors. The molecules, expression and ligands are listed. *, only the L1 ligands involved in immune functions are shown here.

Members of IgSF in the immune system

IgSF members	MAJOR EXPRESSION	LIGANDS
ICAMs		
ICAM-1 (CD54)	Widely expressed: Leukocytes, Endothelial cells, Epithelial cells, Fibroblasts, etc.	CD11a/CD18, CD11b/CD18, Fibrinogen, CD43, Hyaluronan, Rhinovirus, P. falciparum
ICAM-2 (CD102)	Endothelial cells, Leukocytes, Platelets	CD11a/CD18 CD11b/CD18 CD11c/CD18
ICAM-3 (CD50)	Leukocytes	CD11a/CD18 CD11d/CD18 DC-SIGN
ICAM-4 (LW, CD242)	Erythrocytes	CD11a/CD18 CD11b/CD18 CD11c/CD18
ICAM-5 (telencephalin)	Neurons	CD11a/CD18 ICAM-5
Others		
VCAM-1 (CD106)	Endothelial cells	VLA-4 (CD49d/CD29) αXβ2, αDβ2, α4β7
PECAM-1 (CD31)	Endothelial cells, Leukocytes, Platelets	αvβ3 (CD51/CD61) PECAM-1
MAdCAM-1	High endothelial venules (Peyer's patches, mesenteric lymph nodes)	α4β7, L-selectin
CD2	T lymphocytes	LFA-3
LFA-3 (CD58)	Antigen-presenting cells	CD2
ALCAM (CD166)	Activated leukocytes, Thymic epithelial cells	CD6
L1*	Neurons, Glia, Leukocytes	α5β1 αvβ3

In 1987 in Japan, Dr. Kensaku Mori's group found that one monoclonal antibody (mAb) against the dendrodendritic synaptosomes of the rabbit olfactory bulb stained the gray matter in all telencephalic regions. Other brain segments were not stained (Mori et al, 1987). Shortly later, a cell surface glycoprotein was purified with the same antibody from rabbit brain telencephalon. It was named telencephalin (Oka et al,

1990 and subsequently, the mouse (Yoshihara et al, 1994b), rabbit (Yoshihara et al, 1994b), and human (Mizuno et al, 1997) telencephalin cDNAs have been detected.

Structure

Telencephalin is a type I integral cell surface glycoprotein of 130 kDa. It is composed of a large extracellular region, a transmembrane domain and a long cytoplasmic domain (59-64 amino acids) (**Fig 8**). Human telencephalin is very similar to mouse (85%) and rabbit (84%) telencephalin (Mizuno et al, 1997). When telencephalin was cloned, it was found that it displayed homology to the ICAMs and therefore it was renamed as ICAM-5 (Gahmberg, 1997a). ICAM-5 contains nine C2-type Ig domains. Human ICAM-5 contains 15 potential N-glycosylation sites (Mizuno et al, 1997, Ohgomori et al, 2009).

The specific genes for ICAM-1, -3, -4, and -5 are located in human chromosome 19p13.2 (Kilgannon et al, 1998). The ICAM-1 and ICAM-5 genes are situated near to each other, whereas the ICAM-3 gene is located more downstream of ICAM-5.

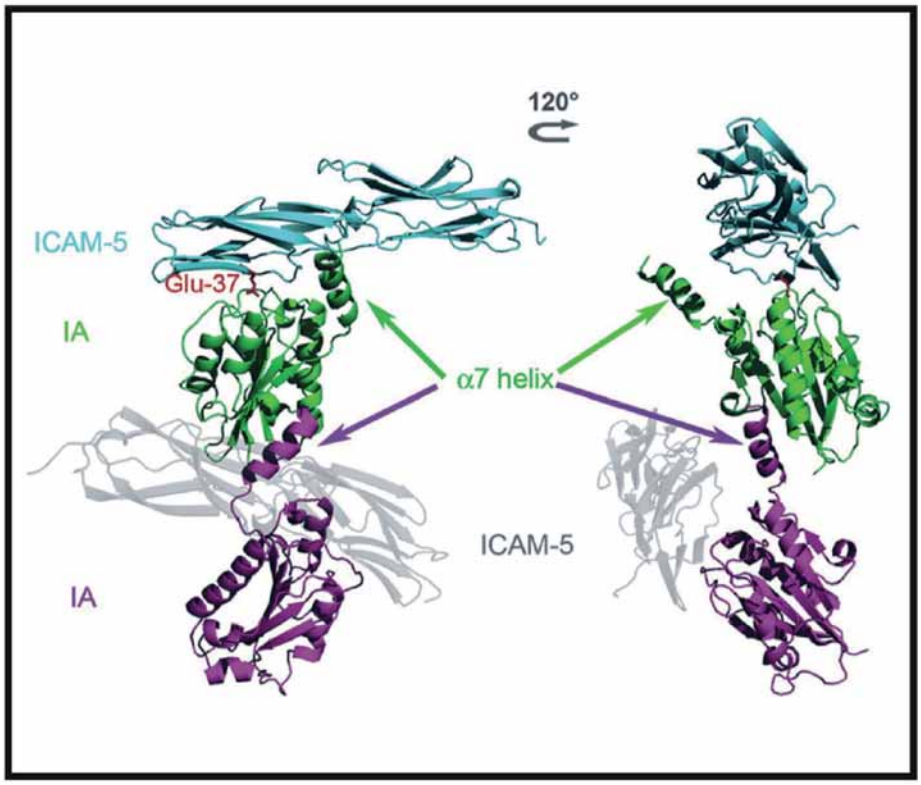

Fig 9. **Ribbon diagram of the ICAM-5-D1-D2/dm-I complex.** Two symmetry-related complexes of the ICAM-5-D1-D2/dm-I are shown. In one complex, ICAM-5 is in cyan and the I domain green. In the other complex, the I domain is in magenta and the ICAM-5 molecule is in shadow for clarity. The C-terminal α7 helix of the magenta I domain inserts into a groove in the green I domain in an upside-down fashion. Glu-37 in ICAM-5 D1 is shown in red as a ball-and-stick model. The figures are all prepared with Pymol (http://www.pymol.org/). Dm, double mutant. Reprinted from *Molecular Cell*, Zhang et al, 2008. Copyright (2008), with permission from Elsevier.

Ligands

ICAM-5 is a cellular ligand for the leukocyte β2 integrin LFA-1 (Tian et al, 1997) (**Table 2**), and domain 1 of ICAM-5 is essential for binding (Tian et al, 2000). In addition, domain 6 may be important for binding to a presently unknown receptor (Tian et al, 2000). X-ray crystallographic studies using the LFA-1-I-domain/ICAM-5-D1-D2 structure have revealed that similar to the other ICAMs, ICAM-5 binds to the LFA-1 I domain. Glu-37 in domain 1 is the main residue mediating binding to LFA-1. The interaction leads to an αL I domain α7 helix outward movement. The α7 helix is able to bind to a neighbouring I domain (**Fig 9**). This allosteric movement is unusual and explains how the weak interaction achieves functional binding (Zhang et al, 2008). ICAM-5 may also bind to β1 integrins (Conant et al, 2010b).

Expression

ICAM-5 is detected only in the brain, more specifically in the telencephalon (Yoshihara and Mori, 1994a). ICAM-5 expression is confined to certain types of neurons, including cortical and hippocampal pyramidal neurons, and olfactory bulb granule cells (Murakami et al, 1991). In the rat hippocampus, ICAM-5 is expressed in excitatory pyramidal neurons during the first stages of dendritic differentiation. It is not expressed in GABA-ergic inhibitory interneurons (Benson et al, 1998). In the cat visual cortex, ICAM-5 expression is low in layer IV neurons but the expression is much higher in neurons in the other cortical layers (Imamura et al, 1990).

ICAM-5 is localized to neuronal cell soma and dendrites. It is not found in axons (Mori et al, 1987, Yoshihara and Mori, 1994a, Benson et al, 1998). Its expression is restricted to dendritic growth cones and filopodia in hippocampal neurons. Dendritic outgrowth is very extensive in neurons expressing ICAM-5 (Benson et al, 1998).

The ICAM-5 expression is dependent on the developmental stages. In rodents, ICAM-5 is expressed at low levels during embryonic development. The expression increases strongly after birth, and continues to be expressed highly in the adult brain (Yoshihara et al, 1994b). In humans, ICAM-5 is expressed in the hippocampus at gestational (embryonic) week 29. In the temporal cortex, ICAM-5 expression starts to increase from the 35th to 39th gestational weeks but high expression levels persist into adulthood (Arii et al, 1999). In the telencephalon, the beginning of ICAM-5 expression temporally parallels dendritic elongation and synaptogenesis.

A soluble form of ICAM-5 (sICAM-5) is found in the cerebrospinal fluid and plasma and its level increases during inflammation and other conditions in the brain, such as acute encephalitis and temporal lobe epilepsy (Lindsberg et al, 2002, Rieckmann et al, 1998).

Cytoplasmic associations

ICAM-5 has been shown to bind to the ERM family members ezrin and radixin (Furutani et al, 2007).

Physiological function

ICAM-5 knockout mice have been produced (Nakamura et al, 2001) and the results indicate that ICAM-5 is not vital since the mutant mice live and reproduce. They display no major abnormalities in their brain anatomy. However, these mice show enhanced LTP and reference memory, for example performance in learning tasks, such as radial maze and water-finding with food rewards, was enhanced. In contrast, the ability to perform Morris water maze and contextual fear conditioning tasks was not enhanced. Thus, it seems that ICAM-5 may be involved in reward-motivated learning and memory. It has also been shown that ICAM-5 knockout mice have enlarged spine heads, which suggests that ICAM-5 regulates the dendritic spine morphology in the brain (Matsuno et al, 2006). Spine maturation is also accelerated in these mice.

ICAM-5 binding to β2 integrins induces relocation of LFA-1 on the microglial cell surface and spreading of microglia (Mizuno et al, 1999). In addition, soluble ICAM-5 may be involved in regulating T cell activation as soluble ICAM-5 is able to weaken T cell activation (Tian et al, 2008, Gahmberg et al, 2008).

4.3. OTHER ICAMS

ICAM-1 (CD54)

ICAM-1 consists of five Ig domains, a transmembrane domain, and a short cytoplasmic domain (Patarroyo et al, 1987, Staunton et al, 1988) (Fig 8). Eight N-glycosylation sites are found in ICAM-1. A soluble form of ICAM-1 (sICAM-1) is found in human serum, due to proteolytic cleavage (Rothlein et al, 1991). Both membrane-bound and soluble ICAM-1 are known to form dimers (Miller et al, 1995, Reilly et al, 1995). The crystal structures have been determined for domains 1-2 (Bella et al, 1998, Casasnovas et al, 1998) and 3-5 of ICAM-1 (Yang et al, 2004, Chen et al, 2007) (Fig 10A, B).

Leukocyte β2 integrins LFA-1 (CD11a/CD18, αLβ2) (Staunton et al, 1990), Mac-1 (CD11b/CD18, αMβ2) (Diamond et al, 1991), and CD11c/CD18 (αXβ2) (Blackford et al, 1996) bind to ICAM-1 (**Table 2**). LFA-1 and Mac-1 bind to domains 1 (Staunton et al, 1990) and 3 (Diamond et al, 1991) of the ICAM-1 dimer, respectively. Two residues Asp-229 and Glu-254, in domain 3 are necessary for the binding of Mac-1 (Diamond et al, 1991). The binding site for CD11c/CD18 on ICAM-1 resides on domain 4 (Frick et al, 2005).

Mutational studies of ICAM-1 indicate that Glu-34 and Gln-73 in domain 1 are important for binding to LFA-1 (Staunton et al, 1990), and the interaction is mediated by Mg^{2+} bound to the I-domain of LFA-1 and the carboxyl group of the Glu-34 residue on ICAM-1 (Bella et al, 1998, Casasnovas et al, 1998) (**Fig 10A, B**).

X-ray crystallographic studies have shown that, Glu-34 in domain 1 is able to form a coordination site with Mg^{2+} in the α I domain metal ion-dependent adhesion site (MIDAS) when the αL I domain is binding to ICAM-1 (Shimaoka et al, 2003). A bend between domains 3 and 4 causes ICAM-1 dimers to become L-shaped, and when it is present on the cell surface, ICAM-1 has been proposed to exist as a bended dimer (Staunton et al, 1990, Wang and Springer, 1998). Dimerization of ICAM-1 increases the avidity of the integrin-ICAM-1 interaction. X-ray crystallographic studies have shown that O-dimers are formed between domains 1 and 4 (**Fig 11A**) though domains 4 and 5 are needed for the formation the dimer. After ICAM-1 binding to LFA-1, conformational changes may occur in ICAM-1 and this leads to the clustering and formation of ICAM-1 "W-dimers" on the cell surface (Grakoui et al, 1999, Yang et al, 2004) (**Fig 11B**). At present, it is not clear how the W-dimers facilitate leukocyte adhesion, transmigration and/or outside-in signaling. Further studies have revealed that the ICAM-1 monomer-dimer transition is usually accompanied with IgSF domain rearrangement (Chen et al, 2007).

Crystal structures have also been determined for the adhesion molecules ICAM-2, VCAM-1, and MAdCAM-1, and the data suggests that an acidic residue in domain 1 is involved in integrin binding. In MadCAM-1 and VCAM-1, the acidic residue is aspartate, and in ICAM-1 and ICAM-2, the residue is glutamate (Wang and Springer, 1998).

ICAM-1 is also a receptor for certain pathogens such as the major group of rhinovirus (Greve et al, 1989, Staunton et al, 1990), coxsackie virus A21 (CAV-21) (Xiao et al, 2001, 2005), and *Plasmodium falciparum* (Berendt et al, 1992) (**Table 2**). According to the results of the crystal structures, residues involved in binding to rhinovirus are located in the BC and FG loops in domain 1. The loops in human ICAM-2 and murine ICAM-1 are very different in their structures, and they do not bind rhinoviruses (Bella et al, 1998, Casasnovas et al, 1998). The binding sites for *Plasmodium falciparum* (Casasnovas et al, 1998) and CAV-21 (Xiao et al, 2001, 2005) are located in domain 1 of ICAM-1. The binding sites for LFA-1, *Plasmodium falciparum*, and rhinovirus are partially overlapping, but not identical in domain 1 of ICAM-1.

The first Ig domain in ICAM-1 also binds to the fibrinogen γ chain (D'Souza et al, 1996). Moreover, ICAM-1 functions as a cellular receptor for hyaluronan (McCourt et al, 1994) and CD43 (Rosenstein et al, 1991) (**Table 2**).

ICAM-1 is expressed on leukocytes, vascular endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, keratinocytes etc. In the CNS, ICAM-1 is expressed in brain endothelial and glial cells (Tian et al, 2008) though its expression level is low. However, it can be strongly increased by various inflammatory cytokines such as interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and lipopolysaccharide (LPS). Thus, ICAM-1 expression can be rapidly upregulated

under immune or inflammatory reactions, and is transcriptionally regulated (Hubbard and Rothlein, 2000).

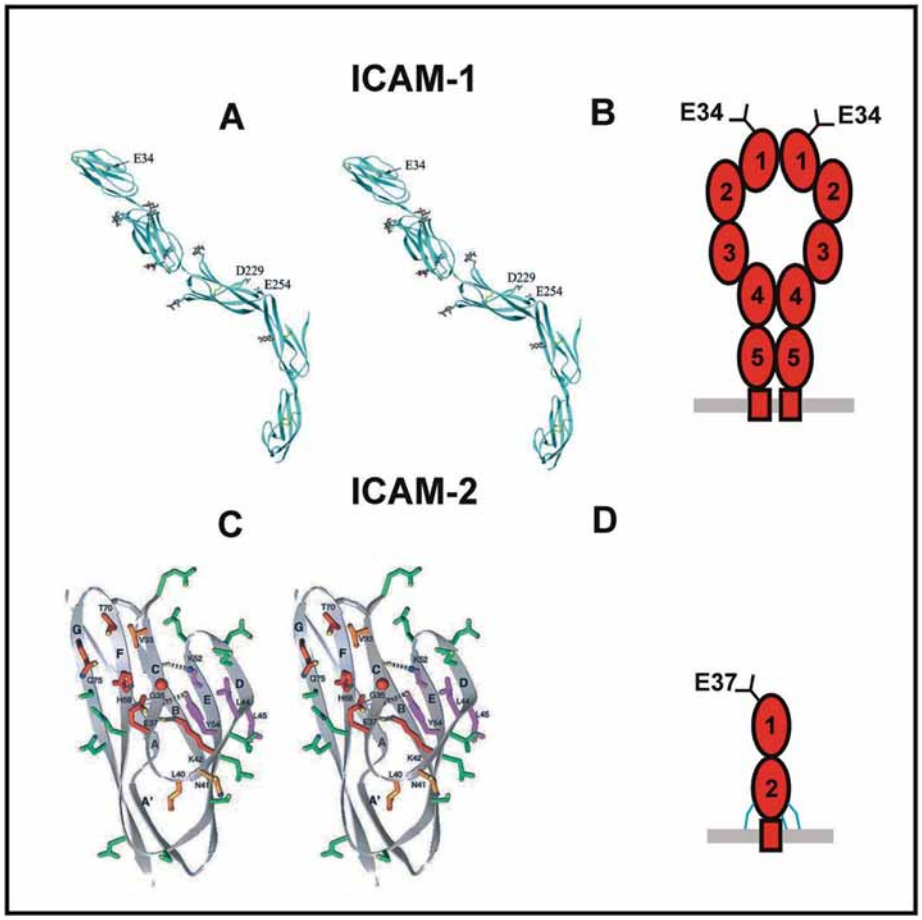

Fig 10. LFA-1/Mac-1 binding regions of ICAM-1 and ICAM-2. The crystal structures of ICAM-1 domains 1-5 (D1-D5) (**A**) and ICAM-2 domain 1 (D1) (**C**) are shown. (**A**) This ICAM-1 D1-D5 model was constructed by linking the known D1-D2 structure and D3-D5 structure reported here at the pivot residue Val-186. The key LFA-1 binding site Glu-34 of D1 and the Mac-1 binding sites Asp-229 and Glu-254 of D3 (all shown in ball-and-stick representation) point upward, available for ligand binding from the opposing cell above. Note that the Glu-34 is on a relatively flat surface, whereas the Asp-229 is on a protruded loop. All seven identified glycans are shown in ball-and-stick representation (prepared with RIBBONS [Carson, 1995]). **A** is reprinted from *Molecular Cell,* Yang et al, 2004. Copyright (2004), with permission from Elsevier. **(C)** The backbone of ICAM-2 is shown in grey as a ribbon diagram with β-strands lettered. Disulfide bonds are shown in grey. Side chains of individual residues tested by mutagenesis are shown as ball and stick. The carbons and bonds of these side chains are color-coded according to the percentage of wildtype binding to LFA-1 after mutagenesis, red, <35%; yellow-orange, 35-70%; green, >70%; magenta, <35% with a possible effect on domain structure. Side chain oxygen and nitrogen atoms are yellow and blue, respectively. **C** is reprinted from *Proceedings of the National Academy of Sciences*, Casasnovas et al, 1999. Copyright (1999), with permission from National Academy of Sciences, U.S.A. **B** and **D** are the ICAM-1 and ICAM-2 schematic models on cell membrane. The tripod-like array of N-glycans in ICAM-2 domain 2 are shown in blue (**D**). Modified from Li Tian's thesis: ICAM-5 (Telencephalin) – a Novel Cell Adhesion Molecule, 2001.

ICAM-1 has a short cytoplasmic domain with only 28 amino acids. It does not contain the tyrosine-containing motif for kinase binding. The ligation of fibrinogen results in tyrosine phosphorylation of the ICAM-1 cytoplasmic domain, which leads to the binding of the Src homology domain 2-containing tyrosine phosphatase 2 (SHP2) (Pluskota et al, 2000). By binding to different adaptor proteins, including growth factor receptor bound protein 2 (Grb2), SOS and Shc, ICAM-1 may act as a signal transducer (Gardiner and D'Souza, 1999). The MAPK pathway is activated by ICAM-1 through downstream tyrosine kinases. LFA-1 binding to ICAM-1 also leads to signal transduction events (**Fig 12**) and cytoskeletal remodeling (**Fig 11A, B**).

ICAM-1 is mainly found to accumulate in membrane projections on some B cell lines, and it colocalizes with a-actinin (Carpén et al, 1992) and ezrin (Heiska et al, 1998). Ezrin and moesin colocalize with ICAM-1 in brain endothelial cells (Romero et al, 2002). Via its membrane proximal region of the intracellular domain, ICAM-1 can bind to a variety of molecules, such as a-actinin (Carpén et al, 1992), b-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Federici et al, 1996), and ERM proteins (Heiska et al, 1998, Mangeat et al, 1999). In the schematic model presented in Figure 11, ezrin and moesin associate with ICAM-1 before clustering and subsequently they dissociate from the ICAM-1 cytoplasmic tail. ICAM-1 has not been reported to bind to the cytoskeletal proteins talin, tensin, or vinculin. There is some data that a Rho GTPase is able to regulate ICAM-1 clustering through binding to ERM proteins (Wojciak-Stothard et al, 1999). The absence of the ICAM-1 cytoplasmic domain blocks T cell migration and polymorphonuclear neutrophil migration (Greenwood et al, 2003, Sans et al, 2001).

α-Actinin-1 and -4 bind to ICAM-1 and this interaction may be involved in neutrophil transmigration. α-Actinin-4 small interfering RNA (siRNA) knockdown was able to decrease neutrophil transmigration (Celli et al, 2006). When LFA-1 on the leukocyte cell surface binds to endothelial ICAM-1, the α-actinin binding site in the ICAM-1 cytoplasmic domain, i.e. the RKIKK sequence, is needed for those endothelial membrane projections that contain ICAM-1 (Oh et al, 2007).

The physiologic role of ICAM-1 has been studied very extensively. ICAM-1 has several functions, including immune cell trafficking, cell adhesion during antigen presentation, viral attachment to cells, and signalling pathways initiated by "outside-in" signalling (Smith et al, 1989). The engagement of ICAM-1 and β2 integrins promotes the adhesion of activated leukocytes to the endothelium and recruits leukocytes towards the site of tissue damage or infection. Binding of fibrinogen to ICAM-1 may also contribute to leukocyte migration towards the inflamed tissue, and in this process, β2 integrins associate with fibrinogen, which further binds to ICAM-1, acting as a mediator between leukocytes and endothelial cells (Altieri et al, 1999). ICAM-1 also has an important function as a costimulatory molecule in both T cells and antigen-presenting cells (APCs). It is able to bind to LFA-1 and promote T cell activation (Lebedeva et al, 2005).

ICAM-1 is involved in the pathogenesis of MS and EAE, the MS animal model. ICAM-1 gene polymorphisms may be associated with a risk of developing MS (Nejentsev et al, 2003, Cournu-Rebeix et al, 2003, Mycko et al, 1998). ICAM-1 expression is increased in endothelial cells, astrocytes and microglia in MS and EAE (McMurray, 1996, Lee and Benveniste, 1999, Matsuda et al, 1994, Gimsa et al, 2001, Sipkins et al, 2000, Carrithers et al, 2000).

Studies on ICAM-1 knockout mice show that they develop quite normally. The ICAM-1 knockout mice having a deletion of the domain 3 or 4 exhibit increased levels of neutrophils, impaired neutrophil emigration, decreased hypersensitivity response and enhanced resistance to septic shock (Sligh et al, 1993, Xu et al, 1994). ICAM-1 knockout mice having a total deletion of all ICAM-1 forms display delayed EAE (Bullard et al, 2007).

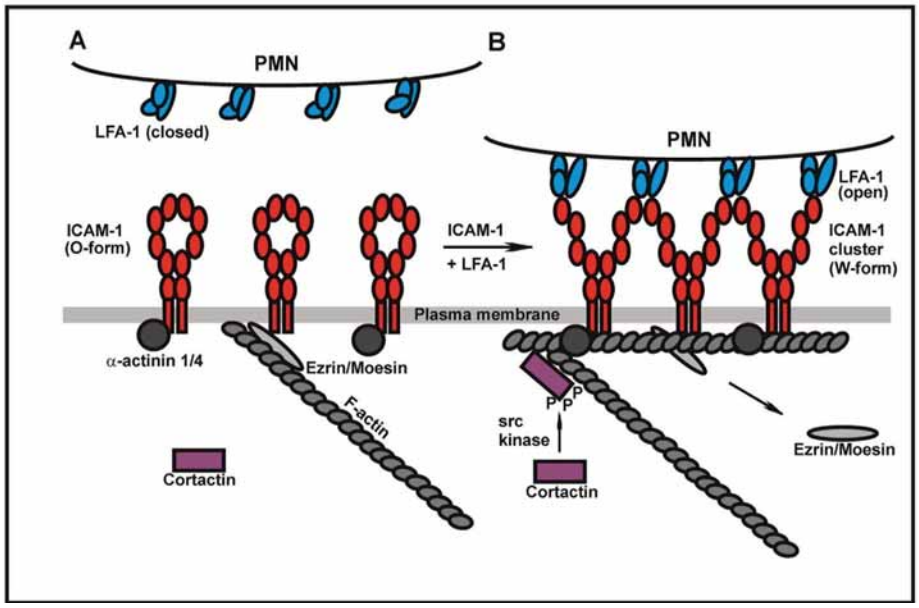

Fig 11. ICAM-1 binding to LFA-1 leads to cytoskeletal reorganization. (**A**) ICAM-1 monomers dimerize to the O-form (Yang et al, 2004). ERM proteins and α-actinin (1 and/or 4) bind to the ICAM-1 cytoplasmic tail. (**B**) ICAM-1 binding to LFA-1 results in conformational changes in both proteins, and this leads to W-form clustering. Ezrin and moesin detach from the ICAM-1 cytoplasmic domain. α-Actinin can still bind, and then cortactin joins the ICAM-1 cluster. The Src-family kinases phosphorylate cortactin. In this way, cytoskeletal reorganization may be the result of ICAM-1 clustering. PMN, polymorphonuclear neutrophil. Modified from *Progress in Inflammation Research,* Adhesion Molecules: Function and Inhibition, Auerbach et al, 2007.

The level of sICAM-1 increases in bacterial sepsis (Berner et al, 1998), type II diabetes (Guler et al, 2002), pre-eclampsia (Coata et al, 2002), atherosclerosis (Blankenberg et al, 2003), and obesity (Brake et al, 2006). Its function is not clear but it may act

as a modulator in inflammation. sICAM-1 may function as an β2 integrin activator. It is also possible that it could inhibit leukocyte adhesion by binding to β2 integrins (Kotovuori et al, 1999).

ICAM-2 (CD102)

ICAM-2 consists of two N-terminal Ig domains with 34% homology to ICAM-1, a transmembrane domain, and a short cytoplasmic tail with 26 amino acids (Nortamo et al, 1991a, Staunton et al, 1989) (**Fig 8**). ICAM-2 domains contain six N-glycosylation sites. The ICAM-2 gene is located on a different chromosome as compared to the other ICAM genes which are clustered in the same chromosome region (Hayflick et al, 1998). ICAM-2 is not known to form dimers (Reilly et al, 1995). Soluble ICAM-2 is found in human serum and its level increases in patients with hemorrhagic fever accompanied by renal syndrome (Qi et al, 2006).

The leukocyte β2 integrins LFA-1 (Staunton et al, 1989), Mac-1 (Xie et al, 1995) and CD11c/CD18 (Sadhu et al, 2007) bind to ICAM-2 (**Table 2)**. Glu-37 in ICAM-2 domain 1 is important for LFA-1 binding (Casasnovas et al, 1999) (**Fig 10C, D**). Furthermore, Mac-1 and its I-domain also bind to the first Ig-domain in ICAM-2 (Xie et al, 1995). An ICAM-2-derived peptide has been shown to bind to LFA-1 and Mac-1 (Li et al, 1993). The specific binding site for CD11c/CD18 has not yet been characterized.

The crystal structure of the ICAM-2 extracellular region has been resolved (Casasnovas et al, 1997). A bend of 35 degrees is found between domains 1 and 2 but the predicted binding region is quite flat, and Glu-37 is located on the CFG face of domain 1 on this flat surface. The three N-glycans are able to form a tripod-like structure in domain 2 (Casasnovas et al, 1997) (**Fig 10D**) which may facilitate binding of β2 integrins to ICAM-2.

ICAM-2 expression is constitutive being expressed by endothelial cells at a high level, and at a lower level on platelets (Diacovo et al, 1994), and most leukocytes (Nortamo et al, 1991a). Cytokines are not able to induce ICAM-2 expression to any major extent (Nortamo et al, 1991b). ICAM-2 tends to be found at endothelial cellular junctions whereas ICAM-1 is found on endothelial cell surface (McLaughlin et al, 1998).

ICAM-2 is found to localize in uropods of natural killer cells, and it colocalizes with ezrin in these regions (Helander et al, 1996). The cell uropod is a membrane projection with related cytoskeletal components at the end of a migrating lymphocyte (Serrador et al, 1997, 2002).In addition, ICAM-2 is known to bind to α-actinin (Heiska et al, 1996), and ERM family proteins (Heiska et al, 1998). The interaction between ICAM-2 and radixin has been confirmed by crystallization studies (Hamada et al, 2003).

ICAM-2 is involved in leukocyte trafficking (Issekutz et al, 1999), immune cell activation (de Fougerolles et al, 1994), and Rac-dependent angiogenesis (Huang et al, 2005). Moreover, ICAM-2 seems to participate in leukocyte transmigration when PECAM-1 is absent (Huang et al, 2006).

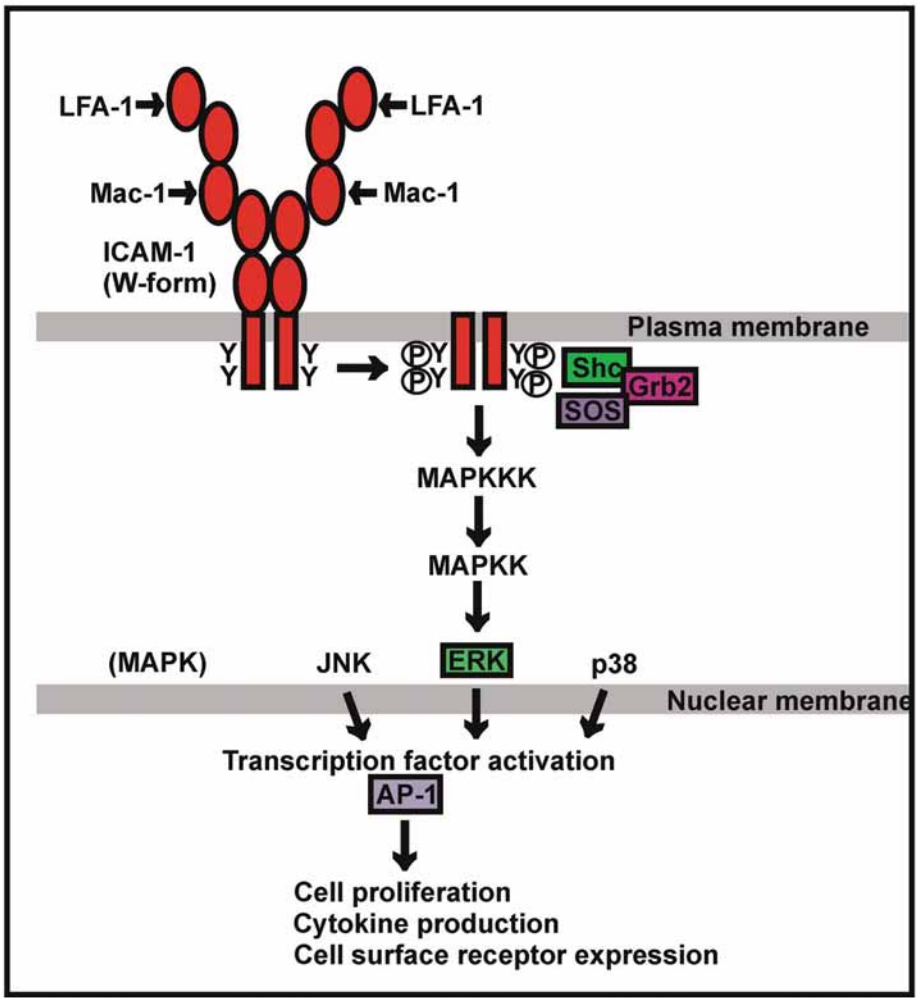

Fig 12. A model of β2 integrin/ICAM-1-mediated signal transduction. ICAM-1 multimers form after binding to β2 integrins (LFA-1, Mac-1). Src-family kinases phosphorylate ICAM-1 cytoplasmic domain. Adaptor proteins, such as Grb2, SOS and Shc help ICAM-1 in signal transduction. The MAPK pathway is activated, and ERKs also become activated. This leads to transcription factor activation, and the results are cell proliferation, cytokine production and increased cell surface receptor expression. Modified from Hubbard and Rothlein, 2000.

ICAM-2 may also be involved in stimulating integrin activity. A P1 peptide from the ICAM-2 domain 1 is able to activate β2 integrins (Li et al, 1995), and it increases the migration and cytotoxicity of natural killer cells (Li et al, 1993). It has been demonstrated that the β2 integrin binding affinity for ICAM-1 is increased by the presence of soluble ICAM-2 (outside-in signalling) (Kotovuori et al, 1999).

ICAM-2 knockout mice have normal amounts of leukocytes (Gerwin et al, 1999). They seem to have a delayed increase in eosinophil accumulation in the airway lumen

during lung inflammation. They also have a prolonged presence of eosinophils in the lung tissue. Thus, ICAM-2 may be involved in eosinophil trafficking from the blood circulation to the airway lumen (Gerwin et al, 1999).

ICAM-3 (CD50)

ICAM-3 consists of five Ig domains, a transmembrane domain, and a short cytoplasmic domain (**Fig 8**). It resembles ICAM-1 to some extent, with 47% amino acid identity (Fawcett et al, 1992). ICAM-3 is known to have 15 N-glycosylation sites. Rotary shadowing electron microscopic studies have shown that there is no bend in the ICAM-3 structure (Vazeux et al, 1992). ICAM-3 appears to exist as a monomer on the cell surface. A soluble form of ICAM-3 can also be found in human serum where its level increases in certain pathological conditions, including systemic lupus erythematosus (Pino-Otin et al, 1995) and MS (Kraus et al, 2000).

LFA-1 (de Fougerolles and Springer, 1992) and CD11d/CD18 ($\alpha D\beta 2$) (Van der Vieren et al, 1995) are binding partners for ICAM-3 (**Table 2**), and binding to LFA-1 occurs through the first Ig-domain in ICAM-3 (Fawcett et al, 1992). It appears that the LFA-1 binding site in ICAM-3 is similar to the corresponding sites in ICAM-1, and -2 (Holness et al, 1995). Interestingly, the LFA-1 binding regions for ICAM-1, -2 and -3 on the LFA-1 I domain seem to be overlapping even though they are not not very similar (Binnerts and van Kooyk, 1999). Differences in the interactions between ICAMs and LFA-1 may contribute to their different affinities towards their ligands. Biophysical studies have shown that the order in LFA-1-binding affinity is ICAM-1>ICAM-2>ICAM-3 (Shimaoka et al, 2001). X-ray crystallographic studies using domain1 of ICAM-3 and LFA-1 I domain have shown that ICAMs possess a similar mechanism for docking to the I domain. Hydrophobic interactions appear to be crucial in the different ICAM ligand binding affinities (Song et al, 2005). Moreover, ICAM-3 deglycosylation results in increased LFA-1 binding affinity (Song et al, 2005).

In addition, a C-type lectin, DC-SIGN, has been shown to function as a high-affinity receptor for ICAM-3. DC-SIGN is found on dendritic cells (DCs) and it binds to the ICAM-3 carbohydrate residues (Geijtenbeek et al, 2000, 2002) (**Table 2**).

ICAM-3 is the major ICAM molecule on resting lymphocytes. It is evidently an important molecule in initiating the immune response. When lymphocytes are activated by exposure to phorbol ester, ICAM-3 expression is not increased (de Fougerolles and Springer, 1992).

ICAM-3 has been postulated to be an important signalling receptor (**Fig 13**). The ICAM-3 cytoplasmic domain consists of 37 amino acids many of which are serine (Hayflick et al, 1997, Lozano et al, 1992) and tyrosine residues (Skubitz et al, 1995). T cell receptor (TCR) crosslinking or phorbol ester treatment was shown to activate the phosphorylation of these residues with PKC appearing to be responsible

for the phosphorylation of serine residues (Hayflick et al, 1997, Lozano et al, 1992). Mutation of serine residues to alanine resulted in loss of T lymphocyte function including cytokine secretion, aggregation of cells and cell spreading (Hayflick et al, 1997). ICAM-3 crosslinking results in tyrosine phosphorylation of different target proteins (Arroyo et al, 1994, Feldhaus et al, 1998, Juan et al, 1994). Increased intracellular calcium level, and activation of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ in leukemia cell lines have also been reported (Juan et al, 1994). The results also revealed an increase in phosphatidylinositol hydrolysis and phospholipase Cγ1 phosphorylation in T lymphocytes (Berney et al, 1999), and PKC-dependent cytoskeletal reorganization in neutrophils (Feldhaus et al, 1998). Furthermore, ICAM-3 overexpression is known to induce phosphorylation of different signalling molecules, including Akt, FAK and phosphoinositide-dependent protein kinase-1 (PDK1), which can lead to enhanced cell proliferation in lung cancer (Kim et al, 2006).

Moesin, a member of the ERM family, interacts with the ICAM-3 cytoplasmic domain in the presence of PIP2. It can be relocated with ICAM-3 to T lymphocyte uropods. Chemokine stimulation has triggered moesin association with ICAM-3 and this seems to depend on the action of Rho GTPases (del Pozo et al, 1999) and/or PI3K (Vicente-Manzanares et al, 1999). In addition, a novel serine-rich motif found in the ICAM-3 cytoplasmic region is important for its subcellular localization in motile lymphocytes. It was found that mutation of serine residues impaired the ICAM-3/moesin interaction and ICAM-3 uropod targeting in migrating lymphocytes (Serrador et al, 2002). In that study, another ERM family member, ezrin, was shown to bind to ICAM-3 *in vitro*. In contrast, earlier studies have failed to detect ezrin binding to ICAM-3 (Heiska et al, 1998).

ICAM-3 is involved in lymphocyte aggregation. The ICAM-3/LFA-1 interaction may facilitate the interaction of resting T cells with antigen-presenting B cells (de Fougerolles and Springer, 1992, Fawcett et al, 1992, Vazeux et al, 1992). Through binding to β2 integrins (Hernandez-Caselles et al, 1993) (**Fig 13A**), or DC-SIGN (Geijtenbeek et al, 2000) (**Fig 13B**), ICAM-3 is involved in T cell activation (Campanero et al, 1993).

The interaction between the APCs and the T cells through ICAM-3 makes it possible for the T cell to recognize the antigenic epitopes on APCs (Montoya et al, 2002). The ICAM-3/LFA-1 interactions may also regulate the ICAM-1/LFA-1-mediated T cell adhesion by increasing LFA-1 accumulation at cell adhesion sites (Bleijs et al, 2000). Additionally, ICAM-3 has been shown to induce apoptotic signals in leukocytes in which the Fas/FasL or TNFα/TNF receptor-activated pathways are not involved (Lopez-Briones et al, 1998, Stucki et al, 2000, Kessel et al, 2006).

ICAM-4 (LW, CD242)

ICAM-4 was originally described as the LW (Landsteiner-Wiener) blood group antigen. Later it was found that it shows sequence similarity to the ICAMs. ICAM-4

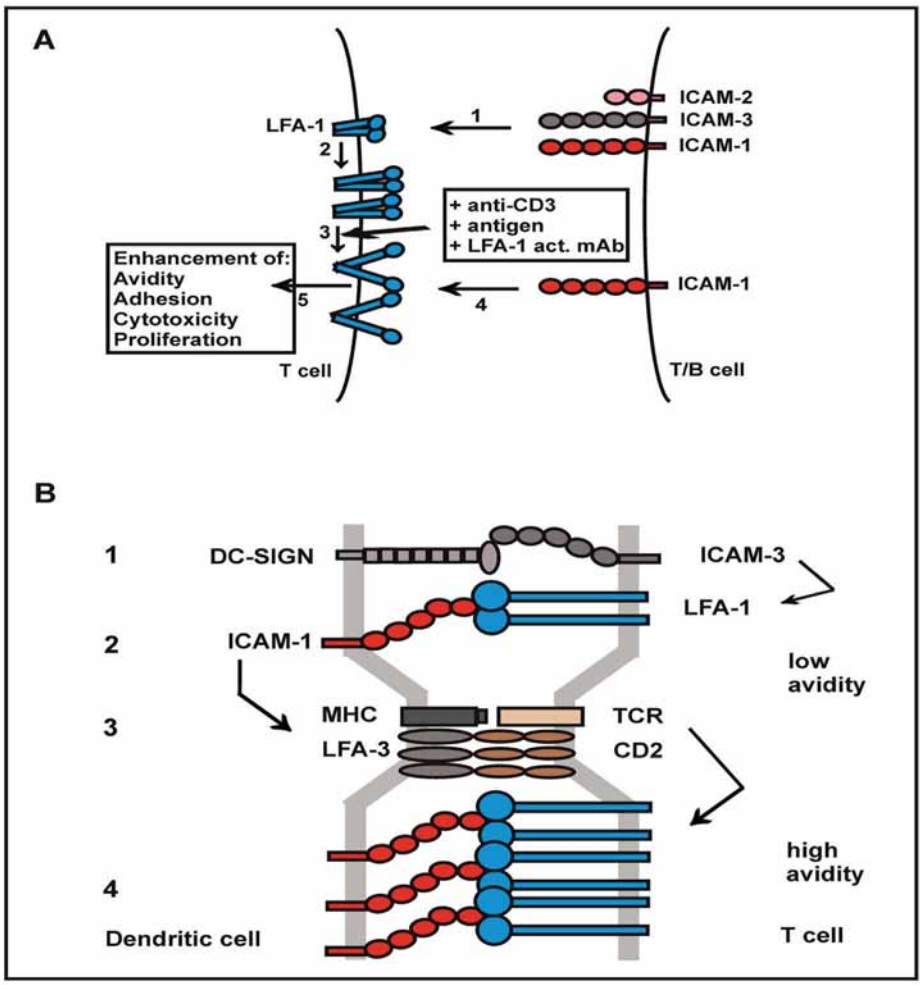

Fig 13. ICAM-mediated signalling models and the involvement of ICAM-3. In **A**, LFA-1 on T cells binds with low affinity to ICAM-3 on the target cell (1). The target cell expresses also ICAM-1 and ICAM-2. LFA-1 binding to ICAM-3 promotes LFA-1 clustering (2). Anti-CD3 triggering or antigen recognition leads to generation of intracellular signals, increased LFA-1 binding affinity for ICAM-1 (3). LFA-1 binds to ICAM-1 to form a stable interaction (4). The LFA-1/ICAM-1 interaction also leads to signal transduction with the result being T cell proliferation, cytotoxicity and gene expression. Modified from Bleijs et al, 2000. In **B**, DC-SIGN on DC and ICAM-3 on T cell bind to each other (1). Low-avidity interactions can form between DCs and T cells. LFA-1/ICAM-1 interaction helps in antigen-independent DC-T cell clustering (2). T cell receptor (TCR) ligation (3) leads to signal transduction, and high-avidity CD2/LFA-3 and LFA-1/ICAM-1 interactions result in immunological synapse stabilization. The LFA-1/ICAM-1 interaction is seen in the outer ring. CD2/LFA-3 and TCR/MHC/peptide are found in the center of the immunological synapse (4). Modified from Geijtenbeek et al, 2000.

consists of two Ig-like domains, a transmembrane domain, and a short cytoplasmic domain with 12 amino acids. ICAM-4 contains four N-glycosylation sites and one O-glycosidic oligosaccharide may also exist (Bailly et al, 1994) (**Fig 8**). Soluble ICAM-4 in human serum has not been described yet.

ICAM-4 binds to LFA-1, Mac-1, (Bailly et al, 1995), and CD11c/CD18 (Ihanus et al, 2007) (**Table 2**). It also binds to the αVβ1, αVβ3 and αVβ5 integrins (Spring et al, 2001, Hermand et al, 2004), and the platelet integrin αIIbβ3 (Hermand et al, 2003). ICAM-4 binds to LFA-1 and Mac-1 with weak affinity (Hermand et al, 2000, Ihanus et al, 2003). It binds more efficiently to CD11c/CD18 (Ihanus et al, 2007). Our group has previously shown that binding occurs through the I domains of β2 integrins (Ihanus et al, 2003, 2007). Interestingly, the αV and αIIbβ3 integrins lack the I domain, i.e. these integrins have a different binding site.

The binding site for LFA-1 in ICAM-4 resides in the first domain (Hermand et al, 2000) but both Ig domains are needed for Mac-1 and CD11c/CD18 binding (Ihanus et al, 2007). In ICAM-4, the most important residue for integrin binding is Arg-52 (Glu-34 in ICAM-1 and Glu-37 in ICAM-2) but even when Arg-52 was mutated back to glutamate, it did not disrupt the LFA-1/ICAM-4 interaction (Hermand et al, 2000). Arg-52 and Thr-91 in the domain 1 are important for Mac-1 and CD11c/CD18 binding. Leu-80 in domain 1 is needed for LFA-1 and Mac-1 binding. Glu-151 and Thr-154 in domain 2 are involved in binding to LFA-1. Glu-166 is involved in CD11c/CD18 binding (Hermand et al, 2000, Toivanen et al, 2008, Ihanus et al, 2007). Thus, the ICAM-4 β2 integrin binding regions are different when compared to the β2 integrin binding regions in other ICAMs. Changes in ICAM-4 glycosylation may regulate its ability to function as an adhesion molecule because when N-glycosylation was inhibited, ICAM-4 adhesion was increased (Mankelow et al, 2004).

ICAM-4 is expressed on erythrocytes with glycophorin A and Rh glycoproteins (Gahmberg et al, 1978, Bony et al, 1999, Southcott et al, 1999, Bailly et al, 1994).

It has been found that the ICAM-4 expressed on sickle cells can be phosphorylated (Zennadi et al, 2004).

The physiological function of ICAM-4 is still not clear. ICAM-4 knockout mice are viable but their erythroblastic island formation was diminished. It is believed that αV integrin and erythroblast ICAM-4 -interactions are important for island formation (Lee et al, 2006). By binding to β2 integrins, ICAM-4 could help to keep the immature red blood cells within the bone marrow. On the other hand, the interaction of ICAM-4 with β2 integrins may also play a role in removing senescent red cells from the circulation in the spleen red pulp by phagocytosis (Ihanus et al, 2007, Toivanen et al, 2008). Moreover, secreted ICAM-4 isoforms could be involved in regulating these interactions (Lee et al, 2003).

5. AIMS OF THE STUDY

ICAM-5 is a unique member of the ICAM subfamily and it is solely expressed in the CNS. The functional role of ICAM-5 has remained unclear. The aims of the present study were:

1) To study mechanism of dendritic outgrowth induced by ICAM-5.

2) To examine the binding of α-actinin to ICAM-5, to map the α-actinin binding site in the ICAM-5 cytodomain and to evaluate the involvement of α-actinin/ICAM-5 interaction in neuronal differentiation.

3) To clarify how activation of NMDA receptors can regulate ICAM-5-dependent dendritic spine development.

4) To characterize the binding sites for ICAM-5 and NR1 in α-actinin, and to determine whether ICAM-5 regulates the localization of NR1 during neuronal differentiation.

6. MATERIALS AND METHODS

Detailed descriptions of the materials and methods are found in the original publications.

Anti-ICAM-5 antibodies	**Recognition site in ICAM-5**
mAb TL-1	D1-2
mAb TL-3	D1
mAb 127E	D1-9
mAb 179B	D1
mAb 179I	D1
mAb 179D	D2
mAb 179K	D2
mAb 246A	D4-5
mAb 246D	D4-5
mAb 246E	D2
mAb 246K	D3
pAb anti-ICAM-5cp	ICAM-5 cytoplasmic domain
1000J	D1-9
Cytoskeletal antibodies	**Specificity**
pAb A2543	α-actinin
mAb MAB1682	α-actinin
mAb AT6/172	α-actinin
mAb clone EA-53	α-actinin
mAb MAB1678	filamin
mAb 8d4	talin
anti-actin pAb	actin
rhodamine-phalloidin	actin
Cy3-phalloidin	actin
Other antibodies	**Specificity**
anti-L1CAM mAb	L1
anti-PSD95 mAb	PSD95
anti-MMP-2 pAb	MMP2
anti-MMP-9 pAb	MMP9
anti-tau mAb	tau on axons
anti-MAP-2 mAb	MAP-2 on dendrites
IgG1 control mAb	-
pAb rabbit anti-mouse IgGs	-
Tetra-His mAb	histidine
HRP-anti-human IgG	-
HRP-streptavidin	biotin
HRP-anti-mouse IgG	-
HRP-anti-rabbit IgG	-
HRP-tetra-His	histidine
Alexa488-anti-mouse IgG	-
Alexa488-anti-rabbit IgG	-
Cy3-anti-mouse and anti-rabbit IgGs	-
Cy5-anti mouse and anti-rabbit IgG	-

ICAM-5 cDNA contructs	Domain structure
Paju-ICAM-5	full-length ICAM-5
Paju-ICAM-5-TM	ICAM-5 ectodomain and transmembrane domain
Paju-ICAM-5-GPI	ICAM-5 ectodomain and a GPI anchor
Paju-ICAM-5-KK/AA	full-length ICAM-5 (lysines 857 and 858 mutated to alanines)
7-9-Fc	D7-9
7-8-Fc	D7-8
6-Fc	D6
5-Fc	D5
4-Fc	D4
4-5-Fc	D4-5
3-5-Fc	D3-5
2-5-Fc	D2-5
1-Fc	D1
1-2-Fc	D1-2
1-3-Fc	D1-3
1-4-Fc	D1-4
1-5-Fc	D1-5
1-9-Fc	D1-9
GST-ICAM-5cyto	ICAM-5 cytoplasmic domain
Other cDNA constructs	**Domain structure**
NCAM-Fc	NCAM
ICAM-1-Fc	ICAM-1
His_6-NR1Acyto	NR1A cytoplasmic domain
Chicken gizzard α-actinin	full-length α-actinin
α-actinin-R1	R1 domain
α-actinin-R2	R2 domain
α-actinin-R4	R4 domain

Peptides	Sequence
$ICAM\text{-}5_{857\text{-}861}$	KKGEY-C
$ICAM\text{-}5\text{-}K_{857}/R$	RKGEY-C
$ICAM\text{-}5\text{-}K_{858}/R$	KRGEY-C
$ICAM\text{-}5\text{-}K_{857}\text{-}K_{858}/A\text{-}A$	AAGEY-C
$ICAM\text{-}5\text{-}K_{857}/A$	AKGEY-C
$ICAM\text{-}5\text{-}K_{858}/A$	KAGEY-C
KKGEY	Biot-GGG-KKGEY
$ICAM\text{-}5_{852\text{-}874}$	Biot-QSTACKKGEYNVQEAESSGEAVC
CTT	CTTHWGFTLC
CTTW/A	CTTHAGFTLC

Inhibitors	Function
MK801	NMDAR antagonist
DNQX	AMPAR antagonist
GM6001	broad spectrum MMP inhibitor
MMP-2/MMP-9 inhibitor II	inhibits MMP-2 and MMP-9
MMP-9 inhibitor I	inhibits MMP-9
CTT peptide	inhibits MMP-2 and MMP-9
Cytochalasin D	disrupts the actin cytoskeleton
Latrunculin A	disrupts the actin cytoskeleton

Methods	**Original publications**
Cell culture	I-IV
Cell fractionation	III
Co-immunoprecipitation	II, IV
Confocal microscopy	II-IV
Enzyme-linked immunosorbent assay (ELISA)	I, IV
Flow cytometry	I
Gelatinase zymography	III
GST fusion protein interaction assays	II, IV
Immobilized metal ion affinity chromatography	IV
Immunofluorescent staining	I-IV
Immunohistochemical staining of tissues	III
Mass spectrometry	III
Mice and rats	I-IV
Native-PAGE	I
Neurite outgrowth assays	I-III
Penetratin peptide treatment	II
Peptide affinity chromatography	II
Recombinant protein production and purification	I, II, IV
SDS-PAGE and immunoblotting	I-IV
siRNA	III
Surface plasmon resonance analysis	II, IV
Transfection	I-IV

7. RESULTS

7.1. THE EFFECT OF ICAM-5 HOMOPHILIC BINDING AND ICAM-5/ α-ACTININ INTERACTION IN NEURITIC OUTGROWTH (I, II)

The rabbit ICAM-5 protein is known to exist as a tetramer (Oka et al, 1990). In this study, it was decided to investigate human ICAM-5 transfected to neuronal Paju cells (**I**). Paju is a neural crest-derived neuroblastoma cell line which is not believed to express ICAM-5 or the expression level is very small (Zhang et al, 1996). Neuroblastoma usually affects children and the neuroblastoma cells are able to differentiate under the influence of the appropriate signals.

Native-PAGE showed that ICAM-5 monomers are converted into multimers in Paju-ICAM-5 cells and in rat brain. ICAM-5 was found to exist as a multimer (550 kDa) in transfected Paju cells (**I**, Fig 5b). When Paju-ICAM-5 cells were incubated with cytochalasin D or soluble ICAM-5 D1-2-Fc protein, ICAM-5 was expressed as a 140 kDa monomer (**I**, Fig 5b). Furthermore, the ICAM-5 expression was examined during the different developmental stages in the rat brain. Just after birth, ICAM-5 was found to exist as a monomer (**I**, Fig 5a) but after the tenth day, a multimer form of 550 kDa appeared (**I**, Fig 5a). This multimer form could have been a tetramer and it is known that ICAM-5 exists in a multimeric form in adult rat brain (**I**, Fig 5a).

There was evidence for homophilic binding, i.e. ICAM-5 colocalized at the contact sites between cells but when the cells were treated with ICAM-5 monoclonal antibodies (mAbs), this colocalization was no longer detected(**I**, Fig 2). In an attempt to clarify the homophilic binding ability of ICAM-5 in more detail, a series of extracellular domain deletion constructs of ICAM-5 being linked at their C-terminals to human IgG1 Fc part were constructed (**I**, Fig 2).

According to the results achieved with flow cytometric analysis, recombinant proteins containing domains 1-4, 1-3, 1-2, 2-5, 3-5, 4-5, 4 and 5, particularly the constructs with domains 1-2 and 4-5, bound strongly to Paju-ICAM-5 cells, but not to Paju-WT cells (**I**, Fig 3). The ICAM-5-Fc constructs with domains 1-9, 1-5, 5-9, 6-9, 7-9, 6, 7-8, NCAM-Fc and ICAM-1-Fc did not bind strongly to Paju-ICAM-5 cells. The results imply that the ICAM-5 homophilic binding is mediated by the binding of domains 1 and 2 to domains 4 and 5 but the longer constructs, ICAM-5 D1-5-Fc and 1-9-Fc, may form tertiary structures less effective in binding.

Further evidence for homophilic binding was obtained when the binding of biotinylated D1-D2-Fc of ICAM-5 was investigated using immobilized truncated ICAM-5-Fc proteins, and it was noticed that direct binding occurred between domains 1-2 and domains 4-5 containing proteins (**I**, Fig 4a). It was also found

that ICAM-5 mAbs 179B, 179I and TL-3 were able to block the binding of domains 1-2 to immobilized domains 1-9 without the Fc part (**I**, Fig 4b), i.e. these antibodies recognize domain 1. mAbs 179D, 179K, and 246E were able to increase binding or they had no effect (**I**, Fig 4b), i.e. they recognize domain 2. Thus, ICAM-5 domain 1 binds homophilicly to domains 4-5.

Based on these studies, it appears that the ICAM-5 multimer forms may inhibit the homophilic interaction, or vice versa. Homophilic binding was seen with the truncated ICAM-5 proteins but no cell aggregation was observed in Paju-ICAM-5 cells. In contrast, mouse ICAM-5-transfected L cells were able to aggregate (Yoshihara et al, 1994b). Moreover, ICAM-5 was mostly localized to the cell uropods (**I**, Fig 1b, f). Only a small portion of ICAM-5 could be found at the cell-cell contact sites (**I**, Fig 1b, f). It could be that ICAM-5 multimers block the homophilic binding of an ICAM-5 monomer expressed in a nearby cell.

The next question was whether the homophilic binding ability of ICAM-5 is involved in neuronal activity. When Paju-WT and Paju-ICAM-5 cells were grown on ICAM-5 D1-9-Fc protein, neurite outgrowth was seen in Paju-ICAM-5 cells (**I**, Fig 6b) but no neurite extension was observed in Paju-WT cells (**I**, Fig 6a). In the cells grown on ICAM-5 D1-2-Fc protein, the neurite outgrowth was even more prominent (**I**, Fig. 6d). mAb 179B was able to block neurite outgrowth in Paju-ICAM-5 cells (I, Fig 6f) but it had no effect on the Paju-WT cells (**I**, Fig 6f). In Paju-WT or Paju-ICAM-5 cells cultured on ICAM-1-Fc, the numbers of neurites were clearly reduced (**I**, Fig. 6g, h; Table II).

After these findings it was decided to determine whether ICAM-5 was involved in dendritic outgrowth *in vivo* and thus rat hippocampal neurons which express ICAM-5 were used with rat cerebellar neurons not expressing ICAM-5 being used as control cells. Microtubule-associated protein 2 (MAP-2) and tau staining of the neurons grown on ICAM-5 D1-2-Fc, D1-9-Fc, laminin, heparin-binding growth-associated molecule (HB-GAM), or poly-DL-ornithine revealed that dendritic outgrowth had been induced (**I**, Fig 7a). MAP-2 is a dendritic marker and tau is an axonal marker. Neurons grown on ICAM-1-Fc did not show neuritic outgrowth (**I**, Fig 7a). The dendritic morphology on ICAM-5 coated surfaces was different when compared to the dendritic morphology caused by other molecules: dendritic arbors were abundant (**I**, Fig 7a, b). Quantitative analysis showed that there was no significant difference in neuritic length induced by the different molecules. However as compared to neuritic length on ICAM-1-Fc coated surfaces, the difference was significant. ICAM-5 colocalized with MAP-2 (yellow) in neuronal dendrites. ICAM-5 (red) staining was rather strong on ICAM-5-coated surfaces and tau (green) staining was not very intense (**I**, Fig 7b). Laminin seemed to promote axonal outgrowth better than ICAM-5 (**I**, Fig 7b, right). ICAM-5 induced dendritic outgrowth (**I**, Fig 7b, left). There was less dendritic outgrowth on cerebellar neurons grown on as

ICAM-5 compared to the hippocampal neurons (**I**, Fig 7c).

In an attempt to determine if ICAM-5 function could be blocked on hippocampal neurons, the neurons were grown on ICAM-5 D1-2-Fc and treated with different antibodies or soluble ICAMs. Dendritic outgrowth was blocked by a polyclonal (pAb) anti-ICAM-5 antibody (1000J) (**I**, Fig 8a) and as was expected, control serum had no effect (**I**, fig 8a). Soluble ICAM-5 D1-2-Fc was also able to block dendritic outgrowth. ICAM-1-Fc did not affect the dendritic outgrowth. The blocking effect was analyzed quantitatively (**I**, Fig, 8b).

The next question was to examine if the ICAM-5 cytoplasmic domain has any cytoskeletel binding partners. A glutathione S-transferase (GST) fusion protein containing the ICAM-5 cytoplasmic domain was produced (**II**, Fig 1A). A GST-pull-down assay was utilized to examine the Paju cells not expressing ICAM-5. By using immunoblotting, it was found that both α-actinin in the Paju cell lysate and purified commercial chicken gizzard α-actinin bound to the ICAM-5 cytoplasmic tail but not to GST (**II**, Fig 1B, a, b). Talin and filamin also associated with ICAM-5 (not shown).

The α-actinin binding region in the ICAM-5 cytoplasmic domain was mapped. For this purpose, a short peptide from the ICAM-5 cytoplasmic domain (ICAM-$5_{857\text{-}861}$) was ordered and used in peptide affinity chromatography with purified α-actinin (**II**, Fig 1A). The KKGEY sequence was chosen because ICAM-1 and ICAM-2 α-actinin binding sites are similar compared to this sequence; they also have positively charged residues (Carpén et al, 1992, Heiska et al, 1996). The ICAM-$5_{857\text{-}861}$ peptide bound to α-actinin, and binding to the scrambled control peptide was much weaker (**II**, Fig 1C, a). According to these present results, ICAM-5 and α-actinin interact directly through the two lysines. The main binding site for α-actinin can be found in the region containing amino acids 857-861 (**II**, Fig 1C, a, b). Surface plasmon resonance (SPR) analysis with a Biacore biosensor revealed that the interaction between the KKGEY peptide and α-actinin was fast and had low binding affinity with a K_D of 22.9 μM (**II**, Fig 1E).

Next the ICAM-5/α-actinin interaction was studied *in vivo*. Coimmunoprecipitation experiments showed that ICAM-5 coprecipitated with α-actinin from Paju-ICAM-5 cell lysates (**II**, Fig 2A) and vice versa, α-actinin also coprecipitated with ICAM-5 from rat brain homogenates (**II**, Fig 2B, C). Therefore, α-actinin and ICAM-5 associate with each other *in vivo*.

After this, it was decided to study the colocalization of ICAM-5, α-actinin and F-actin in cells. Different truncated Paju-ICAM-5 cell lines were created and subjected to confocal microscopy (**II**, Fig 3). In Paju-ICAM-5 cells, ICAM-5 localized mostly at the cell uropods and cell-cell contact sites (**II**, Fig 4D, d). Colocalization with α-actinin (**II**, Fig 4F) and F-actin (**II**, Fig 4f) was seen. Paju-neo cells did not seem to produce ICAM-5 (**II**, Fig 4A, a). In the truncated Paju-ICAM-5 cell lines,

Paju-ICAM-5-TM (the cytoplasmic domain of ICAM-5 has been deleted) and Paju-ICAM-5-GPI (ICAM-5 transmembrane domain and the cytoplasmic domain have been replaced with a GPI anchor), weak colocalization of ICAM-5 and α-actinin was seen in both cases (**II**, Fig 4I, L, I, l). Colocalization was totally abolished in the mutant Paju-ICAM-5-KK/AA (lysines replaced with alanine) cell line (**II**, Fig 4O, o). Thus it appears that the lysines are important in the interaction between ICAM-5 and α-actinin. More dying cells were observed when ICAM-5-KK/AA cDNA was transfected to Paju cells. The growth of the cells was very slow and they displayed a different morphology (data not shown). The colocalization was also studied in rat hippocampal neurons where ICAM-5 and α-actinin colocalized in 50% of dendritic filopodia at day 7 (**II**, Fig 5F), and in 80% or more mature dendritic spines at day 14 (**II**, Fig 5L).

Next it was decided to examine how the interaction between ICAM-5 and the actin cytoskeleton affects neuronal differentiation. The different truncated Paju-ICAM-5 cell lines were activated with 100 nM phorbol 12,13-dibutyrate (PdBu), and the cells were allowed to grow (2-3 days) until neuritic outgrowth was observed. ICAM-5, α-actinin and F-actin immunostaining was done, and confocal microscopy performed. In Paju-ICAM-5-TM (**II**, Fig 6A, e-h) and Paju-ICAM-5-KK/AA (**II**, Fig 6A, i-l) cells, the colocalization was not so obvious as in Paju-ICAM-5 cells (**II**, Fig 6A, a-d). In Paju-ICAM-5-TM (average length 36 μm/neurite) (**II**, Fig 6B) or in Paju-ICAM-5-KK/AA cells (average length 35 μm/neurite) (**II**, Fig 6B) the neurites were shorter than in Paju-ICAM-5 cells (average length 73 μm/neurite) (**II**, Fig 6B) ($P < 0.001$).

Interestingly, when Paju-ICAM-5 cells were treated with the penetratin-coupled $ICAM\text{-}5_{857\text{-}861}$ peptide containing the sequence KKGEY, morphological changes could clearly be seen. The Paju-ICAM-5 cells became much more round-shaped compared to the normal Paju cells (**II**, Fig 7A-B). Penetratin 1 is a peptide containing 16 amino acids which is able to pass through biological membranes (Prochiantz, 1996, Derossi et al, 1996).

7.2. THE ROLE OF ICAM-5 IN DENDRITIC SPINE MATURATION AND NR1 LOCALIZATION (III, IV)

Matrix metalloproteinases (MMPs) are enzymes that are involved in tissue remodelling. MMP-2 and MMP-9 expression is strong in the developing brain (Szklarzyk et al, 2002, Ayoub et al, 2005). NMDAR activation and LTP affect the MMP-9 expression level and activity (Meighan et al, 2006, Nagy et al, 2006). MMP-9 may also be associated with dendritic spine remodeling, synaptic plasticity, and memory formation (Meighan et al, 2006, Nagy et al, 2006). Therefore, it was

considered important to study if ICAM-5 could be cleaved by MMPs and whether this affects the dendritic spine development.

The next experiment examined if ICAM-5 would be cleaved in hippocampal neurons during neuronal maturation under physiological conditions. It is known that sICAM-5 can be found in different pathological conditions. Full-length ICAM-5 (130 kDa) expression in neurons increased from 7 days *in vitro* (DIV) forward (**III**, Fig 1A, B, right). sICAM-5 (85-110 kDa) could be detected at 14-21 DIV (**III**, Fig 1A, B, left). Usually at this time, dendritic spines have formed and synaptic connections are made. Thus, the release of the ICAM-5 ectodomain may be involved in dendritic spine maturation.

Since GluRs are involved in dendritic spine development, it was interesting to elucidate the effect of NMDA and AMPA on ICAM-5 enzymatic cleavage in cultured rat hippocampal neurons. It was observed that NMDA and AMPA could induce ICAM-5 cleavage from hippocampal neurons (**III**, Fig 1C) and this was blocked by the NMDAR antagonist MK801, the AMPA receptor (AMPAR) antagonist DNQX (6,7,-dinitroquinoxaline-2,3[1H,4H]-dione) (**III**, Fig 1C, D), MMP-2- and -9 inhibitors (**III**, Fig 2A, B) and siRNAs (**III**, Fig 2C). Thus, it seems that NMDAR activation stimulates MMP-2 and MMP-9, leading to ICAM-5 cleavage.

NMDARs and AMPARs are known to be involved in regulating actin cytoskeleton dynamics. Based on this knowledge, it was decided to examine whether ICAM-5 cleavage induced by NMDAR activation depended on anchoring to the actin cytoskeleton. The results showed that cytochalasin D and latrunculin A were able to significantly increase the ICAM-5 cleavage in hippocampal neurons (**III**, Fig 3A, B). When the neurons were incubated with 20 μM NMDA, ICAM-5 was found in the soluble fraction of the neuronal lysates in large amounts (**III**, Fig 3C, D). Paju-ICAM-5-TM cells also exhibited an increase of ICAM-5 cleavage, in comparison to Paju-ICAM-5 cells (**III**, Fig 3E, F). Thus, it seems that when ICAM-5 dissociates from the actin cytoskeleton, it is more susceptible to MMP cleavage.

To find out the role of MMP-2 and MMP-9 in ICAM-5 proteolytic cleavage, ICAM-5 expression in MMP knockout mice was monitored during postnatal development (from postnatal 1 d to 10 wk). It was found that full-length ICAM-5 expression was higher in newborn MMP-2- and MMP-9 knockout mice as compared to the wild-type (WT) mice (**III**, Fig 4A, C). In contrast, L1 expression was diminished (**III**, Fig 4A, C). Gelatinase zymography further clarified the genotypical identity of the mice. It was also shown that MMP-2 activity was diminished during the postnatal brain development (**III**, Fig 4B).

Earlier findings have shown that ICAM-5 can be detected in dendritic filopodia. The ICAM-5 expression starts at the same time as synaptogenesis (Matsuno et al, 2006). To investigate ICAM-5 expression in dendritic spines at different stages, 10-17 DIV hippocampal neurons were transfected with enhanced green fluorescent

protein (EGFP) being used as a dendritic marker, and dendritic protrusions were studied. ICAM-5 was found in dendritic filopodia (**III**, Fig 5A, arrowheads) and immature thin spines (**III**, Fig 5A, arrowheads). In the mushroom spines, there was less ICAM-5 (**III**, Fig 5A, asterisks). It was also found that NMDA activation could reduce ICAM-5/F-actin colocalization and the number of mushroom spines increased (**III**, Fig 6A, B).

The effects of MMP inhibitors on NMDAR activated ICAM-5 cleavage from spines were studied. Rat hippocampal neurons were incubated with 5 μM NMDA and different MMP inhibitors. It was observed that after NMDA treatment, MMP inhibitors were able to block ICAM-5 cleavage in dendritic shafts but not in spines (**III**, Fig 7A-C). The function of ICAM-5 in thin spines was studied by comparing the responses of NMDA-stimulated EGFP-transfected 15 DIV ICAM-5 knockout and WT hippocampal neurons with time lapse imaging. It was noted that spine heads retracted after NMDA treatment in ICAM-5 knockout mice (**III**, Fig 8B, arrowheads). Thus, it seems that ICAM-5 was crucial for thin spine motility.

It was found that soluble ICAM-5 was able to induce dendritic filopodia elongation from wild-type neurons but not in ICAM-5 knockout mice (**III**, Fig 9A-C). Thus, MMPs are involved in ICAM-5-mediated dendritic spine maturation.

Next it was considered important to study whether ICAM-5 could regulate the localization of NMDA receptors, especially NR1 in neurons. From previous studies it was known that ICAM-5 was able to bind to α-actinin (**II**). NR1 and NR2B, the subunits of the NMDA receptor for glutamate, are also binding partners for α-actinin (Wyszynski et al, 1997). Therefore, it was investigated if ICAM-5 and NR1 have overlapping binding regions in α-actinin.

In an attempt to define the region in α-actinin which binds to the ICAM-5 and NR1 cytoplasmic domains, a biotinylated cytoplasmic ICAM-$5_{852\text{-}874}$ peptide (**IV**, Fig 1A) or a His_6-NR1Acyto protein representing the C-terminal cytoplasmic domain of NR1 (C0-C1-C2 region) (**IV**, Fig 1B) was used in the interaction studies with the different α-actinin constructs in enzyme-linked immunosorbent assay (ELISA) (Gilmore et al, 1994). The KKGEY sequence which is the main α-actinin binding region was included in the ICAM-$5_{852\text{-}874}$ peptide (**IV**, Fig 1A) (**II**). It was found that full-length α-actinin ($P < 0.001$), and α-actinin construct R2 ($P < 0.001$) mediated the most efficient binding to the cytoplasmic ICAM-$5_{852\text{-}874}$ peptide (**IV**, Fig 1D). Full-length α-actinin ($P < 0.001$), and α-actinin construct R2 ($P < 0.001$) also bound strongly to the NR1 cytodomain and the binding was significant (**IV**, Fig 1E). α-Actinin R4 domain ($P < 0.05$) also bound to the NR1 cytodomain. So it seems that ICAM-5 and NR1 share a binding site in the α-actinin R2 domain.

An SPR analysis was performed to determine binding affinities between α-actinin and its R2 and R4 domains with ICAM-5 and NR1 cytoplasmic domains. The ICAM-5 cytodomain had very high affinity for full-length α-actinin, R2 and R1-R4 domains

of α-actinin, the K_D values were 361 nM, 161 nM and 478 nM, respectively (**IV**, Fig 2A). In contrast, the NR1A cytodomain only bound with low affinity to full-length α-actinin (K_D 2.97 μM), α-actinin R2 (K_D 2.02 μM) and R1-R4 domains (K_D 6.27 μM) (**IV**, Fig 2A). These results show that both NR1 and ICAM-5 can bind to the α-actinin R2 domain in the rod domain region, but ICAM-5 binding is preferred because it has higher binding affinity.

Competition assays were done to study if ICAM-5 and NR1 could compete for binding to α-actinin. A GST/α-actinin R1-R4 protein containing the rod domain region was preincubated with ICAM-5 or NR1 cytoplasmic domain. Subsequently, ICAM-5 or NR1 cytodomain was added at 1 μM concentration and it was found that NR1 competed with ICAM-5 for α-actinin binding (**IV**, Fig. 2B, C). However, ICAM-5 inhibited the binding of NR1 to α-actinin very efficiently (**IV**, Fig 2D, E**) (**$P < 0.001$). These findings imply that NR1 and ICAM-5 share an overlapping binding area in the α-actinin rod domain.

To analyze the consequences of this competition in more detail, NR1 distribution was studied in 9 DIV and 15 DIV WT and ICAM-5 knockout mouse hippocampal neurons (**IV**, Fig 3A-C) with EGFP being used as a dendritic marker. The NR1 punctae size was quantified (**IV**, Fig 3C). There was no difference in the NR1 expression pattern between the WT and ICAM-5 knockout mice during the early stage of neuronal development (**IV**, Fig 3A). The NR1 distribution was very punctuated in 15 DIV neurons from ICAM-5 knockout mice (**IV**, Fig 3B, green circles). The NR1 puncta in the ICAM-5 knockout 15 DIV neurons were also much larger when compared to the WT mice (**IV**, Fig 3B, C). These results suggest that ICAM-5 may be involved in regulating NR1 localization.

It is known from previous studies that release of the ICAM-5 ectodomain leads to ICAM-5 dissociation from the actin cytoskeleton (**III**). In an attempt to investigate whether ICAM-5 is able to dissociate from α–actinin after NMDA receptor activation, mouse cortical WT and ICAM-5 knockout 13-DIV neurons were cultured with and without NMDA for 24 h. α-Actinin was immunoprecipitated from neuronal cell lysates. The results indicated that NR1 could coprecipitate with α-actinin more strongly after treating the neurons with NMDA (**IV**, Fig 4A, B). The amount of NR1 in ICAM-5 knockout neurons treated with and without NMDA remained at the same level under both conditions (**IV**, Fig 4A, B).

8. DISCUSSION

8.1. ICAM-5 HOMOPHILIC BINDING AND INTERACTION BETWEEN ICAM-5 AND α-ACTININ ARE INVOLVED IN REGULATING NEURITIC/DENDRITIC OUTGROWTH

By localization, there are two types of neuronal cell adhesion molecules: some are expressed in axons and some are found in dendrites. They are called the axon-associated cell adhesion molecules (AxCAMs) and dendrite-associated cell adhesion molecules (DenCAMs). L1 and NCAM belong to AxCAMs (Yoshihara and Mori, 1994a, Brummendorf and Rathjen, 1996). The functions of AxCAMs have been studied in detail and they are now known to be involved in axonal outgrowth and growth cone guidance through different intracellular signalling pathways (Walsh and Doherty, 1997). Not only do DenCAMs participate in dendritic outgrowth but they are also involved in cell adhesion by acting as counter-receptors for AxCAMs during synapse formation, and in this way they can stabilize the synaptic connections.

ICAM-5 is a DenCAM because it is found in the cell soma and neuronal dendrites (**Fig 14**), but it is not expressed in axons (Yoshihara and Mori, 1994a, Benson et al, 1998). During the postnatal development, its expression begins at the same time as dendritic elongation and synapse formation are increased. Earlier studies have shown that ICAM-5 can induce neuritic outgrowth in embryonic hippocampal neurons (Tamada et al, 1998). It is also involved in hippocampal LTP (Sakurai et al, 1998). LTP underlies synaptic plasticity and is associated with memory formation and learning.

As a ligand for leukocyte integrins, ICAM-5 participates in regulating the immune response in the CNS, especially T cell activation (Tian et al, 2008, 2009). ICAM-5 may be involved in immunological synapse function through weakening T cell activation (Gahmberg et al, 2008). Furthermore, it has been shown that ICAM-5 induces spreading of microglia and at the same time LFA-1 clustering is observed. LFA-1 signalling induced by ICAM-5 binding is believed to lead to signal transduction. Subsequently, microglia spreading is enhanced as is LFA-1 relocation. ICAM-5 may be involved in regulating functions of microglia under different physiological and pathological conditions (Mizuno et al, 1999). In addition to its role as a leukocyte-binding protein in brain, ICAM-5 may still be involved in other neuronal functions. It is strongly expressed in the telencephalon, especially in the hippocampus (Mizuno et al, 1997, Tian et al, 1997, 2000).

These studies reveal that the homophilic ICAM-5 binding induces dendritic arborization in Paju-ICAM-5 cells and rat hippocampal neurons. Different mAbs against ICAM-5 were able to inhibit dendritic outgrowth. The ICAM-5 clustering

at cell-cell contact sites in Paju-ICAM-5 cells implies that ICAM-5 monomers may bind to each other through homophilic adhesion. The experiment with recombinant ICAM-5-Fc proteins from the ICAM-5 ectodomain revealed that domains 1 and 2, especially domain 1, bind to domains 4-5. Interestingly, it was found that the pI values between these domains were different: domains 1 and 2 were basic (pI=11.3) whereas domains 4 and 5 were acidic (pI=4.3). The homophilic binding between these domains may occur through electrostatic interactions. ICAM-5 D1-9-Fc bound very poorly to domains 1 and 2. One reason for this phenomenon could be that it may form multimers. Other neuronal cell adhesion molecules also show homophilic binding, including NCAM, L1 and N-cadherin (Soroka et al, 2003, Zhao and Siu, 1995, Tamura et al, 1998).

The possibility was also studied that ICAM-5 might form multimers. The ICAM-5 clustering in Paju cells at the cell uropods indicated that it may be possible to form multimers when the protein amount is high. Indeed, ICAM-5 is expressed as a monomer early in the postnatal development as seen in Paju-ICAM-5 cells and rat brain. After day ten of postnatal development, the monomers turned partly into multimers of 550 kDa, perhaps a tetrameric form. In the adult rat brain, there were only multimers. By disrupting the actin cytoskeleton with cytochalasin D in Paju-ICAM-5 cells, it was concluded that ICAM-5 existed as monomers. This implies that binding to the cytoskeletal proteins regulates ICAM-5 multimerization.

The monomer/multimer transition may have important functions regarding dendritogenesis and synaptogenesis. When monomers turn into multimers, it parallels dendritogenesis just after birth in the rodent CNS. At the beginning of dendritogenesis, the homophilic binding of the ICAM-5 monomeric form may induce dendritic outgrowth. During the later stages of dendritogenesis, ICAM-5 multimers probably could no longer induce dendritic elongation because homophilic binding would be abolished.

Since these studies implied that ICAM-5 is linked to the cytoskeletal proteins, it was decided to investigate the interactions of ICAM-5 with different cytoskeletal proteins. Indeed, it was found that ICAM-5 could bind to α-actinin, an F-actin binding protein. This was studied in GST pull down assays by using a GST fusion protein containing the ICAM-5 cytoplasmic domain. Coprecipitation assays confirmed the *in vivo* interaction between the two proteins. Peptide affinity chromatography revealed that positively charged amino acids in the ICAM-5 cytoplasmic domain are important for α-actinin binding. The KKGEY sequence at residues 857-861 has two basic lysines (amino acids 857 and 858) ICAM-5/α-actinin colocalization could be seen in Paju-ICAM-5 cells and in dendritic filopodia and spines of hippocampal neurons. The colocalization was not seen in the mutant Paju-ICAM-5-KK/AA cells, i.e. it seems that the lysines are important for α-actinin binding. Thus, the main binding site for α-actinin in ICAM-5 cytoplasmic domain resides at residues 857-

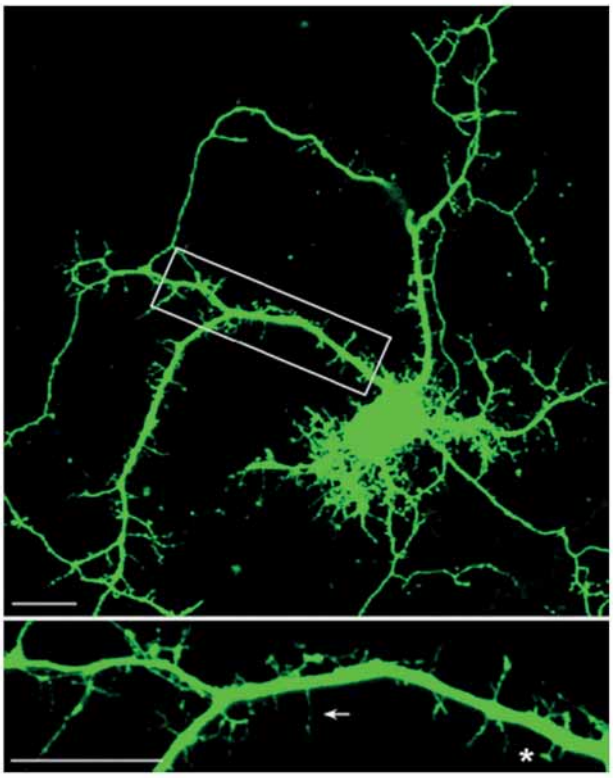

Fig 14. ICAM-5 expression in a mouse hippocampal neuron. The neuron was immunofluorescently labelled with an anti-ICAM-5 antibody and visualized by a confocal microscope. In the enlarged figure (below) a filopodium is marked with an arrow and a mature spine with an asterisk. Bars, 10 μM. Reprinted from *Immunology Letters*, Gahmberg et al, 2008. Copyright (2008), with permission from Elsevier.

861. It resembles the α-actinin binding sites in other ICAMs, especially in ICAM-1 and ICAM-2 (Carpén et al, 1992, Heiska et al, 1996). The binding sites in these ICAMs also contain charged residues. The α-actinin binding site in ICAM-1 (RKIKK) resembles the corresponding site in ICAM-5 (Carpén et al, 1992). In addition other cell surface proteins bind to α-actinin through positively charged basic residues. SPR analysis indicated that the binding affinity between the ICAM-5 KKGEY peptide and α-actinin was not very high.

Next the physiological relevance of the ICAM-5/α-actinin interaction was examined. To achieve this goal, truncated Paju-ICAM-5 cell lines were used. α-Actinin was strongly expressed at the same regions as full-length ICAM-5. ICAM-5 homophilic binding is probably responsible for this phenomenon. In other truncated Paju-ICAM-5 cell lines, the codistribution pattern was no longer so obvious. ICAM-5 localization is probably regulated through α-actinin crosslinking between the

ICAM-5 cytoplasmic domain and the actin cytoskeleton. Morphological changes occurred when Paju-ICAM-5 cells were treated with the penetratin-coupled KKGEY peptide, probably due to changes in cytoskeletal structure, i.e. the cells looked more rounded. The peptide may thus compete for α-actinin binding.

Finally, it was possible to show that the ICAM-5/α-actinin interaction plays a role in neuritc outgrowth. After phorbol ester activation, the neurite length was shorter in the truncated Paju-ICAM-5-TM and Paju-ICAM-5-KK/AA cells when compared to neuritic length in Paju-ICAM-5 cells. ICAM-5 and α-actinin may both be involved in synaptic plasticity and synapse formation as α-actinin is strongly expressed in the postsynaptic dendritic spines (Shirao and Sekino, 2001). F-actin is also needed for synaptic development and maintenance and α-actinin can also bind to F-actin (Zhang and Benson, 2001).

ICAM-5 and α-actinin may also be involved in AD pathogenesis. Many cytoskeletal proteins, including actin, α-actinin, vinculin and amyloid beta-protein precursor, are found as inclusion bodies in the brain of AD patients (Maciver and Harrington, 1995). Furthermore, ICAM-5 is known to associate with presenilins 1 and 2 (Annaert et al, 2001). Presenilins belong to the γ-secretase complex which is involved in AD. Presenilin 1 is involved in regulating the ICAM-5 protein level through autophagy, which is a protein degradation pathway inside the cells (Esselens et al, 2004).

8.2. NMDAR ACTIVATION INDUCES ICAM-5 ECTODOMAIN CLEAVAGE LEADING TO DENDRITIC SPINE DEVELOPMENT AND ICAM-5 IS INVOLVED IN REGULATING NR1 ASSOCIATION WITH THE CYTOSKELETON

The next experiment examined the association between NMDARs and ICAM-5. It is known that NMDAR stimulation and LTP lead to MMP-9 activation (Meighan et al, 2006, Nagy et al, 2006). MMPs, especially MMP-2 and MMP-9 are proteolytic enzymes that have a wide distribution in the developing brain (Szklarczyk et al, 2002, Ayoub et al, 2005). The present studies showed that either NMDAR or AMPAR activation can evoke ICAM-5 ectodomain cleavage in rat hippocampal neurons. Because the MMP-2 and MMP-9 inhibitors and siRNAs blocked ICAM-5 cleavage and ICAM-5 expression was more abundant in newborn MMP-2 or MMP-9 knockout mice and also later, it is likely that MMP-2 and MMP-9 are mainly responsible for cleaving the ICAM-5 ectodomain. Another neuronal cell adhesion molecule, L1 was expressed at high levels early in development in these knockout mice whereas the expression level decreased during the postnatal development. It

appears that L1 functions more in the early stages of brain development whereas ICAM-5 is more important in the more mature brain. ICAM-5 dissociation from the actin cytoskeleton is also important for its cleavage. These results provide evidence that the ICAM-5 cytoplasmic domain is involved in NMDAR-dependent dendritic spine morphogenesis through regulating ICAM-5 cleavage by MMPs. NMDARs are able to induce dendritic spine formation and stabilization (Lin et al, 2004).

Finally, these studies showed that ICAM-5 is involved in dendritic spine development. First, ICAM-5 was found in dendritic filopodia and the immature thin spines. Secondly, soluble ICAM-5 was able to induce filopodia elongation. sICAM-5 was also released at day 14 which is the period when spine formation occurs. It has also been shown that ICAM-5 can act as a negative regulator for spine development through its binding to ERM proteins (Matsuno et al, 2006, Furutani et al, 2007). Retraction of thin spine heads in ICAM-5 knockout neurons was seen after NMDA treatment. ICAM-5 knockout mice are believed to have larger mature spine heads (Matsuno et al, 2006). In normal neurons, maturing spines have more abundant NMDARs that sensitize the NMDA-induced MMP activation and ICAM-5 cleavage, which explains the reduction of ICAM-5 expression in mature spines. In ICAM-5 knockout neurons, the absence of ICAM-5 in immature spines may lead to a reduced actin cytoskeletal dynamics in response to NMDA stimulation, whereas the absence of ICAM-5 in mature spines may cause a long-term lingering and accumulation of NMDARs in spine heads due to a lack of competition from ICAM-5 for cytoskeletal anchorage, which explains the larger spine heads in ICAM-5 knockout neurons.

It has also been shown that LTP causes MMP-mediated ICAM-5 cleavage indicating that MMPs are involved in synaptic plasticity (Conant et al, 2010a). Moreover, metamphetamine, a psychostimulant associated with addiction, can stimulate MMPs to cleave the ICAM-5 ectodomain. The ICAM-5 external part may bind to β1 integrins and it is able to stimulate cofilin phosphorylation depending on β1 integrin association, and these events could be involved in dendritic spine expansion (Conant et al, 2010b).

These present results and earlier reports on ICAM-5 (Tian et al, 2000, Matsuno et al, 2006) were the foundation for creation of a schematic model describing the role of ICAM-5 in dendritic spine development (**Fig 15**). NMDAR or AMPAR activation is able to increase the proteolytic activity of MMP-2 and MMP-9 in neuronal cells, and this leads to the release of the ICAM-5 ectodomain from immature spines and ICAM-5 dissociation from the cytoplasmic tail. Since there is less ICAM-5 on the cell membrane, membrane and cytoskeletal reorganization is easier facilitating dendritic spine remodelling.

In the last part, the binding between NMDARs, ICAM-5 and α-actinin was evaluated to determine if ICAM-5 is involved in the regulation of the localization of NR1. The results showed that ICAM-5 and NR1 share the same binding region

in α-actinin, the R2 domain. It seems as if these molecules were also competing for binding to this region but they also had different binding affinities, i.e. ICAM-5 bound to α-actinin with higher affinity. Earlier studies showed that the binding affinity between the ICAM-5 KKGEY peptide and α-actinin was low, and the reason for the high binding affinity between the ICAM-5 cytoplasmic domain and α-actinin probably is that strong binding to α-actinin may involve several binding regions in the ICAM-5 cytodomain. NR1 may inhibit ICAM-5 binding to α-actinin when it is used at higher molar ratios.

In ICAM-5 knockout mice, the NR1 distribution was totally different compared to the WT mice, the staining was more punctate-like and the NR1 puncta were quite large. ICAM-5 may have a regulatory role in NR1 localization. It also seemed that the NR1 expression increased in ICAM-5 knockout mice as the neuronal maturation continued. The reason might be an increase in overall protein expression level or because the NR1 membrane trafficking to the dendritic shafts was increased. NR1 was also able to coprecipitate with α-actinin more efficiently after NMDA stimulation implying that NR1 binding to α-actinin could be enhanced.

Thus, these findings imply that usually ICAM-5 binding to α-actinin is preferred because it has higher binding affinity than NR1 for α-actinin. After GluR activation, MMP activity results in ICAM-5 cleavage and the ICAM-5 cytoplasmic tail dissociates from the cytoskeleton (**Fig 15)** and possibly from α-actinin. After this, NR1 is able to bind to α-actinin. In conclusion, ICAM-5 appears to be involved in regulating the NR1 association with the actin cytoskeleton.

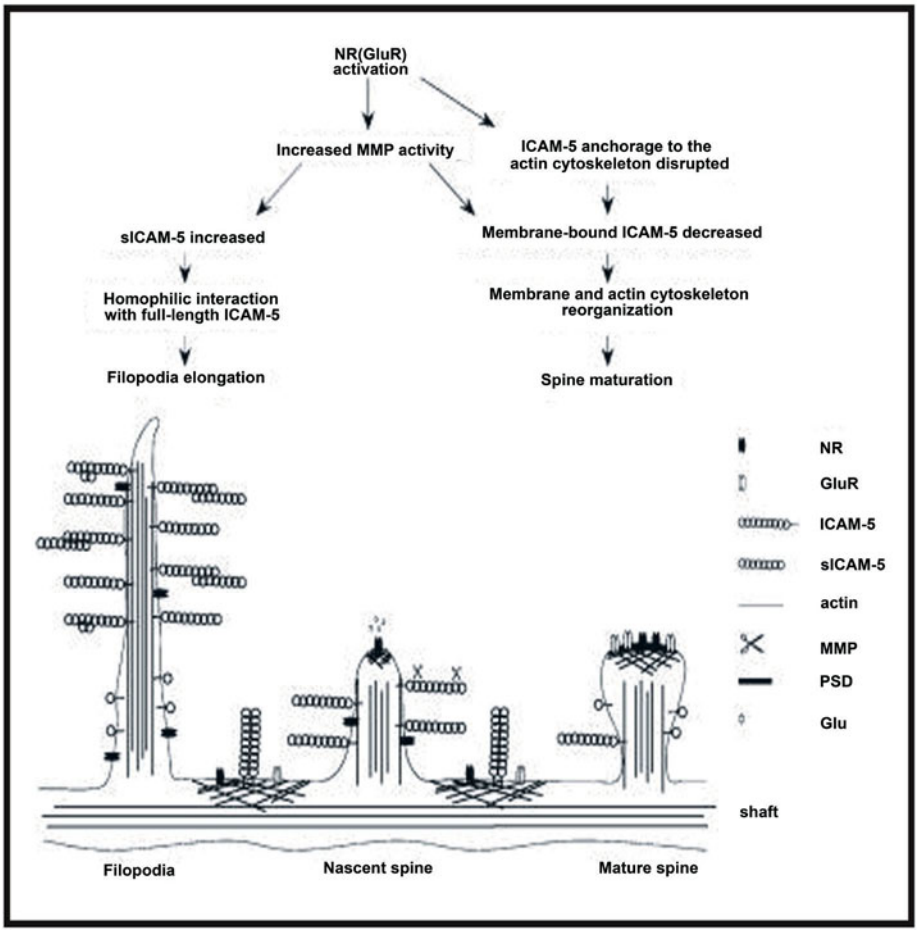

Fig 15. Schematic model of ICAM-5 involvement in spine maturation and filopodia elongation through activation of glutamate receptors. The activation of NRs or GluRs in neurons induces MMP-2 and -9 activities, which cleave the ectodomains of ICAM-5 from nascent spines, and results in dissociation of ICAM-5 from the actin cytoskeleton. The remaining C-terminal fragment (CTF) of ICAM-5 may compete and disrupt the anchorage of full-length ICAM-5 to the actin cytoskeleton, which further promotes its cleavage from spines by MMPs. Reduced membrane levels of ICAM-5 may facilitate local membrane and cytoskeleton reorganization, which induces the maturation of dendritic spines. Concomitantly, the sICAM-5 fragments produced by MMPs can bind in a homophilic manner to the full-length ICAM-5 in the neighborhood filopodia and promote their elongation. Reprinted from *Journal of Cell Biology*, Tian et al, 2007. doi:10.1083/jcb.200612097. Copyright (2007), with permission from Rockefeller University Press.

CONCLUDING REMARKS

ICAM-5 is a very interesting molecule in many ways. In the CNS, it is found only in the telencephalon and in dendrites of neurons. It is believed to be involved in the immune response in the CNS through its binding to the leukocyte-specific β2 integrin LFA-1. However, it may take part in other activities, including cognitive functions, such as memory formation and learning and it may also participate in synapse formation. This thesis demonstrated that ICAM-5 participates in neuritic outgrowth through homophilic interactions. Binding to α-actinin is involved in this process. Importantly, ERM protein binding to the ICAM-5 cytoplasmic domain also affects neuritic development and is involved in the formation of dendritic filopodia. NMDAR activation produces soluble ICAM-5 by MMP cleavage which is important for dendritic spine maturation. This result further supports its role as a regulator in synaptogenesis. In the future, it will also be important to determine the role of ICAM-5 in different neurological diseases. Its interactions with the amyloid precursor protein and presenilins imply that it may affect the developmental stages of AD. It is postulated that ICAM-5 has more interacting partners in the brain but their identification will need more investigations. However, this is an important topic since it will deepen understanding of the molecular complexity of neurons and the brain.

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Helsinki, January 14th, 2011

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