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ANU AONURM-HELM

Depression-like phenotype and altered intracellular signalling in neural cell adhesion molecule (NCAM)-deficient mice

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II. **Aonurm-Helm A**, Zharkovsky T, Jürgenson M, Kalda A, Zharkovsky A. (2008) Dysregulated CREB signalling pathway in the brain of neural cell adhesion molecule (NCAM)-deficient mice. Brain Res 1243; 104–112.

III. **Aonurm-Helm A**, Berezin V, Bock E, Zharkovsky A. (2010) NCAM-mimetic, FGL peptide, restores disrupted fibroblast growth factor receptor (FGFR) phosphorylation and FGFR mediated signalling in neural cell adhesion molecule (NCAM)-deficient mice. Brain Res 1309; 1–8.

Author's contribution:

I. The author was the main person in behavioural and immunohistochemical studies, and in manuscript writing.

II. The author was the main person responsible for tissue processing, data analyzing and writing the manuscript.

III. The author was participating in study design and was the main responsible person in experimental processes, data analysis, interpreting the data and writing the manuscript.

ABBREVIATIONS

BrdU	5-bromodeoxyuridine
CAM's	cell adhesion molecules
CaMK (II, IV)	Ca/calmodulin dependent kinase type II, IV
CREB	cAMP response element binding protein
ERK1/2	extracellularly regulated kinases 1 and 2
FAK	focal adhesion kinase
FGFR	fibroblast growth factor receptor
Fyn	tyrosine specific phospho-transferase
MAPK	mitogen activated protein kinase
MEK1/2	MAP and ERK kinases 1 and 2
NCAM	neural cell adhesion molecule
pAkt	phosphorylated protein kinase B
pCaMKII	phosphorylated CaMKII
pCaMKIV	phosphorylated CaMKIV
pCREB	phosphorylated CREB
pERK	phosphorylated ERK
pMEK	phosphorylated MEK
PSA-NCAM	polysialic acid linked NCAM
SPT	sucrose preference test
ST8Sia (II, IV)	polysialyltransferases (II, IV)
TST	tail suspension test

INTRODUCTION

Recent theories of the pathogenesis of depression link the development of this disease with reduced brain plasticity. Brain plasticity refers to the ability of the brain to adapt to environmental or physiological stimuli and the ability to add or remove neuronal connections. In the central nervous system, plasticity and connectivity in the brain are mediated by the neural cell adhesion molecule (NCAM) and its polysialylated form PSA-NCAM. Therefore, according to the plasticity theory of depression, NCAM may have a crucial role in the development of this condition. The NCAM is able to bind to itself and to a series of counter-receptors including tyrosine kinase receptors, such as fibroblast growth factor receptor (FGFR), and through the binding, activate the downstream signalling pathways, which all lead to the activation of cyclic-AMP-response element binding protein (CREB). To study the role of NCAM in brain plasticity, mice deficient in all isoforms of NCAM protein have been developed. Previous studies have shown that these mice demonstrate impairment in long-term plasticity at the mossy fibre synapses, and also disrupted spatial learning and impaired contextual and auditory-cued fear conditioning.

The aim of this study was to investigate whether mice with the constitutive deficiency in NCAM exhibited dysfunctional neuronal plasticity and whether it results in the depressive-like phenotype. By using NCAM-deficient mice, NCAM interaction partners and downstream signalling pathways were studied in detail. Also to investigate, whether a synthetic peptide FGL, which mimics the actions of NCAM, is able to reverse emerged disturbances and restore the activation of intracellular signalling cascades in NCAM deficient mice.

REVIEW OF THE LITERATURE

1. Cell adhesion molecules

Cell adhesion molecules (CAMs) are cell membrane-associated proteins required for the dynamic connection of cells to each other or to the extracellular matrix components in the process of cell adhesion. Cell adhesion is required for tissue formation, maintenance and functioning during development and adulthood. Hundreds of adhesion molecules exist, all belonging to four main families: the immunoglobulin (Ig) superfamily, the integrins, the catherins and the selectins. This study is focused on the Ig superfamily. The external domain regions of Ig-like CAMs have a modular structure in which Ig-like domains are located near the membrane-distal N-terminus (Williams et al., 1988). Diverse protein modules (most commonly fibronectin type III repeats) provide linkers to the plasma membrane (Cunningham, 1995) and the presence of a catalytic cytoplasmic domain (Crossin and Krushel, 2000).

1.1. Neural cell adhesion molecule (NCAM)

1.1.1. Structure of NCAM

The NCAM was the first Ig-like CAM to be isolated and characterized in detail (Brackenbury et al., 1977; Cunningham et al., 1987). All NCAM isoforms are composed of five regions at the amino terminus which are homologous to Ig domains followed by two fibronectin type III (FN3) repeats (Cunningham et al., 1987). The NCAM is expressed in several isoforms, which are all generated via alternative splicing of a primary transcript from a single gene, *Ncam1* (Jorgensen and Bock, 1974). *Ncam1* gene is consisting twenty major exons and six additional small exons in mice (Walmod et al., 2004) which is located on chromosome 11 in humans (Nguyen et al., 1986), on chromosome 9 in mice (D'Eustachio et al., 1985) and on chromosome 8 in rats (Yasue et al., 1992).

Three major isoforms of NCAM are named by their approximate molecular weight: two isoforms are transmembrane forms of 180 kD (named NCAM-A or –ld, large cytoplasmic domain) and 140 kD (NCAM-B or –sd, short cytoplasmic domain) and a third, 120 kD (NCAM-C or –ssd, short surface domain), which is attached to the cell membrane by a glycosylphosphatidylinositole anchor (Walmod et al., 2004) (Fig. 1). In addition to three main isoforms of NCAM, the molecule also exists in a secreted form produced by the expression of the small SEC-exon located between exons 12 and 13. This exon contains a stop codon thereby producing a truncated form of the extracellular part of NCAM with a molecular weight of about 115 kD (Bock et al., 1987; Gower et al., 1988). Soluble NCAM can also exist in a shedded form. This

phenomenon can appear following the enzymatic removal of NCAM-120 from the membrane by phosphatidylinositol specific phospholipase C or by proteolytic cleavage of the extracellular part of any of the three major isoforms (He et al., 1986), but transmembrane isoforms of NCAM have also been found in a soluble form in cerebrospinal fluid (Olsen et al., 1993).

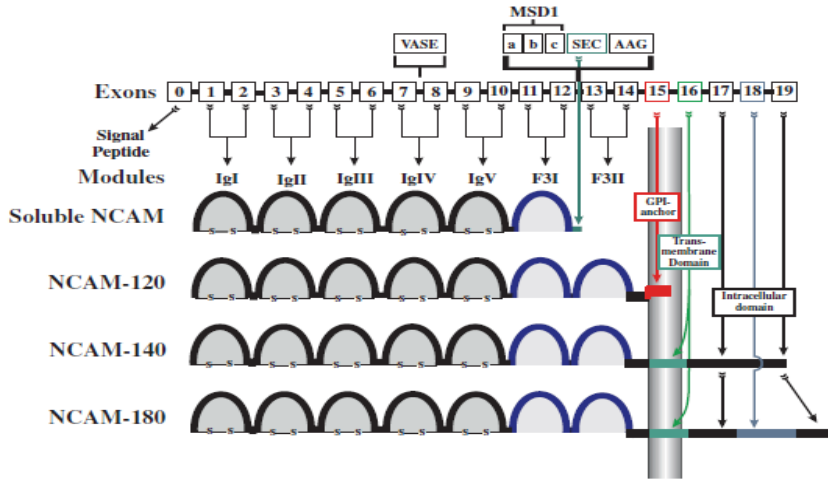

Figure 1. NCAM isoforms. This figure shows the exons constituting the NCAM gene and how the translation of the respective exons is related to the different isoforms of NCAM. IgI-V indicates immunoglobulin homology modules. F3I-II indicates fibronectin type III homology modules (Walmod et al., 2004).

1.1.2. Localization of NCAM

The NCAM has been found in almost all tissues, with the highest expression in the central and peripherial nervous tissue. It is expressed in the nervous system during neural tube closure and persists into adulthood on both neurons and glia (Maness and Schachner, 2007). Predominantly expressed in neurons in late development, NCAM-180 contains a 40 kD cytoplasmic domain insert that distinguishes it from NCAM-140. The NCAM-140 is localized to migratory growth cones and axons shafts of developing neurons and mediates neurite outgrowth responses, whereas NCAM-180 is enriched at sites of cell contact and postsynaptic densities of mature neurons (Persohn et al., 1989). Maturation of vesicle cycling has been ascribed to NCAM-140 (Polo-Parada et al., 2004). Whereas NCAM-140 is expressed both by neurons and glia, NCAM-120 is predominantly expressed by glial cells (Maness and Scachner, 2007).

I.2. Polysialic acid (PSA)-linked NCAM

It was reported (Finne, 1982) that in the developing mammalian brain, large amounts of a long α-2,8-linked polymer of sialic acid, called polysialic acid (PSA), is present. The cell surface molecule NCAM, which activates cell to cell and cell to extracellular matrix adhesion as well as intracellular signalling, was found to have a high content of PSA (Hoffman et al., 1982; Finne et al., 1983). Studies on PSA-NCAM binding characteristics led to the finding that PSA reduces NCAM-mediated adhesion (Cunningham et al., 1983). The long, negatively charged, linear glycopolymer of PSA at the cell surface mediates unusual physical properties. For two cell membranes to approach each other, the water between them needs to be removed. Polysialic acid has a particularly high degree of hydration and thus occupies a much larger volume than its carrier NCAM. Basically, the water is trapped by its association with a membrane-bound component and the contact between the cells is reduced (Rutishauser, 2008). The glycosylation of NCAM takes place in the endoplasmatic reticulum or Golgi compartment (Kiss and Rougon, 1997). Most carbohydrates are attached to a variety of proteins, whereas long chains of PSA are attached exclusively to the last of NCAM's five amino-terminal immunoglobulin domains and are adjacent to the two fibronectin type III domains (Finne et al., 1983; Rutishauser, 2008).

Polysialylated NCAM (PSA-NCAM) is abundant in the embryonic brain, while most NCAM in the adult brain does not contain PSA (Rothbard et al., 1982). However, PSA-NCAM is continuously present in restricted areas such as the olfactory bulb and hippocampus, where neural generation and neural plasticity persists in the adult (Rutishauser and Landmesser, 1996).

The most significant advances in the molecular biology of PSA have been the identification and characterization of two polysialyltransferases – ST8SiaIV and ST8SiaII – either of which enables the synthesis of PSA chains to NCAM (Eckhardt et al., 1995; Kojima et al., 1996). Although NCAM is the preferred substrate for polysialyltransferases, there is evidence that other cell-surface components (e.g the voltage-sensitive sodium channel and neuropilin) have detectable levels of polysialylation (James and Agnew, 1987; Curreli et al., 2007). However, it is not known whether the polymer length and content that is obtained in these cases is sufficient to produce a similar regulatory effect that is obtained with NCAM.

1.3. NCAM interactions

1.3.1. Homophilic NCAM interactions

It has long been known that NCAM is involved in homophilic trans-interactions: NCAM molecules on one cell surface interact with NCAM molecules on the opposing cell surface (Rutishauser et al., 1982). Studies undertaken on chicken NCAM demonstrated that all five immunoglobulin modules of NCAM were individually able to bind NCAM and further indicated that IgI binds to IgV, IgII to IgIV and IgIII to IgIII. This finding led to a model for homophilic NCAM trans-interactions involving all five immunoglobulin modules (Ranheim et al., 1996). However, based on the structural and functional studies made by Soroka et al. (2003) a double-zipper-model has been proposed. The first hypothetical zipper is formed between NCAM cis-dimers (one NCAM molecule interacts with another NCAM molecule on the same cell surface) from one cell surface interacting with NCAM cis-dimers on the other cell surface through IgII and IgIII contacts. The second zipper is formed between NCAM cis-dimers from one cell surface interacting through IgI and IgIII or IgII and IgII on the other cell surface.

1.3.2. Heterophilic NCAM interactions

NCAM is also able to bind to some other molecules on the cell surface and conceive heterophilic interactions. For example, NCAM has been shown to bind to its closely related adhesion molecule L1, probably with cis-interaction, which induces phosphorylation of tyrosine and serine residues in L1 (Heiland et al., 1998). Like NCAM, L1 also belongs to the immunoglobulin superfamily and has six Ig-like domains and four or five fibronectin III-type repeats in the extracellular region and a conserved cytoplasmic domain that is able to link to actin (Schmid and Maness, 2008). It has been shown that L1 is able to regulate the growth of axons in developing neurons, cell migration and neurite outgrowth by functional interaction with β1 integrins, and that it is able to bind to components of the cytoskeleton, including members of the ancyrin family of adaptor proteins (Davis and Bennett, 1994). Previous studies demonstrated that NCAM binds to L1 through oligomannosidic glycans present in L1 (Kadmon et al., 1990). It was suggested that NCAM homophilic binding inhibits L1 binding and prevents L1-L1 induced neurite outgrowth (Kristiansen et al., 1999). Adhesion molecule L1 and β1 integrins associate with low affinity on the cell surface and activate common intracellular signalling pathways. These pathways involve the activation of nonreceptor tyrosine kinase c-Src, phosphatidylinositide 3-kinase (PI3 kinase), MEK and the MAP kinases ERK 1 and 2 (Schmid et al., 2000), similarly to NCAM. NCAM has been also reported to

bind another neuronal immunoglobulin superfamily receptor TAG-1 (Milev et al., 1996).

Immunoprecipitates of NCAM have been demonstrated to possess Ca^{2+}–Mg^{2+}–dependent ATP hydrolysing activity, indicating that NCAM is tightly associated with ATPase (Dzhandzhugazyan and Bock, 1993). It has also been demonstrated that ATP can bind to NCAM directly (Dzhandzhugazyan and Bock, 1997). Interestingly, the binding of ATP to NCAM inhibits cellular aggregation and neurite outgrowth induced by homophilic NCAM interactions, suggesting that the binding of ATP to NCAM interferes with homophilic NCAM interactions (Skladchikova et al., 1999). The ATP binding site has been localized on the FN3II domain of NCAM, indicating that the effects of ATP on homophilic NCAM interactions are most likely the result of structural alterations in the extracellular part of NCAM, rather than direct sterical interference between interacting NCAM Ig-homology modules involved in homophilic binding (Kiselyov et al., 2003).

NCAM interacts also with several extracellular matrix components. It is known that NCAM binds glycosaminoglycan heparin (Herndon et al., 1999). This interaction is believed to involve the heparin binding domain, which comprises a 17 amino acid long-sequence in the IgII module of NCAM (Cole et al., 1985; Cole and Glaser, 1986; Cole and Akeson, 1989). This suggestion is supported by the fact that adhesion of neurons to immobilized recombinant IgII motifs is reduced by the addition of heparin (Frey et al., 1992). A number of chondroitin sulphate proteoglycans and heparin sulphate proteoglycans are also bound by NCAM (Cole et al., 1985; Burg et al., 1995). One heparin sulphate proteoglycan named agrin, proposed to be important for synaptogenesis and axonal growth (Kröger and Schröder, 2002), has been suggested to interact with NCAM via the NCAM heparin binding domain in the IgII module and via PSA on the IgV module (Storms and Rutishauser, 1998).

NCAM has also been reported to bind collagens I – IV and IX. However, the interaction can be reduced in a concentration-dependent manner by heparin and chondroitin sulphate, and it has been proposed that the interaction between NCAM and collagen occurs indirectly via heparin sulphate (Kiselyov et al., 1997; Probstmeier et al., 1989).

1.3.3. Interactions with fibroblast growth factor receptor (FGFR)

Fibroblast growth factor receptor (FGFR) is an immunoglobulin superfamily receptor tyrosine kinase and it has been demonstrated to interact in a heterophilic manner with NCAM. It was demonstrated that FGFR contained a CAM homology domain with homology to VASE-sequence of NCAM. Treatment with a synthetic peptide corresponding to this domain, or with antibodies against it, abrogated NCAM-mediated neurite outgrowth (Williams et al., 1994). It has long been known that FGFR is involved in NCAM signalling

through direct or indirect interaction, but only recently it was shown that direct interaction occurs between FGFR1 and NCAM, demonstrated by Kiselyov et al. (2003). Using nuclear magnetic resonance (NMR), it has been shown that the FG loop region of the second FN3 module of NCAM is involved in binding to FGFR, and a peptide corresponding to this region has been shown to induce phosphorylation of FGFR, neurite outgrowth and neuronal survival in primary neurons (Kiselyov et al., 2003; Neiiendam et al., 2004). Homophilic NCAM interactions lead to phosphorylation of FGFR (Saffell et al., 1997), indicating that NCAM binds and stimulates FGFR. It has been demonstrated that the ATP-binding site on NCAM overlaps with the FGFR binding site, and that ATP can inhibit NCAM-induced signalling mediated through FGFR (Kiselyov et al., 2003).

An essential role for FGFR in NCAM-mediated neurite extension has been shown by employing pharmacological inhibitors of FGFR in cerebellar granule neurons stimulated with NCAM mimetic peptide FGL (Neiiendam et al., 2004).

Since the signalling pathways that are activated by NCAM are in a wide range shared by the signalling pathways activated by the cognate FGFR ligands (Doherty and Walsh, 1996), the question arises as to whether NCAM is mimicking FGF in its actions in the stimulation of FGFR.

1.3.4. NCAM-dependent cell signalling

Intracellular signalling mediated by NCAM depends on the induction of down-stream signal transduction pathways through the direct or indirect interaction of NCAM with the intra- and extracellular ligands.

According to the FGFR hypothesis, suggested by Doherty and Walsh (1996), the ability of NCAM to induce differentiation is attributed to its ability to interact with FGFR. The phosphorylated residues on the cytoplasmic part of FGFR dock several proteins, including phospholipase C (PLCγ), which becomes activated upon binding. The substrate for PLCγ is phosphatidylinositol-diphosphate (PIP_2), which is cleaved to generate two other secondary messengers, phosphatidylinositol-3-kinase (IP_3) and diacylglycerol (DAG). The IP_3 diffuses through the cytosol and binds to intracellular Ca^{2+} channels, leading to the release of Ca^{2+} from intracellular stores and an increase in $[Ca^{2+}]_i$. The DAG remains a part of the plasma membrane and can either activate protein kinase C (PKC) or be converted by DAG-lipase to 2-arachidonylglycerol (2-AG) and arachidonic acid (AA) (Walmod et al., 2004). The FGFR hypothesis is supported by several findings. It has been demonstrated that NCAM interacts directly with FGFR, inducing autophosphorylation of FGFR (Kiselyov et al., 2003). NCAM-mediated neurite outgrowth, induced by homophilic NCAM trans-interactions or by the addition of the NCAM peptide ligand, is impaired in PC12 cells as well as in neurons from transgenic mice expressing dnFGFR (Rønn et al., 2000). The involvement of PLCγ is supported

by studies demonstrating that inhibitors of PLCγ block NCAM-mediated neuritogenesis (Saffell et al., 1997; Kolkova et al., 2000), and NCAM-mediated neuritogenesis can also be blocked with an inhibitor of DAG-lipase, indicating an involvement of 2-AG and AA in this process (Williams et al., 1994). Arachidonic acid (AA) regulates specific Ca^{2+} channels in the plasma membrane (Shuttleworth and Mignen, 2003), and treatment of cells with AA has been shown to induce an influx of Ca^{2+} leading to an increase in $[Ca^{2+}]_i$ and an induction of neurite outgrowth (Williams et al., 1994). In addition to signalling through the receptor tyrosine kinase FGFR, NCAM also mediates signalling via non-receptor tyrosine kinases, leading to the stimulation of the MAPK pathway. The two non-receptor tyrosine kinases Fyn and focal adhesion kinase (FAK) interact with NCAM, and experiments have demonstrated that antibody-mediated cross-linking of NCAM molecules on the surface of COS7 cells or B35 neuroblastoma cells results in an increase in phosphorylation of both of these kinases (Beggs et al., 1997). FAK is known to activate the MAPK pathway through the small GTPase Ras. NCAM-mediated neuritogenic responses are inhibited in neurons treated with an inhibitor of MAPK (Schmid et al., 1999). The NCAM is also able to induce the phosphorylation of the MAPKs ERK1 and 2 downstream of MEK1/2, and of the transcription factor CREB, which is activated either via the kinase Rsk downstream of ERK1/2 (Schmid et al., 1999) or via mitogen- and stress-activated protein kinase (MSK1), also downstream of ERK2 (Deak et al., 1998). Disruption of the MSK1 gene in mouse embryonic stem cells has been shown to prevent mitogen-stimulated phosphorylation of CREB, whereas phosphorylation of CREB by protein kinase A (PKA) is unaffected (Arthur and Cohen, 2000).

As NCAM can also interact with glial cell-derived neurotrophic factor (GDNF) and GDNF family receptor alpha1 (GFRα), it can thereby activate downstream signalling components including Fyn, FAK and ERK1/2, leading to neurite outgrowth and cell migration in FGFR independent manner (Paratcha et al., 2003).

One of the targets of PKC is the calmodulin-binding, growth-associated protein GAP-43, which is activated in response to NCAM stimulation of the FGFR-PLCγ pathway (Meiri et al., 1998). It is known that GAP-43 is important for neural development and plasticity, and it has been proposed that GAP-43 sequesters the Ca^{2+} binding protein calmodulin at synaptic membranes and that it modulates the cytoskeleton (Benowitz and Routtenberg, 1997). Another effect of the NCAM-mediated increase in $[Ca^{2+}]_i$ appears to be an activation of CaMKII, since NCAM-mediated neuritogenesis can be blocked by the CaMK inhibitor KN-62 (Williams et al., 1995). It is well recognized that CaMKII is a multifunctional kinase that transduces numerous neuronal Ca^{2+} signals (Braun and Schulman, 1995). The cascade of CaMKs comprises CaMKIV and its upstream activator CaMKK. The expression of CaMKIV occurs at high levels in neurons, T-cells and testis, is highly localized in the nucleus and is also present in the cytosol. Consistent with its nuclear localization, CaMKIV is

thought to regulate Ca^{2+} stimulated gene expression through phosphorylation of multiple transcription factors (Soderling, 1999).

Phosphatidylinositol-3-kinase (PI3-K) and protein kinase B (PKB/Akt) were also demonstrated to be downstream transducers of NCAM-mediated signalling. Stimulation of NCAM-mediated signalling by synthetic peptides led to the phosphorylation of PKB/Akt and to PI3-K-dependent neurite outgrowth (Ditlevsen et al., 2003). It is likely that PI3-K is activated by FGFR via FRS2-Grb2-Gab1 complex (Ong et al., 2001; Steelman et al., 2004). The activation of PI3-K may also be mediated through other routes, and it can also be stimulated by Ras (Rodriguez-Viciana et al., 1994). The targets of PKB/Akt responsible for NCAM-mediated neuritogenesis probably include CREB and nuclear factor kappa B (NFκB) (Du and Montminy, 1998; Kane et al., 1999), the latter of which is also known to be regulated by NCAM. Transcription of an NFκB-driven reporter construct is activated in response to stimulation of NCAM in astrocytes and cerebellar neurons (Krushel et al., 1999). Also, studies with various inhibitors indicate that NCAM-mediated activation of NFκB (in astrocytes) is dependent on signalling via PLCγ, PKC and CaMKII (Choi et al., 2001).

If NCAM is important in cellular signalling, the question arises as to which are the possible alterations in NCAM-mediated cell signalling in mice with a constitutional loss of NCAM?

2. The roles of NCAM/PSA-NCAM in brain plasticity

The main issue in neurosciences is that of synaptic plasticity. During development of the nervous system, cells need to adhere to themselves and to their environment in order to provide stable connections between cells, and at the same time the cells need to move to their correct positions, extend the axons and remodel synaptic networks. Adhesion molecules are the key players in the aforementioned processes and are critical for the proper functioning of the mature nervous system. Therefore the question arises of how NCAM or PSA-NCAM affects synaptic plasticity. The most credible function for NCAM or PSA-NCAM in this process is related to the dynamic balance between stability and plasticity of synaptic contacts. This is consistent with the developmental expression pattern of NCAM and PSA-NCAM whereby PSA-NCAM is expressed on axons and dendrites before the contact formation, but it is rapidly down-regulated when the contacts are formed (Bruses and Rutishauser, 2001). The PSA-NCAM is widely expressed in the embryonic and early postnatal brain but in the adult brain it is expressed in only a few areas where a high level of structural remodeling persists, including the olfactory system and mossy fibre system in hippocampal formation (Ni Dhuill et al., 1999; Seki and Arai et al., 1993). It has been shown that PSA-NCAM acts as a plasticity promoting molecule, decreasing the overall cell-adhesion and thereby allowing structural

remodeling (Rutishauser and Landmesser, 1996), and that regeneration of central and peripherial neuronal fibres is associated with an up-regulation of PSA-NCAM expression (Daniloff et al., 1986; Müller et al., 1994). These findings suggest the hypothesis that PSA may be involved in structural remodeling of neuronal connections in the mature nervous system. Evidence suggests that PSA-NCAM is also necessary for subtle structural remodeling of synaptic connections associated with long-term memory. It was demonstrated that the polysialylation of NCAM-180 isoform was increased during the acquisition and consolidation of a passive avoidance response in rats (Doyle et al., 1992). An increase in polysialylation is localized to a population of hippocampal dentate granule neurons and to neurons in the entorhinal cortex that form the cortico-hippocampal pathway, which is involved in learning (Murphy et al., 1996; O'Connell et al., 1997). Also, it has been shown that the polysialylation of the granule cells in hippocampal formation decreases with age (Regan and Fox, 1995). This decrease in PSA-expression may contribute to the age-related decrease in regeneration processes. Further evidence that NCAM is necessary in learning processes was provided from experiments that involved the intracerebroventricular injection of antibodies against NCAM, which impaired learning in rats and chicks (Doyle et al., 1992; Scholey et al., 1993), and from the enzymatic removal of PSA which was found to inhibit spatial learning in rats (Becker et al., 1996), suggesting that PSA-NCAM expression is necessary for learning to occur. To understand the role of NCAM in learning, a model of synaptic plasticity, the induction of long-term potentiation (LTP) in the hippocampus, has been implemented. Indication of a role of NCAM in LTP was demonstrated by the amount of soluble NCAM isoforms in the extracellular space of the dentate gyrus, which increased following induction of LTP *in vivo* (Fazeli et al., 1994), presumably due to an increased proteolytic activity that allows structural remodelling (Fazeli et al., 1990).

The PSA also has a role in the timing of cell differentiation: newly generated granule cell precursors in the hippocampus express prodigious amounts of PSA which is associated with cell migration, and the removal of PSA inflicts upon their differentiation into mature neurons (Seki et al., 2007). Moreover, removal of PSA from the cell surface of neuroblastoma cells *in vitro* led to reduced proliferative activity and activation of ERK, causing an increased survival and differentiation (Seidenfaden et al., 2003). This finding permits the hypothesis that PSA-NCAM is essential for the proliferation and migration of cells and that NCAM is essential for survival and differentiation of the neurons.

Furthermore, the role of PSA in the processes of myelinization during development must be mentioned. It has been shown that down-regulation of PSA during oligodendrocyte differentiation is a presumption for adequate myelination by mature oligodendrocytes (Fewou et al., 2007).

2.1. Neural plasticity hypothesis of depression

Brain plasticity refers to the brain's ability to change its structure and function during maturation, learning, environmental changes or pathology (Lledo et al., 2006). Multiple dissociable plastic changes in the adult brain involve not only functional plasticity, as evidenced by the synaptic strength, but also structural plasticity, which involves changes in the number of synapses, axonal fibre densities, axonal and dendritic branching and neurogenesis (Butz et al., 2009). One hypothesis links depression with brain plasticity and adult neurogenesis (Jacobs et al., 2000). Neurogenesis occurs throughout adulthood in all mammals (Taupin and Gage, 2002). It occurs primarily in two regions of the adult brain, the subventricular zone and the dentate gyrus of the hippocampus, including humans (Eriksson et al., 1998). According to this theory, depression is not only due to the changes in neurotransmitter concentrations and receptor dysfunction, but also to the impairment in brain plasticity, tissue remodelling and reduced adult hippocampal neurogenesis (Duman et al., 1999; Jacobs et al., 2000; Czeh et al., 2001; Santarelli et al., 2003; Jaako-Movits and Zharkovsky, 2005). Although there are numerous data showing increased neurogenesis following antidepressant treatment (Santarelli et al., 2003; Sahay and Hen, 2007; Jaako-Movits et al., 2006; Wang et al., 2008; Boldrini et al., 2009), no alterations in hippocampal neurogenesis in the patients with depression was demonstrated so far. Only in one *post mortem* study (Boldrini et al., 200) a 50% reduction in the number of dividing cells was found in the dentate gyrus of untreatead patients with major depressive disorder as compared with controls, but the data did not reach the levels of significance. On the other hand, several authors have demonstrated a decreased neurogenesis in stress- or olfactory bulbectomy-induced models of depression in rodents (Kempermann and Kronenberg, 2003; Jaako-Movits and Zharkovsky, 2005). However, blocking cell replication by irradiation does not induce depression-like behaviour in mice (Santarelli et al., 2003). The hippocampus is a key limbic structure that modulates the effective responses to contextual change (Bannerman et al., 2004). In clinical studies it has been found that depression is often associated with a decrease in the volume of the hippocampus (McEwen, 1997; Sheline, 2003). Chronic stress, which is a major risk for depression, reduces hippocampal volume in monkeys and rats by suppressing neurogenesis in the dentate gyrus and causing dendrite atrophy and neuronal death in the CA3 subregion (Coe et al., 2003; Pham et al., 2003). These negative effects of stress are mediated by adrenal steroid elevation (Cameron et al., 1998) and a decrease in brain-derived neurotrophic factor (BDNF) levels (Tsankova et al., 2006). Stress also has a suppressive effect on cell proliferation in the prefrontal cortex (PFC) (Czeh et al., 2007) where structural impairment and secondary cognitive deficits related to clinical depression have been reported (Bremner et al., 2004). There are also a variety of other factors which suppress hippocampal neurogenesis, such as genetic vulnerability (Lemaire et al., 1999), alcohol abuse (Nixon and Crews, 2004),

inflammation (Monje et al., 2003), infection (Guan and Fang, 2006) and neurodegenerative disorders (Zhang et al., 2007).

Concerning the functionality of adult hippocampal neurogenesis, persistent disruption of this might diminish the plasticity and finally enhance the likelihood of mood and memory disorders (Jacobs et al., 2000; Jacobs, 2002). In the regions that are important in memory formation and emotional behaviour, the structural alterations in response to stress in animals and in rodents with mood disorders have been shown (McEwen, 1997; Duman et al., 1999; Sheline et al., 1999). Animal studies have shown that prolonged stress reduced adult hippocampal neurogenesis and that clinically active antidepressants induced the increase in the proliferation rate of neuronal progenitors, enhancing their maturation into neurons in the hippocampus after chronic administration of these drugs (Duman et al., 1999; Gould and Tanapat, 1999; Malberg et al., 2000). Furthermore, recent studies also demonstrated reduced neurogenesis in rats with the surgical removal of olfactory bulbs, which has been considered as the most validated animal model of depression (Jaako-Movits and Zharkovsky, 2005; Jaako-Movits et al., 2006).

2.2. Theoretical rationale for the involvement of NCAM in depression

Depression is a widespread complex disorder with several physical, mental and socio-economical consequences. According to the American Psychiatric Association, depression is defined as a loss of energy and interest, accompanied with feelings of guilt or worthlessness, and loss of pleasure in nearly all activities previously considered pleasurable (American Psychiatric Association, 2000).

One prevailing hypothesis is that deficits in noradrenalin and serotonin are the major cause of depression. In some patients, the deficiency in monoaminergic system functionality may be associated with hippocampal atrophy, neuronal loss and dendritic reorganization, resulting in decreased synaptic connectivity (Schmidt and Duman, 2007; Sandi and Bisaz, 2007) and reduced brain plasticity in this region. Other structures such as the frontal and prefrontal cortex and the amygdala have been shown to be involved in the formation of the depression-like phenotype. All these regions express high levels of PSA-NCAM (Cox et al., 2009; Varea et al., 2007; Nacher et al., 2002). There are reciprocal interactions between the serotoninergic system and PSA-NCAM: serotonin increases the levels of PSA-NCAM (Brennaman and Maness, 2008) whereas restoration of serotoninergic innervation in animals with lesioned serotoninergic nerve fibres occurs in the presence of PSA-NCAM (Brezun and Daszuta, 2000). Recently it was found that chronic antidepressant (fluoxetine) treatment increases the expression of PSA-NCAM in the medial prefrontal cortex of rats and thereby enhances neuronal plasticity (Varea et al., 2007). Several authors

have shown that NCAM–/– mice have increased levels of stress-induced corticosteroids and show increased inter-male aggression, anxiety, decreased LTP and plasticity, and decreased learning and memory (Stork et al., 1997; Stork et al., 1999; Becker at al., 1996; Muller et al., 1996; Cremer et al., 1994). Chronic restraint stress or early postnatal stress are associated with increased levels of corticosteroids and reduced levels of NCAM-140 isoform mRNA in the hippocampus and PFC, while the levels of PSA-NCAM are increased (Sandi and Loscertales, 1999; Sandi et al., 2001; Venero et al., 2002; Tsoory et al., 2008).

In depressed patients increased levels of soluble NCAM isoforms in the CSF have been shown (Jorgensen, 1988; Poltorak et al., 1996). Soluble NCAM can disrupt the homophilic or heterophilic interactions required for synaptic plasticity and a decrease in NCAM expression or function could result in synapse instability. Therefore, depression may be a consequence of the inability of the brain to adapt to synaptic and structural changes.

The changes in NCAM expression have been also postulated as a candidate for bipolar disorder. Vawter et al. (2000) and Arai et al. (2004) reported that three single nucleotide polymorphisms in *NCAM1* gene and haplotype, located in the linkage disequilibrium block, are strongly associated with bipolar affective disorder. In patients with bipolar disorder, an increase in the levels of the secreted form of NCAM (108–115 kD) in the hippocampus has been shown (Vawter et al., 1999). Soluble NCAM isoforms are elevated in the hippocampus, PFC and CSF of effected patients compared to healthy controls (Vawter et al., 1999; Poltorak et al., 1996; Vawter et al., 1998).

Other lines of evidence show that NCAM has important functions in the regulation of activity of FGFR and dysregulation of the interaction between NCAM and FGFR might be implicated in the mechanisms of depression.

The FGF-family ligands and receptors have been shown to be dysregulated in post-mortem studies of individuals with major depressive disorder, specifically in the prefrontal cortex and hippocampus (Evans et al., 2004). Furthermore, administration of antidepressants increased the levels of FGF in hippocampal and cortical areas (Mallei et al., 2002). Turner et al. (2008) showed that administration of FGF2 resulted in an antidepressant-like action and was accompanied by an increase in FGFR1 levels, specifically in the dentate gyrus of the hippocampus. Another possible interaction partner for NCAM/PSA-NCAM is the brain derived neurotrophic factor (BDNF) (Muller et al., 2000; Vutskits et al., 2001). The BDNF plays a critical role in the development and maintenance of the nervous system, and in neuronal survival and proliferation, including synaptic reorganization and neurogenesis (Murer et al., 2001; McAllister et al., 1999; Lindvall et al., 1994). Several authors have shown that in humans, brain BDNF levels were reduced in postmortem samples from depressed patients and that antidepressants were able to restore the levels of BDNF (Castren, 2004; Chen et al., 2001; Karege et al., 2005). Recent studies indicate that single nucleotide polymorphisms of the BDNF gene play an

important role in the predisposition to depression and cognitive deficits (Phillips et al., 2003; Pezawas et al., 2004; Egan et al., 2003).

Keeping in mind that NCAM is major regulatory molecule implicated in the regulation of brain plasticity, and that dysfunctionl neuronal plasticity might have an important role in the mechanisms of depression, it is reasonable to more precisely study the role of NCAM in the development of mood disorders.

2.3. Mice deficient in NCAM

To enable greater understanding of the roles of NCAM, several NCAM-related transgenic mice have been generated (Cremer et al., 1994; Seki and Rutishauser, 1998; Polo-Parada et al., 2004; Pillai-Nair et al., 2005). This methodology has greatly increased knowledge of the roles and functions of NCAM *in vivo.*

2.3.1. Targeting the NCAM gene

Targeted mutant mice are produced by first inducing gene disruptions (an *Ncam* genomic clone is isolated and cloned into a vector. The vector is able to remove a 500-bp fragment, whose 5' end is within exon 13 and the 3' end downstream in the intron, from the *Ncam* gene) into embryonic stem (ES) cells via homologous recombination between the exogenous (targeting) DNA and the endogenous (target) gene. The genetically-modified ES cells are then microinjected into host embryos at the eight-cell blastocyst stage. These embryos are transferred to pseudopregnant host females which then bear chimeric progeny. The chimeric progeny carrying the targeted mutation in their germ line are then bred to establish a line. If the newly established line has a disrupted or deleted gene, it is called a knockout line. A congenic strain is produced by backcrossing mice carrying the locus of interest to a recipient strain, identifying the offspring with the locus of interest and backcrossing them to the recipient, and repeating this procedure for a minimum of five to ten generations. Each successive generation retains the locus of interest but has increasingly less genomic material from the donor. A strain is considered an incipient congenic after five to nine backcross cycles (N5 to N9) and a full congenic after ten backcross cycles (N10) (Rabinowitz et al., 1996; JaxMice Database).

2.3.2. Phenotype of NCAM–/– mice

Cremer et al. (1994) have demonstrated that mice deficient in all isoforms of NCAM are fertile and healthy and show only minor defects such as smaller bodyweight, smaller olfactory bulbs and a total brain of about 10% less than in

control animals. Mutant mice can distinguish between different odours and their motor abilities are not affected. Also, a slightly laminated organization of the CA3 region of the hippocampus (Cremer et al., 1998), an enlarged rostral migratory pathway and gliosis in this region (Chazal et al., 2000) were seen in NCAM deficient mice. Functionally, NCAM–/– mice showed impairment in the CA3 region LTP, which was probably related to the abnormal development of mossy fibre projections (Cremer et al., 1998). It has also been shown that NCAM deficiency, but not a deficiency in the polysialylated form of NCAM, induced impairment in the LTP of the dentate gyrus and it is proposed that NCAM is necessary for synaptic plasticity in identified synapses *in vivo*, and suggests that polysialylated NCAM expressed by immature granule cells supports the development of basal excitatory transmission in this region (Stoenica et al., 2006). According to Rafuse et al. (2000) NCAM deficient mice have smaller neuromuscular junctions, reduced synaptic efficacy in muscles during repetitive stimuli and NCAM–/– synapses were unable to sustain transmitter output during repetitive stimuli.

The NCAM–/– mice showed impairment in cognitive behaviour, particularly in spatial learning which was revealed in the Morris water maze task, deficits in contextual and cued fear conditioning (Stork et al., 2000) and impairment in exploratory behaviour (Cremer et al., 1994). These mice also demonstrated anxiety-like behaviour, increased responses to serotonin 1A receptor stimulation, higher inter-male aggression and increased corticosterone levels after the presentation of an intruder in the home cage (Stork et al., 1997; 1999). Similar behavioural effects like deficits in reversal and spatial learning were observed in mice with conditional ablation of NCAM (Bisaz et al., 2009).

The mutant mice, which are characterized by an increased expression of the soluble form of NCAM, showed a striking reduction in synaptic puncta of GABAergic interneurons, reduction in the density of excitatory synapses, higher locomotion and enhanced responses to amphetamine (Pillai-Nair et al., 2005).

3. NCAM-derived peptide, FGL

Recently, the FN3 modules of NCAM have, by surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) analyses, been demonstrated to bind FGFRs 1 and 2, and the NCAM binding site for the FGFR has been identified (Kiselyov et al., 2003; Christensen et al., 2006). A 15 amino acid-long peptide, termed FGL, encompassing the Fβ-strand, the Gβ-strand and the interconnecting loop in the second FN3 module of NCAM, has been shown to bind and activate the FGFR, inducing neurite outgrowth and promoting neuron survival *in vitro* (Kiselyov et al., 2003; Neiiendam et al., 2004).

3.1. The design of FGL peptide

A peptide sequence, EVYVVAENQQGKSKA, corresponding to Glu^{681} – Ala^{695} of the human, mouse and rat NCAM and encompassing the Fβ-strand, the F-G-turn and the Gβ-strand (termed the FG-loop peptide or FGL) of the second F3 module of NCAM has been identified as part of the NCAM binding site for the extracellular IgII-III fragment of FGFR (Kiselyov et al., 2003). The FGL contains a sequence, A686ENQQGKS693, homologous to a common nucleotide binding motif, also termed the Walker motif (Berezin and Bock, 2004). The FGL is synthesized on TentaGel resin using 9-fluorenyl-methoxycarbonyl-(Fmoc) protected amino acids. The dimeric form of the peptide is composed of two monomers coupled to aminodiacetic acid (N-(carboxymethyl)glycine (Loke Diagnostics, Risskov, Denmark).

3.2. The actions of FGL peptide

The FGL peptide has been shown to bind to FGFR (Kiselyov et al., 2003) and furthermore, not only to FGFR1 but also to FGFR2 splice variant IIIc (Christensen et al., 2006). Interaction with FGFR results in the phosphorylation of the receptor.

In vitro, FGL has been demonstrated to induce neurite outgrowth in primary rat dopaminergic neurons and hippocampal neurons. It also increases neuronal survival and reduces DNA fragmentation when apoptosis is induced. These effects are dependent on the activation of FGFR, MAP and ERK kinases, and MEK and PI3K (Neiiendam et al., 2004). The FGL also protects neurons in dissociated rat hippocampal cultures and cultures of hippocampal slices following oxygen and glucose deprivation (Skibo et al., 2005). The FGL has also been demonstrated to promote synapse formation and enhance presynaptic functioning in hippocampal cultures by facilitating transmitter release in an FGFR activation-dependent manner (Cambon et al., 2004), and also to attenuate interleukin-1β production and enhance interleukin-4 release in mixed glial cell cultures, indicating that FGL might have anti-inflammatory potential (Downer et al., 2008).

In vivo, FGL has been shown to induce improvement of memory in contextual fear conditioning and in the water maze task (Cambon et al., 2004). In a rat model of β-amyloid peptide-induced neurotoxicity, FGL has been demonstrated to reduce all signs of β-amyloid-induced neuropathology and cognitive impairment. It also prevents and ameliorates the neurotoxic effects of β-amyloid (Klementiev et al., 2007). In newborn rats, intranasal administration of FGL accelerates early postnatal development of coordination skills, and in adult animals, subcutaneous (s.c) administration of FGL results in prolonged retention of social memory (Secher et al., 2006). It has been also shown that s.c treatment with FGL in aged rats attenuates the impairment in LTP and age-

related changes in CD200 and the markers of activated microglia (Downer et al., 2010), and also induces structural alterations in synapses and dendritic spines (Popov et al., 2008). In aged rats treatment with FGL during four weeks of continuous stress, followed by intermittent stress once a week during the subsequent six months, has been shown to prevent stress-induced cognitive impairment (Borcel et al., 2008).

FGL rapidly penetrates the blood-brain barrier and is detectable in blood and CSF for up to five hours after either s.c or intranasal administration (Secher et al., 2006).

THE AIMS OF THE STUDY

I. To evaluate the possible depression-like behaviour and alterations in hippocampal plasticity in NCAM–/– mice.
II. To determine whether FGL peptide is able to ameliorate the depressive-like behaviour and altered hippocampal neurogenesis in NCAM–/– mice.
III. To detect which NCAM interaction partners and NCAM-dependent signalling pathways are being dysregulated in NCAM-deficient mice.
IV. To evaluate the capability of FGL peptide to affect dysregulated NCAM-dependent signalling pathways in NCAM–/– mice.

MATERIALS AND METHODS

1. Animals

All the experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (Directive 86/609/EEC). The experiments also conformed to local guidelines on the ethical use of animals and all efforts were made to minimize the number of animals and their suffering. All the experiments were carried out by licensed individuals. NCAM–/– mice and NCAM+/+ mice used for this study were obtained by crossing C57BL/6-Ncam$^{tm1Cgn+/–}$ heterozygotic mice (purchased from Jackson Laboratories, Maine, USA). An F2 generation of NCAM–/– mice and their wild-type (NCAM+/+) littermates of age 4 to 6 months and with an average weight of 22.0 g were used. All animals were housed under standard housing conditions, namely, the mice were group-housed (five mice per cage) under a 12 h light/dark cycle (lights on at 9.00/lights off 21.00). All mice had free access to food and water.

2. Drug treatment

NCAM+/+ and NCAM–/– mice were divided into subgroups and were administered one of the following: vehicle, citalopram, amitriptyline or FGL, all dissolved in 0.9% NaCl solution (vehicle). FGL was administered either acutely or repeatedly, repeated administration lasted for 21 days, administered every second day. All drugs were administered subcutaneously (s.c) or intraperitoneously (i.p) in a dose of 10 mg/kg.

3. Behavioural testing

3.1. General locomotor activity

Locomotor activity was determined in a rectangular wooden cage (50 × 50 × 50 cm) uniformly illuminated with dim lighting. A light sensitive video camera, connected to the computer, was mounted about 1 m above the observation cage. The locomotor activity of an animal was monitored and analysed using VideoMot2 software (TSE Systems, Germany) during a 30 min observation period.

3.2. Open field activity

Open field activity was determined between 9.00 a.m. and 11.00 a.m. The open-field apparatus consisted of a four-sided 52 × 52 × 50 cm (L x W x H) wooden box, which was covered inside with folium to increase the reflectivity of the walls. The floor of the box was divided into 16 squares. A 60 W light bulb was positioned 90 cm above the base of the apparatus, and was the only source of illumination in the room. Each animal was tested for a 5 min period. Animals were placed in the centre of the test cage and allowed to explore freely for 5 min. During the test time the number of passed squares, time spent on the central squares and the number of vertical activity were measured. After each animal, the test apparatus was cleaned with a 10% ethanol solution and water to remove any olfactory cues.

3.3. Rotarod

Motor coordination and balance were tested using an accelerating rotarod. The rotarod test was performed by placing a mouse on a rotating drum and measuring the time period for which each animal was able to maintain its balance, walking on top of the rod. The speed of the rotarod accelerated from 10 to 40 rpm.

3.4. Tail suspension test (TST)

TST is the analogue to the forced swim test and is based on the fact that mice suspended by the tail alternate periods of struggle and immobility. Mice were suspended by the tail using an adhesive tape, approximately 1 cm from top of the tail, to a wooden beam and the total duration of immobility during a 6 min test period was measured. Immobility was defined as complete lack of movement besides respiration.

3.5. Sucrose preference

For the testing of sucrose preference, each mouse was placed in a separate cage for 18 days. During the test mice were given a free choice between two graduated bottles, one with 0.8% sucrose solution and another with tap water. To prevent habituation to side preference in drinking, the position of the bottles was changed every 24 h. No previous food or water deprivation was applied prior to testing. The consumption of both liquids was estimated every day at the same time by measuring the level of liquid for 18 consecutive days. The sucrose

preference was calculated as the percentage of sucrose solution intake of the total amount of liquid drunk.

3.6. Taste aversion

To elucidate whether NCAM–/– mice can discriminate between different taste, the taste aversion test was performed. During the test the mice from both genotypes were given a free choice between two graduated bottles, one with taste solution (100 mM HCl) and another with tap water. To prevent habituation to side preference in drinking, the position of the bottles was changed after 24 h. No previous food or water deprivation was applied prior to testing. The consumption of both liquids was estimated every day at the same time by measuring the level of liquid. The liquid preference was calculated as the percentage of taste solution intake of the total amount of liquid drunk.

4. Neurogenesis assay

4.1. Administration of 5-bromodeoxyuridine (BrdU)

To assess the survival and differentiation of the newly born cells, all groups of NCAM+/+ and NCAM–/– mice received three intraperitoneal (i.p) injections (100 mg/kg per injection, total dose 300 mg/kg) of 5-bromodeoxyuridine (BrdU) (Sigma-Aldrich, Germany) with a 2 h interval and were sacrificed 4 weeks thereafter.

4.2. BrdU immunohistochemistry and quantification of BrdU positive cells

The mice were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and transcardially perfused using 0.9% saline and then 4% paraformaldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.4). After a post-fixation of the brain in paraformaldehyde/PBS solution for 24 h, sections 40 μm thick were cut on a vibromicrotome (Leica VT1000S, Germany), collected in PBS and kept at 4ºC until further processing.

For cell proliferation assessment, the Ki-67 immunohistochemistry was used. Free-floating sections were incubated in 1% H_2O_2 in TBS for 30 min, followed by unmasking with 0.01 M citrate buffer (pH 6.0) in a water bath at 84ºC for 30 min. Incubation in blocking solution containing 2% normal goat serum and 0.25% Triton X-100 for 1 h was followed by 48 h of incubation at 4ºC with a polyclonal antibody to Ki-67 (1:75, goat polyclonal IgG, Santa Cruz

Biotechnology Inc., Germany) diluted in blocking solution. After being washed in PBS, sections were incubated in anti-goat antibody (1:300 biotinylated anti-goat IgG (H+L), Vector Laboratories, CA, USA) diluted in blocking solution for 1 h. Ki-67-positive cells were visualized using the peroxydase method (ABC system and diaminobenzidine as chromogen, Vector Laboratories, CA, USA). The sections were dried, cleared with xylol and cover-slipped with mounting medium (Vector Laboratories, CA, USA).

The survival of the newly born cells in the dentate gyrus of the hippocampus was assessed using BrdU immunohistochemistry 3 weeks (survival time) following BrdU (300 mg/kg, i.p.) administration. For BrdU immunohisto-chemistry, the free-floating sections were incubated in 0.3% H_2O_2 in PBS for 30 min, followed by incubation with 0.1 M Tris-HCl consisting of 0.025% trypsine and 0.1% $CaCl_2$ for 10 min, followed by incubation in 2 N HCl at 37ºC for 30 min. Incubation with blocking solution containing 2% normal goat serum and 0.25% Triton X-100 for 1 h was followed by overnight incubation at 4ºC with a rat monoclonal antibody to BrdU (1:200 Rat MAB Anti BrdU, Accurate Chemicals, USA) followed by incubation in biotinylated goat anti-rat antibody (1:400 Biotinylated anti-rat IgG (H+L), Vector Laboratories, CA, USA) for 1 h. BrdU-positive cells were visualized using the peroxydase method (ABC system and diaminobenzidine as chromogen, Vector Laboratories, CA, USA). The sections were dried, cleared with xylol and cover-slipped with mounting medium (Vector Laboratories, CA, USA).

All counting of the Ki-67 and BrdU-positive cells was done according to the method described previously by Malberg and Duman (2003). For each animal, positive cells (peroxydase stained) were counted in one-in-sixth sections (240 μm apart) within the dentate gyrus (granule cell layer and hilus). All counts were performed using an Olympus BX-51 microscope equipped with X 60 magnification (oil) objective to achieve optimal optical sectioning of the tissue. An average of eight sections were analysed from each animal. To estimate the total number of Ki-67 and BrdU-positive cells in a given region, the sum of cell counts from eight sections was then multiplied by six. Left and right dentate gyri were analysed separately and the estimates were averaged for each animal.

4.3. Determination of the phenotype of BrdU positive cells

Between four and six sections from each animal, surviving 4 weeks after the BrdU injection, were analysed for co-expression of BrdU and neuronal or glial markers. For immunofluorescent double-labelling, sections were incubated with a mixture of anti-BrdU monoclonal antibody (1:300 Rat MAB Anti BrdU, Accurate Chemicals, USA) and one of the following: antibody against Tuj-1 (1:300 mouse anti-tubulin, beta III isoform, Chemicon International Inc., USA), a marker for young post-mitotic neurons; anti-calbindin antibody (1:800 rabbit anti-calbindin, Chemicon International Inc., USA), a marker for mature neurons

or anti-glial fibrillary acidic protein (1:800 mouse anti-GFAP, Chemicon International Inc., USA), a marker for astrocytes. Secondary antibodies were TexasRed (1:300 TexasRed®, dye-conjugated, Jackson Immuno Research Laboratories Inc., PA, USA), anti-mouse Alexa-488 (1:300 Alexa Fluor®488, goat anti-mouse IgG_1 Molecular Probes Inc., OR, USA) and anti-rabbit Alexa-488 (1:300 Alexa Fluor®488, goat anti-rabbit IgG (H+L), Molecular Probes Inc., OR, USA). Fluorescent signals were detected with a confocal microscope MRC-1024 (Olympus⁄Bio-Rad, Germany) equipped with an argon–krypton laser. Three-dimensional images were constructed from a series (12–15) of scans of the dentate gyri at 1 μm intervals taken using X 40 (water) objective and further analysed for the co-localization of the BrdU signal with the signals of neuronal or glial markers. The data were expressed as a percentage of BrdU-positive cells found in the dentate gyrus that expressed phenotype marker calbindin, Tuj-1 or GFAP.

5. Determination of pCREB positive cells in different brain regions

For pCREB immunohistochemistry, the free-floating sections were incubated in 0.3% H_2O_2 in PBS for 30 min, followed by unmasking with 0.01 M citrate buffer (pH 6.0) in a water bath at 84 ºC for 30 min. Incubation in blocking solution for 1 h was followed by 24 h incubation at room temperature with goat polyclonal antibody to pCREB (1:200; Santa Cruz Biotechnology Inc., Germany) diluted in blocking solution. After being washed in PBS, sections were incubated in biotinylated rabbit anti-goat antibody (1:200; Vector Laboratories, UK) diluted in blocking solution for 1 h. pCREB-positive cells were visualized using the peroxydase method (ABC system and diamino-benzidine as chromogen, Vector Laboratories, UK). The sections were dried, cleared with xylol and cover-slipped with mounting medium (Vector Laboratories, UK). The number of pCREB positive nuclei were counted in the following brain areas according to Paxinos and Franklin (2001) according to bregma: the prefrontal cortex (PFC) and frontal cortex (FC), from 2.96 mm to 2.58 mm; the basolateral nucleus of the amygdala (BLA) and basomedial nucleus of the amygdala (BMA), from –1.06 mm to –1.58 mm and hippo-campus and piriform cortex (Pir), from –1.82 mm to –2.46 mm. For each structure, four random sections per mouse were taken and positive nuclei were counted manually according to the optical fractionation method (West, 1993), where the number of counting frames in the delineated region were applied randomly by CAST program (Olympus, Denmark). Counting was performed using an Olympus BX-51 microscope. Immunoreactivity was expressed as the number of positive nuclei per 0.1 mm^2 of brain region. Throughout all stages of assessment, the experimenter was blind to the experimental groupings.

6. Cell staining and cell density analysis

For quantification of the total cell density in different brain regions, every sixth section throughout the region was incubated in a 0.1 M TRIS HCl buffer, containing 0.025% trypsin and 0.1% $CaCl_2$, for 10 min, followed by incubation in acid-alcohol (HCl 1% in 70% ethanol) solution for 10 sec. The slides were stained using haematoxylin-eosine, washed in PBS and cover-slipped with a water-based mounting medium (Vector Laboratories, UK). Cell numbers were quantified according to the optical fractionation method (West, 1993). The stereology system consisted of an Olympus BX-51 microscope, a microcator (Heidenhain, DN 281) and the Computer-Assisted Stereological Toolbox (CAST)-Grid system (Olympus, Denmark). Numerical density (Nv) was calculated according to the formula $Nv= \Sigma Q/\Sigma v$ (dis), where ΣQ is the number of cells counted and Σv (dis) is the volume of dissectors.

7. Western blotting

Adult (4 months old) NCAM+/+ and NCAM–/– mice were sacrificed by decapitation for immunoblotting analysis. The brain was removed from the scull on ice and in a cold room (4°C). Olfactory bulbs and cerebellum were removed and the PFC and FC were dissected out approximately 1 mm from the rostral part of the hemispheres according to the following coordinates: bregma from 3.56 mm to 2.58 mm (Paxinos and Franclin, 2001). The PFC and FC were pooled for each probe. Hippocampi were dissected as described in the published protocol (Madison and Edson, 1997). Briefly, the brain was bisected with a scalpel along the midline. The hemibrain was turned so that the medial surface was facing up and the neocortex was peeled off, exposing the hippocampus. When the hippocampus was totally exposed it was taken out. The dissected brain tissues were placed immediately into liquid nitrogen and stored at –80ºC until further processing.

Tissues were lysed in 10 vol RIP-A lysis buffer: 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA containing protease and phosphatase inhibitors, homogenized manually, incubated for 20 min on ice and centrifuged (13000 rpm for 20 min at 4ºC). The supernatants were resolved by electrophoresis on 10% or 12% SDS-polyacrylamide gel. Proteins were transferred onto Hybond™-P PVDF Transfer Membranes (Amersham Biosciences, UK) in 0.1 M Tris-base, 0.192 M glycine and 20% (w/w) methanol using an electrophoretic transfer system. The membranes were blocked with 0.1% (w/w) Tween-20/TBS containing 5% (w/w) non-fat dried milk powder at room temperature for 1 h. After blocking, the membranes were incubated overnight at 4°C with one of the following polyclonal antibodies: rabbit anti-pFGFR1(Tyr645) (1:1000), rabbit anti-FGFR1 (1:1000), rabbit anti-

pFyn(Tyr530) (1:1000), rabbit anti-Fyn (1:1000) (purchased from AbCam, US), goat anti-pCREB (1:4000), rabbit anti-CREB (1:2000), goat anti-pMEK1/2 (1:800), rabbit anti-MEK (1:800), goat anti-pERK (1:800), rabbit anti-ERK (1:800), goat anti-pCaMKII (1:800), rabbit anti-CaMKII (1:800), rabbit anti-pCaMKIV (1:800), goat anti-CaMKIV (1:800), rabbit anti-pAkt (1:800), rabbit anti-Akt (1:800), goat anti-Raf1(Ser338) (1:800) and rabbit anti-Raf1 (1:800) (purchased from SantaCruz Biotechnology Inc., Germany), followed by incubation with secondary antibodies: anti-goat IgG (1:10000; Vector Laboratories, UK) and anti-rabbit-HRP (1:2000; Pierce, US), respectively for 1 h at room temperature, followed by incubation with ABC system (Vector Laboratories, UK).

The membranes were incubated with ECL detection reagent (ECL, Amersham, UK) for 5 min to visualize proteins and then exposed to auto-radiography X-ray film (Amersham hyperfilm ECL, UK). To normalize the immunoreactivity of the proteins, the β-actin protein was measured on the same blot with a mouse monoclonal anti-β-actin antibody (1:10000; Sigma, St.Louis, USA) followed by anti-mouse HRP secondary antibody (1:2000; Pierce, US) for 1 h at room temperature as an internal control for loading. The blots probed for proteins of interest were densitometrically analysed using a QuantityOne 710 System (BioRad). The proteins optical density ratios were calculated. The ratio of phosphorylated and non-phosphorylated protein was calculated.

8. Data analysis

All data are given as mean ± SEM. Statistical analysis was performed using unpaired Student's t-test, two-way ANOVA or Kruskal-Wallis nonparametric test, where appropriate. Post-hoc comparisons were made using Bonferroni or Dunn's test. Every p value, less than 0.05 ($p<0.05$) was considered statistically significant

RESULTS

1. NCAM deficient mice

1.1. Behaviour of NCAM–/– mice

To evaluate depression-like behaviour in terms of face validity, we employed tail suspension and sucrose preference tests. The tail suspension test (TST) in mice is similar to the behavioural despair test (Porsolt test) in mice and reflects the ability of an animal to cope with stress. In TST, mice were suspended by the tail from a horizontal bar for 6 min using adhesive tape, and the immobility time during the 6 min test was determined. As shown in Figure 2, NCAM–/– mice had a significantly higher immobility time than control NCAM+/+ mice (Student's t-test, $p<0.01$, $df=16$, $n=8$). To rule out the possibility that the increased immobility time in the tail suspension test was due to the impairment of locomotion or coordination, we subjected the mice to locomotor activity and rotarod tests. NCAM–/– mice had higher locomotor activity (Student's t-test, $p<0.01$, $df=19$, $n=8$) than wild-type littermates, while no impaired coordination in the rotarod test was observed in NCAM–/– mice. Also, the higher locomotor activity was observed in NCAM–/– mice in the open field test, where the animals crossed more squares compared to wild-type littermates (Fig. 2).

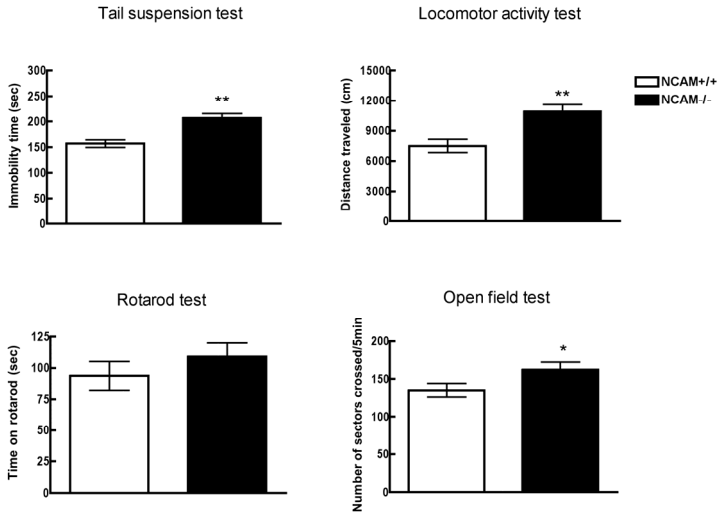

Figure 2. The effects of NCAM deficiency on the immobility time in the tail suspension test (upper left panel), locomotor activity (upper right panel), coordination on the rotarod (lower left panel) and open field test (lower right panel). Groups of 6 to 8 mice from both genotypes were used in the tests. * $p<0.05$; ** $p<0.01$ as compared to wild-type littermates (Student's t-test).

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To assess the level of anhedonia, wild-type and NCAM–/– mice were subjected to a sucrose preference tests (SPT), which is commonly used to determine the level of anhedonia and motivational deficits (Rygula et al., 2005). Wild-type mice demonstrated a clear preference for sucrose and approximately 75% of the solution consumed was sucrose. In contrast, NCAM–/– mice had no preference for sucrose and approximately 50% of the solution consumed was sucrose. The amount of total fluid consumed on the basis of body weight did not differ between wild-type and NCAM–/– mice (Fig. 3). Since taste receptors in the taste buds develop in the microenvironment provided by NCAM (Miura, et al., 2005), the reduced preference for sucrose might result from the global impairment of the development of taste cells and receptors due to NCAM deficiency. To explore this possibility, NCAM+/+ and NCAM–/– mice were also tested for the preference of sour solution. When animals were given free choices between water and 100 mM HCl solution, both NCAM+/+ and NCAM–/– mice demonstrated low preference for the 100 mM HCl solution. The 100 mM HCl solution preferences in NCAM +/+ and NCAM–/– mice were 4.8±1.1% and 4.1±0.8% (n=5, not significant), respectively. These data show that taste processing is not impaired in NCAM–/– mice.

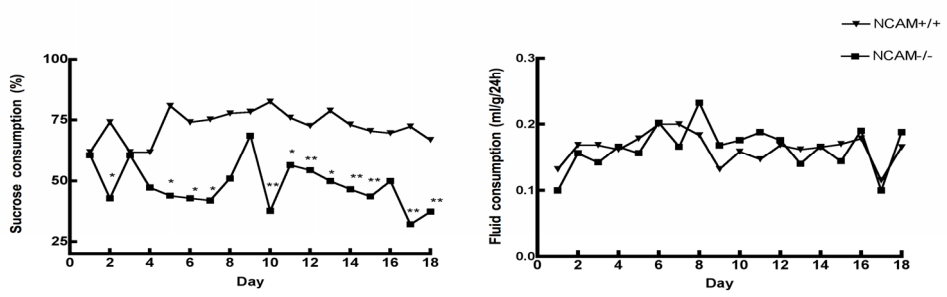

Figure 3. Sucrose consumption by NCAM–/– and NCAM+/+ mice in the SPT. Left panel shows a percentage of fluid volume (in ml) consumed as sucrose solution. Right panel shows total liquid consumption (in ml). Each group consisted of 12 mice. * $p<0.05$; ** $p<0.01$ (ANOVA for repeated measures, followed by Bonferroni post-hoc test).

1.2. Effects of FGL peptide and antidepressants on the depression-like behaviour of NCAM–/– mice

We next studied the effect of NCAM mimetic peptide FGL on the immobility time by employing TST in NCAM–/– mice and compared its effect with the effects of the known antidepressants amitriptyline (non-selective monoamine re-uptake inhibitor) and citalopram (selective serotonin re-uptake inhibitor) (Fig. 4). The antidepressants and FGL peptide were given in a dose of 10 mg/kg and injected i.p. and s.c, respectively. The animals were subjected to the TST

2 h following the injection. The data are shown in Figure 4. The two-way ANOVA revealed a significant effect of genotype ($F_{1.36}$=38.1, $p<0.001$), a significant effect of amitriptylline ($F_{1.36}$=15.4, $p<0.001$) and a significant interaction between genotype and amitriptyline ($F_{1.36}$=7.3, $p<0.01$). Post-hoc analysis revealed that amitriptylline strongly (Bonferroni post-hoc test**,** $p<0.001$) reduced the immobility time in NCAM–/– mice, and revealed a slight but significant reduction in the immobility time ($p<0.05$) in wild-type mice (Fig. 4). In contrast, no significant effects of citalopram on the immobility time were found in the NCAM+/+ and NCAM–/– mice. The low effectiveness of antidepressants in NCAM+/+ mice is most probably due to the genetic background of mice used in this study. Previous studies have demonstrated that C57Bl6 mice are the least responsive to the effects of antidepressants in TST (Crowley et al., 2005). The two-way ANOVA also demonstrated the significant effect of FGL ($F_{1.30}$ =14.4, $p<0.001$) and the significant effect of genotype x drug interaction ($F_{1.30}$=10.2, $p<0.01$). Post-hoc analysis showed that FGL treatment induced a significant reduction in the immobility time in NCAM–/– mice but not in NCAM+/+ mice.

Repeated administration of FGL in a dose of 10 mg/kg, s.c. every second day for 21 days also induced a reduction in the immobility time (Two-way ANOVA, followed by Bonferroni post-hoc test, $F_{1.20}$=24.82, $p<0.01$; n=6) in TST and this effect could be observed in NCAM–/– but not in NCAM+/+ mice (Fig. 5).

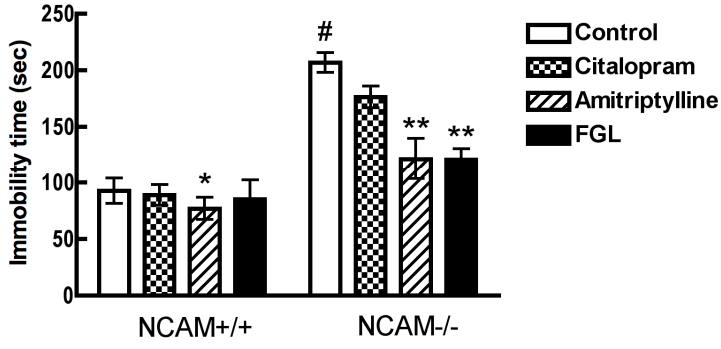

Figure 4. The effects of acute administration of citalopram, amitriptyline and FGL (all drugs administered in a dose of 10 mg/kg) on the immobility time in TST in NCAM–/– mice and their wild-type littermates. Each group consisted of 8 mice. # $p<0.05$ as compared to wild-type littermates; * $p<0.05$, ** $p<0.01$ as compared to vehicle controls. (Bonferroni post-hoc test)

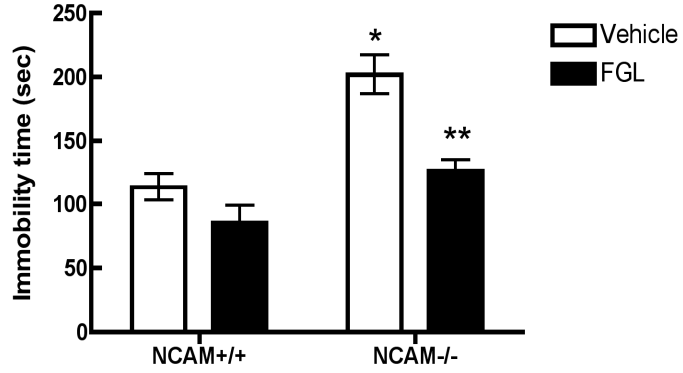

Figure 5. The effects of repeated administration of FGL (administered in a dose of 10 mg/kg, s.c, every second day) on the immobility time in the TST in NCAM–/– mice and their wild-type littermates. Each group consisted of 7 mice. * $p<0.05$, ** $p<0.01$ as compared to vehicle controls.

2. Neurogenesis in NCAM–/– mice and the effects of FGL peptide thereon

Although the exact mechanisms of depression remain largely unknown, an attractive hypothesis links depression with reduced hippocampal neurogenesis (Duman et al., 1999; Santarelli et al., 2003; Jaako-Movits and Zharkovsky, 2005; Jaako-Movits et al., 2006). Therefore it was of interest to study whether NCAM deficiency affects adult hippocampal neurogenesis. To do this, NCAM –/– mice and their wild-type littermates were i.p. administered proliferation marker BrdU in a dose of 300 mg/kg in order to label proliferating cells, and following this both NCAM+/+ and NCAM–/– mice were divided into two subgroups and treated either with vehicle or FGL (10 mg/kg, i.p every second day for 27 days). Two hours after the last treatment the mice were transcardially perfused. The brains were sectioned and processed for immunohistochemistry with antibodies against the proliferation marker Ki-67 to assess proliferation, or against BrdU to assess the survival of the BrdU labelled cells. To study the differentiation pattern of the newly generated cells, additional sections were processed for co-localization of BrdU with neuronal or glial markers such as calbindin for mature neurons, Tuj1 for young, postmitotic neurons and GFAP for astroglial cells. Ki-67 immunohistochemistry revealed dividing cells in the dentate gyri of both NCAM–/– and wild-type mice. In both groups of mice, most of the Ki-67-positive cells were found in the border between the granule cell layer and the hilus. The quantification of the Ki-67-immunopositive cells did not reveal any differences between NCAM–/– and NCAM+/+ mice, indicating that NCAM deficiency does not affect proliferation of the progenitor

cells. Repeated FGL administration also did not change the number of Ki-67 positive cells in either the NCAM–/– or NCAM+/+ mice (Fig. 6).

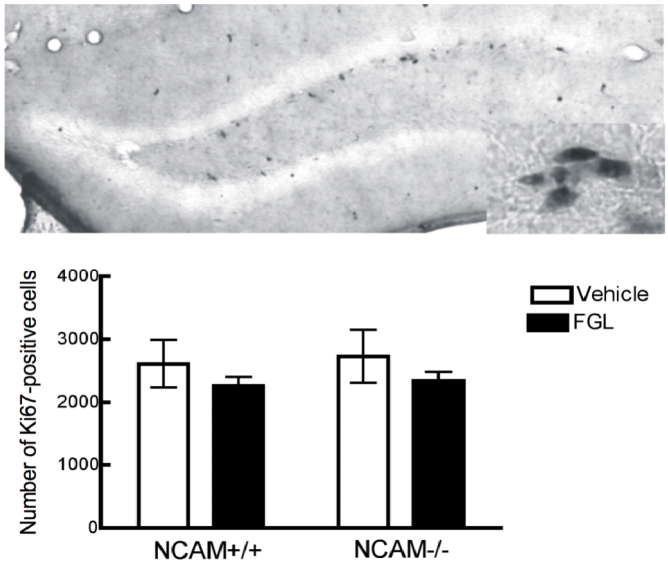

Figure 6. Ki-67-positive cells in the dentate gyrus of NCAM+/+ and NCAM–/– mice after every second day of treatment during 4 weeks with either vehicle or FGL (10 mg/kg, s.c.). No changes were detected in the number of Ki-67-immunoreactive cells in NCAM–/– mice as compared to wild-type littermates. Each group consisted of 8 mice. Upper panel demonstrates distribution of immunoreactive cells within the dentate gyrus (x100 magnification; insert: Ki-67-positive cells at x600 magnification).

Next it was studied whether the survival of the newly born BrdU labelled cells within the dentate gyrus were affected by NCAM deficiency. The survival study revealed that in NCAM+/+ mice, many BrdU-positive cells had migrated to the granule cell layer of the dentate gyrus. In NCAM–/– dentate gyri, BdrU positive cells failed to migrate and were found almost exclusively in the border between the granule cell layer and the hilus. An estimation of BrdU-positive cells revealed that NCAM–/– mice had a significantly (Student's t-test, $p<0.01$) lower number of BrdU-positive cells (Table 1). No reduction in the total number of cells in the granule cell layer or in volume of the dentate gyrus was observed in NCAM–/– mice when compared with wild-type mice (data not shown). To determine whether NCAM deficiency effects the differentiation of the newly generated cells that survived, we performed double-fluorescence immunohistochemistry for BrdU and either neuronal or glial markers (Fig. 7). Co-localization experiments revealed that the differentiation pattern of the cells that survived in the NCAM–/– mice was altered. Whilst in the wild-type mice

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21.3±4.4% of BrdU-labelled cells co-localized with a marker for adult granule neurons, calbindin, NCAM–/– mice had a significantly lower number of BrdU positive cells (Student's t-test, 16.7 ± 1.7%; $p<0.001$) which had differentiated into adult (calbindin-positive) neurons. At the same time point, a larger proportion of young postmitotic neurons expressing Tuj1 were found in NCAM–/– mice (Table 1). No changes in the percentage of BrdU-positive cells, differentiated into the glial phenotype and co-expressing glial fibrillary acidic protein (GFAP), were found. The repeated FGL administration had an effect on the survival of the newly generated cells (Table 1). The two-way ANOVA revealed a significant FGL x genotype interaction ($F_{1,29}=4.2$, $p<0.05$). Post-hoc analysis showed that FGL treatment did not affect the survival of the newly generated cells in the NCAM+/+ mice but increased their number in NCAM–/– mice (Table 1). Analysis of the phenotype of the newly generated cells revealed that FGL treatment did not affect the proportion of BrdU-positive cells expressing neuronal markers in either the NCAM+/+ or NCAM–/– mice. A small but significant increase in the BrdU-positive cells expressing astroglial marker GFAP was observed in both genotypes following FGL treatment (Table 1).

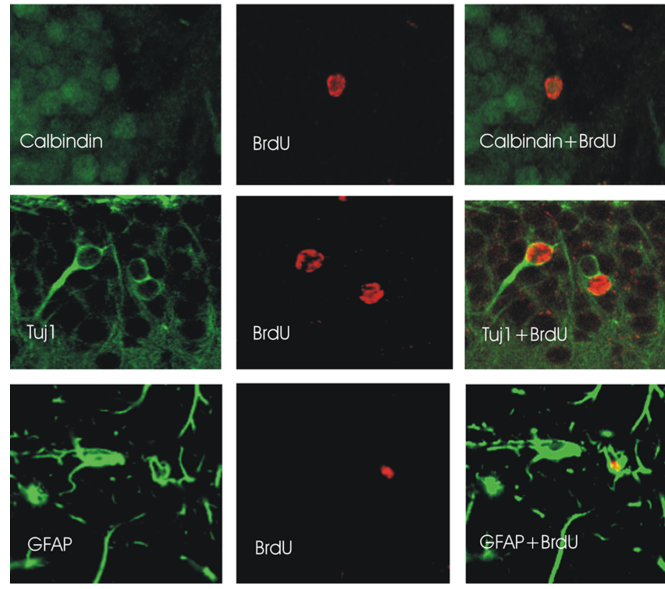

Figure 7. Laser scanning images of sections double-labelled by BrdU (red) and marker for mature neurons calbindin (green) (upper panel), marker for postmitotic young neurons Tuj1 (green) (middle panel) and marker for astrocytes GFAP (green) (bottom panel).

Table 1. Effects of repeated administration of FGL peptide (10 mg/kg, s.c., over-day, 21 days) on the survival and differentiation of newly born cells in the dentate gyrus of NCAM+/+ and NCAM–/– mice 3 weeks following administration of BrdU (300 mg/kg, i.p.). The differentiation pattern of the newly born cells labeled with BrdU was determined using neuronal (Tuj1, calbindin) and glial (GFAP) markers. The differentiation of the newly born cells was calculated as a percentage of BrdU positive cells co-localized with neuronal or glial markers to the total number of BrdU-positive cells. The number of animals in each group is given in parenthesis. Data are expressed as mean ± SEM. * $p<0.05$; as compared with wild type littermates; #$p<0.05$; ## $p<0.01$ as compared with corresponding vehicle group (Two way ANOVA followed by Bonferroni post-hoc test).

	NCAM +/+ Vehicle (n=8)	NCAM +/+ FGL (n=9)	NCAM –/– Vehicle (n=7)	NCAM –/– FGL (n=9)
Number of survived BrdU positive cells	1378 ± 104	1298 ± 161	982 ± 83 *	1522 ± 192 #
% co-localization with calbindin	32.5 ± 1.9	25.0 ± 3.6	19.8 ± 2.9 *	21.0 ± 4.6
% co-localization with Tuj1	34.4 ± 2.6	43.7 ± 6.0	37.0 ± 6.0	45.4 ± 4.0
% co-localization with GFAP	5.8 ± 1.0	9.0 ± 0.8 #	9.0 ± 0.7 *	14.7 ± 1.5 ##
Volume (mm^3) of GCL	0.2 ± 0.02	–	0.2 ± 0.02	–

3. NCAM-mediated signalling pathways in NCAM–/– and NCAM+/+ mice

3.1. Reduced FGFR phosphorylation levels in NCAM deficient mice

Signalling pathways involved in the CREB phosphorylation depend on the interaction between NCAM and its interaction partners FGFR, Raf kinase and Src-family nonreceptor tyrosine kinase Fyn. Possible differences between NCAM–/– mice and their wild-type littermates in the phosphorylation of these interaction partners were investigated. Fyn kinase contains two phosphorylation sites which play opposing roles in the regulation of its activity. While autophosphorylation of the kinase at Tyr419 increases its activity, phosphorylation at Tyr530 reduces the activity of the kinase (Tsujikawa et al., 2002). The same holds true for Raf1 which can either be inhibited by phosphorylation at Ser364 or activated by phosphorylation at Tyr491/Ser494 and Tyr341 or Ser338 (Chong et al., 2001). Therefore, the phosphorylation levels of activated FGFR1, inactivated Fyn and activated Raf1 kinases were

measured. In NCAM-deficient mice the levels of pFGFR1 were significantly lower in the hippocampus compared to their wild-type littermates. However, no changes were observed in the levels of pFyn(Tyr530) and Raf1 kinase (Fig. 8). Similar results were obtained from pooled prefrontal/frontal cortical tissues from NCAM–/– and control mice. In cortical tissues the reduction in pFGFR levels were also seen in NCAM-deficient mice compared to their wild-type littermates and no changes were seen in pFyn or pRaf1 levels (data not shown).

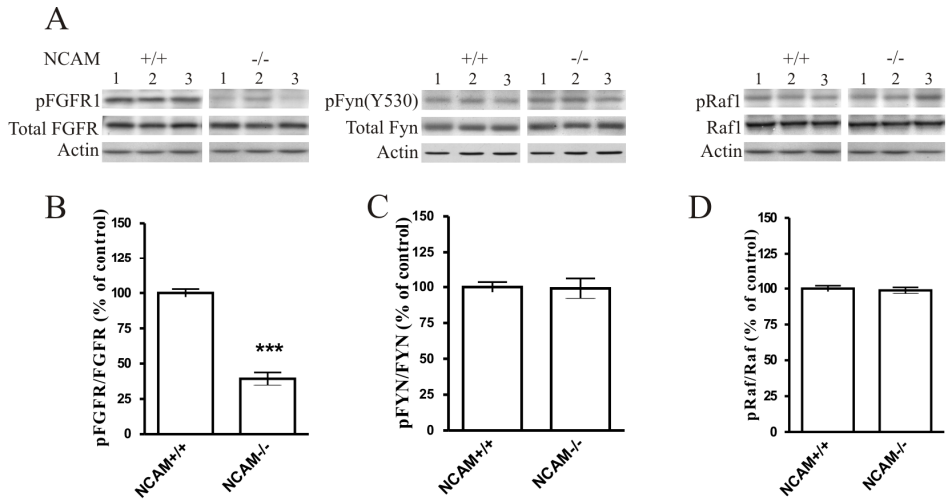

Figure 8. Representative Western blotting images of pFGFR, FGFR, pFyn(Tyr530), Fyn, pRaf1 and Raf1 proteins in the hippocampus of NCAM–/– mice and their wild-type littermates. The bands were obtained from a single animal and the bands from 3 animals for each genotype are presented (A). The ratio of pFGFR and total FGFR (B); pFyn(Tyr530) and total Fyn (C) and pRaf1 and unphosphorylated Raf1 proteins (D) in the hippocampal tissue of control and NCAM–/– mice. Data are expressed as % of control ± SEM. The immunoreactivity in NCAM+/+ mice is taken as 100%. Each experimental group consisted of 6 mice: ***$p<0.0001$ (Student's t-test).

3.2. Altered signalling pathways in the hippocampus and PFC/FC in the brain of NCAM–/– mice

To understand which signalling pathways were involved in the reduced phosphorylation of CREB in NCAM–/– mice, Western Blot analysis was performed on tissue lysates obtained from the hippocampus and cortex (pooled PFC/FC) of NCAM+/+ and NCAM–/– mice for MAPK and calcium-calmodulin-dependent protein kinase (CaMK) signalling pathways, which are dependent on NCAM signalling (Ditlevsen et al., 2008).

No differences were observed in the levels of pMEK1, pMEK2 and MEK, pERK and ERK, pAkt and Akt immunoreactivities (Student's t-test, unpaired,

two-tailed; p>0.05) (Figures not shown). By contrast, a significant decrease was observed in pCaMKII and pCaMKIV immunoreactivities (Student's t-test, unpaired, two-tailed) (Fig. 9 and 10) in NCAM–/– mice compared to their wild-type littermates, whereas no differences were observed in unphosphorylated CaMKII and CaMKIV between NCAM+/+ and NCAM–/– mice.

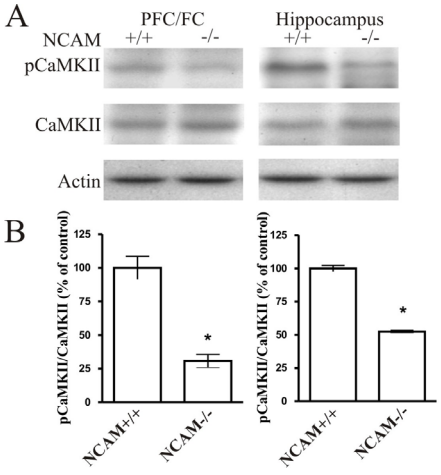

Figure 9. Western blot analysis highlighting the levels of phosphorylated and unphosphorylated CaMKII protein in PFC/FC and hippocampus of NCAM+/+ and NCAM–/– mice (A). pCaMKII/ CaMKII ratio as a % of control in PFC/FC and hippocampus of NCAM+/+ and NCAM–/– mice (B). Immunoreactivity in NCAM+/+ mice was taken as 100%; *p<0.05 (Student's t-test; n=6).

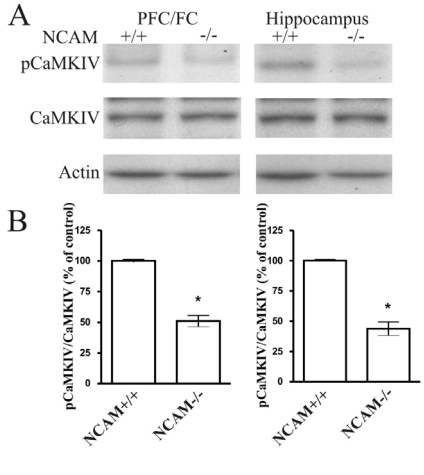

Figure 10. Western blot analysis highlighting the levels of phosphorylated and unphosphorylated CaMKIV protein in PFC/FC and hippocampus of NCAM+/+ and NCAM–/– mice (A). pCaMKIV/ CaMKIV ratio as a % of control in PFC/FC and hippocampus of NCAM+/+ and NCAM–/– mice(B). Immunoreactivity in NCAM+/+ mice was taken as 100%; *p<0.05 (Student's t-test; n=6).

3.3. Increased levels of L1 adhesion molecule in the brains of NCAM–/– mice

Since L1 adhesion molecule also interacts with Src-family kinases and Raf kinase, the levels of expression of L1 protein in NCAM–/– mice were measured. As can be shown in Figure 11, the levels of all isoforms of L1 protein were significantly higher in the hippocampus of NCAM–/– mice as compared with NCAM+/+ mice (Student's t-test L1 210 kD: p<0.0001; L1 140 kD: p<0.0001; L1 80 kD: p<0.0001).

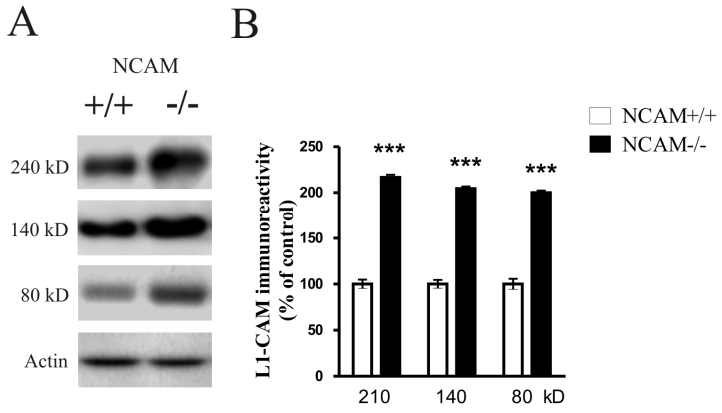

Figure 11. Representative western blotting images of L1-CAM immunoreactivity in the hippocampus of NCAM+/+ and NCAM–/– mice (A). Immunoreactivity of L1-CAM as % of control ± SEM (B). Each experimental group consisted of 6 mice; Immunoreactivity in NCAM+/+ mice was taken as 100%; ***p<0.0001 (Student's t-test).

3.4. Effects of repeated administration of FGL peptide on the altered signalling pathways in the brain of NCAM–/– mice

No differences were observed in the levels of pFyn(Tyr539) and Fyn, pRaf1 and Raf1, pMEK1, pMEK2 and MEK, pERK and ERK, pAkt and Akt protein levels between NCAM–/– and NCAM+/+ mice. The levels of these proteins in NCAM–/– and NCAM+/+ mice (figures not shown) were not affected by FGL either. Given the role of CaMKII and CaMKIV in brain plasticity, it was studied whether repeated administration of FGL could affect the phosphorylation levels of kinases constituting the CaMK pathways (CaMKII and CaMKIV) in the hippocampus and cortex of NCAM-knockout mice and their wild-type littermates.

It was found that the levels of the phosphorylated FGFR1, as well as CaMKII and CaMKIV, were reduced in the hippocampus of NCAM–/– mice and that repeated administration of FGL could ameliorate the observed reduction. For FGFR the two-way ANOVA revealed a significant effect of genotype ($F_{1.20}$=107.5, p<0.0001), a significant effect of repeated administration of FGL ($F_{1.20}$=13.32, p<0.01) and a significant interaction between genotype and repeated administration of FGL ($F_{1.20}$=24.64, p<0.0001). For CaMKII the two-way ANOVA revealed a significant effect of genotype ($F_{1.20}$=109.6, p<0.0001), a significant effect of repeated administration of FGL ($F_{1.20}$=28.87, p<0.01) and a significant interaction between genotype and repeated administration of FGL ($F_{1.20}$=7.367, p<0.05). Post-hoc analysis revealed reduced levels of pFGFR1 and pCaMKII in the hippocampus of NCAM–/–

mice compared to their wild-type littermates, whereas repeated administration of FGL restored the levels of pFGFR1 as well as pCaMKII. Similar results were found with pCaMKIV, where the two-way ANOVA also revealed a significant effect of genotype ($F_{1.20}$=27.05, p<0.001), a significant effect of repeated administration of FGL ($F_{1.20}$=12.06, p<0.01) and a significant interaction between genotype and repeated administration of FGL ($F_{1.20}$=13.17, p<0.01). Post-hoc analysis revealed a reduction in the levels of pCaMKIV in the hippocampus of NCAM–/– mice compared to their wild-type littermates, and repeated administration of FGL was able to restore the levels of pCaMKIV (Fig. 12). No effect of FGL on the levels of phosphorylated FGFR1, CaMKII and CaMKIV were observed in wild-type mice. No changes in FGFR1, CaMKII and CaMKIV levels in the hippocampus of NCAM–/– and NCAM+/+ mice and no effect of repeated administration of FGL were observed (Fig. 12). Similar results were obtained in the pooled prefrontal and frontal cortical tissues (data not shown).

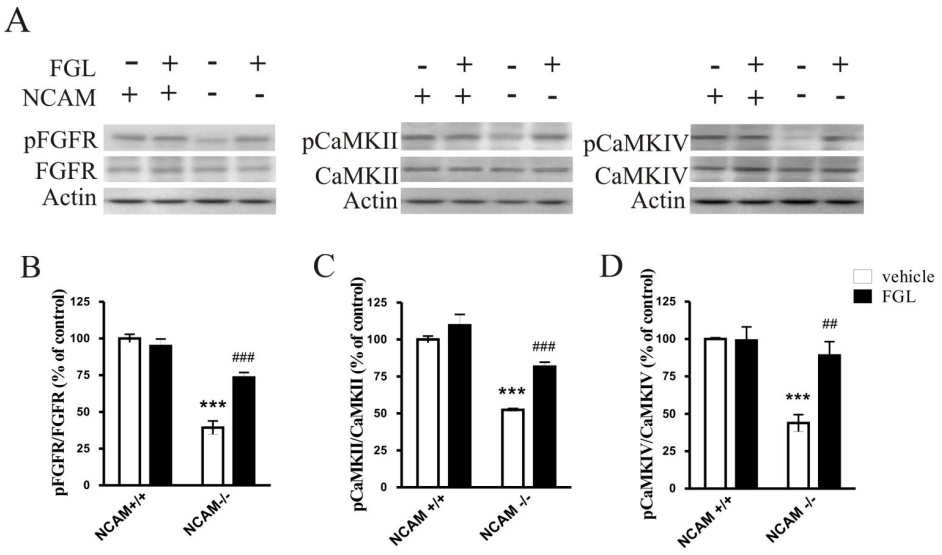

Figure 12. Representative Western blotting images of pFGFR1, FGFR1, pCaMKII, CaMKII, pCaMKIV and CaMKIV proteins in the hippocampus of NCAM–/– mice and their wild-type littermates treated either with vehicle or FGL (A). The ratio of pFGFR1 and FGFR1 (B); pCaMKII and CaMKII (C) and pCaMKIV and CaMKIV proteins (D) in the hippocampus of control and NCAM–/– mice. Data expressed as % of control ± SEM. Each experimental group consisted of 6 mice. The immunoreactivity in NCAM+/+ mice was taken as 100%; ***p<0.0001 as compared to wild-type mice. ##p<0.001; ###p<0.0001 as compared to vehicle control (two-way ANOVA, followed by Bonferroni post-hoc test).

3.5. Phosphorylation of CREB in the brain of NCAM–/– mice and the effects of FGL peptide

The levels of CREB phosphorylation were estimated by two methods which were quantitative immunohistochemistry, using phosphospecific antibodies raised against pCREB at Ser133, and semiquantitative Western immunoblotting of pCREB and CREB. The pCREB immunoreactive cells in several brain regions of NCAM–/– mice and their wild type littermates were counted. Cell counts for pCREB positive cells in the frontal cortex (FC), prefrontal cortex (PFC), basolateral nucleus of the amygdala (BLA) and CA3 subregion of the hippocampus (CA3) were significantly lower in NCAM–/– mice as compared to their wild-type littermates (Student's t-test PFC $p<0.001$; FC $p<0.001$; BLA $p<0.0001$; CA3 $p<0.0001$) (Fig.13, Table 2). In contrast, pCREB positive cell counts in the basomedial nucleus of the amygdala (BMA), dentate gyrus of the hippocampus (DG), CA1 subregion of the hippocampus (CA1) and piriform cortex (Pir) did not differ in NCAM+/+ and NCAM–/– mice (Table 2).

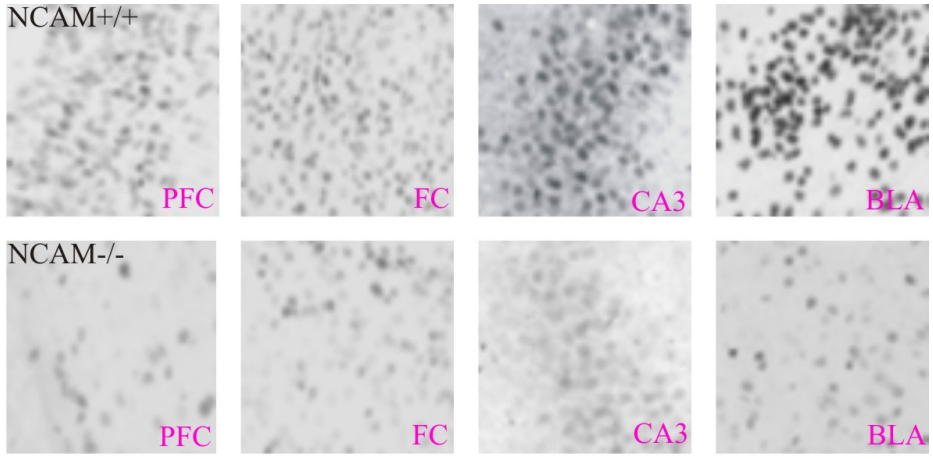

Figure 13. Representative microphotographs demonstrating pCREB-positive cells in the prefrontal cortex (PFC), frontal cortex (FC), CA3 subregion of hippocampus (CA3) and basolateral amygdala (BLA) of NCAM+/+ and NCAM–/– mice (x200 magnification).

Table 2. Number of pCREB positive cells in the brain regions of NCAM+/+ and NCAM–/– mice. The data are expressed as mean ± SEM per 0.1 mm^2. ** $p<0.01$; *** $p<0.001$ (Student's t-test; n=5).

Brain region	NCAM+/+	NCAM–/–
PFC	45.4 ± 2.6	20.2 ± 0.7 ***
FC	45.4 ± 3.3	18.4 ± 0.4 **
DG	102.2 ± 4.4	100.7 ± 4.6
CA1	49.5 ± 18.6	31.2 ± 4.9
CA3	193.6 ± 21.2	25.0 ± 2.2 ***
BLA	59.1 ± 1.1	21.6 ± 1.0 ***
BMA	48.7 ± 1.9	46.9 ± 3.3
Pir	70.2 ± 4.2	65.7 ± 3.1

Abbreviations: PFC – prefrontal cortex, FC – frontal cortex, DG – dentate gyrus of hippocampus. CA1 – CA1 subregion of hippocampus, CA3 – CA3 subregion of hippocampus, BLA – basolateral nucleus of amygdala, BMA – basomedial nucleus of amygdala, Pir – piriform cortex

Since the observed reduction of pCREB expression in NCAM–/– mice might be due to either the decreased phosphorylation of CREB or to the reduced expression of CREB, we performed Western blot analysis with lysates obtained from pooled PFC/FC and the hippocampus of NCAM+/+ and NCAM–/– mice. Figure 14 shows representative immunoblots of pCREB and CREB and the effects of FGL peptide on the levels of these proteins in the hippocampus of NCAM–/– mice and their wild-type littermates. No differences in the immunoreactivity of CREB were observed in NCAM+/+ and NCAM–/– mice.

The two-way ANOVA, followed by Bonferroni post-hoc test, revealed a significant effect of genotype ($F_{1.20}=47.58$, $p<0.0001$), a significant effect of acute administration of FGL ($F_{1.20}=5.148$, $p<0.05$), a significant effect of repeated administration of FGL ($F_{1.20}=11.41$, $p<0.01$), a significant interaction between genotype and acute administration of FGL ($F_{1.20}=14.51$, $p<0.001$) and a significant interaction between genotype and repeated administration of FGL ($F_{1.20}=20.43$, $p<0.001$) on the levels of pCREB in the hippocampus of NCAM–/– mice. Post-hoc analysis revealed reduced levels of pCREB in the hippocampus of NCAM–/– mice compared to their wild-type littermates, whereas both acute and repeated FGL administrations restored the levels of pCREB to the levels observed in wild-type mice. No changes in total CREB levels were observed in the hippocampus of NCAM–/– and NCAM+/+ mice neither in untreated nor FGL-treated animals (Fig. 14).

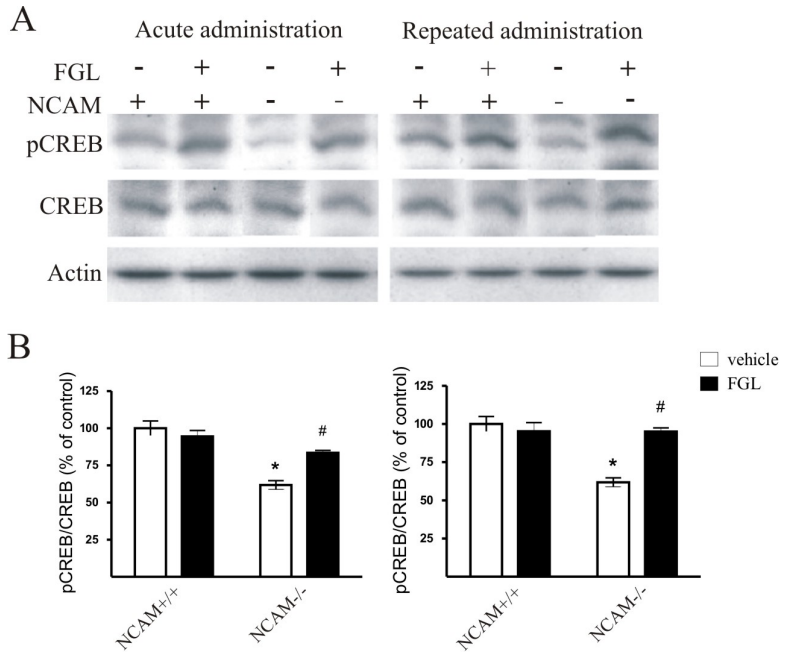

Figure 14. Western blot analysis highlighting phosphorylation levels of CREB and unphosphorylated protein (A), pCREB/CREB immunoreactivity ratio as a % of control (B) in the hippocampus of NCAM+/+ and NCAM–/– mice after administration of vehicle or FGL (acutely or repeatedly); unfilled bar: saline treated mice; filled bar: FGL treated mice. The immunoreactivity in NCAM+/+ mice is taken as 100%; *$p<0.05$ as compared to wild-type littermates, $^{\#}p<0.05$ as compared to saline control. (Bonferroni post-hoc test; n=6).

In the pooled PFC/FC, similar effects were found in NCAM–/– mice and their wild-type littermates. The two-way ANOVA, followed by Bonferroni post-hoc test, revealed a significant effect of genotype ($F_{1.20}=15.78$, $p<0.001$), a significant effect of acute administration of FGL ($F_{1.20}=4.535$, $p<0.05$), a significant effect of repeated administration of FGL ($F_{1.20}=5.053$, $p<0.05$), a significant interaction between genotype and acute administration of FGL ($F_{1.20}=8.581$, $p<0.01$) and a significant interaction between genotype and repeated administration of FGL ($F_{1.20}=15.57$, $p<0.001$) on the levels of pCREB in the PFC/FC of NCAM–/– mice. Post-hoc analysis revealed reduced levels of pCREB in the PFC/FC of NCAM–/– mice compared to their wild-type littermates, whereas both acute and repeated FGL administrations restored the levels of pCREB to the levels observed in wild-type mice. No changes in total CREB levels were observed in the PFC/FC of NCAM–/– and NCAM+/+ mice neither in untreated nor FGL-treated animals (data not shown).

DISCUSSION

1. Behaviour and neurogenesis in NCAM–/– mice

1.1. Depression-like phenotype in NCAM–/– mice

The results of the present study demonstrate that constitutive deficiency of NCAM in mice leads to an increased immobility time in TST and a reduced preference for sucrose intake in the SPT. TST and SPT are used to detect depressive-like behaviours in rodents and for screening of the effects of anti-depressants (Steru et al., 1985; Rygula et al., 2005). The increased immobility time in TST was not due to impaired mobility or motor coordination of NCAM–/– mice. Similarly, a lack of preference for sucrose consumption was not due to impairment in the taste cell development or alterations of gustatory receptors, since NCAM–/– mice demonstrated avoidance for the bitter solution. The data can be interpreted as the reduced ability of NCAM–/– mice to cope with stress and anhedonia, respectively.

These experiments also demonstrate that the known antidepressants amitriptyline, and to a lesser extent, citalopram, reduce immobility time in TST and increase sucrose consumption in SPT in NCAM–/– mice. In NCAM+/+ mice these effects of antidepressants were less pronounced as compared to NCAM –/– mice. The results of our study demonstrate that NCAM deficiency is associated with impaired adult hippocampal neurogenesis. Detailed analysis demonstrated that although the proliferation of the neuronal precursors was not affected by NCAM deficiency, the survival of the newborn cells in the dentate gyrus was reduced. The differentiation pattern of the cells that survived demonstrated a decrease in the proportion of cells which differentiated into mature neurons. Previous studies employing cultures of the neuronal progenitor cells isolated from a rat or mouse hippocampus have demonstrated that the addition of the soluble NCAM reduced cell proliferation and induced differentiation of the progenitor cells into the neuronal lineage (Amoureux et al., 2000). The similar promoting effects on the differentiation of the neuronal precursors was seen when soluble NCAM was added to the cultures of the hippocampal cells prepared from NCAM-knockout mice, suggesting that NCAM promotes neuronal differentiation by the heterophilic binding with some other receptors located on the cell surface (Amoureux et al., 2000). Our experiments on NCAM–/– mice confirm these data and also show that NCAM deficiency retards survival and differentiation of the newly generated cells without affecting their generation. In contrast, other experiments employing cultures of the neuronal precursors prepared from 1-day-old NCAM-knockout mice have demonstrated a reduced number of the BrdU-positive cells as well as a reduced number of cells expressing neuronal marker ß-tubulin (Vutskits et al., 2006). From this study however, it is not clear whether this was due to reduced

proliferation or enhanced death of the neuronal precursors resulting from impaired differentiation because neuronal death was not measured. Previous studies have suggested that impaired neurogenesis could be linked to the depressive-like state (Duman et al., 1999; Santarelli et al., 2003; Jaako-Movits and Zharkovsky, 2005). Our data confirm this theory and also demonstrate that the depression-like phenotype is associated with impaired neurogenesis in NCAM deficient mice.

Taken together, our data clearly show that impaired plasticity due to NCAM deficiency leads to the development of a depression-like phenotype, which is sensitive to antidepressant treatment and associated with impaired neurogenesis.

1.2. Effects of FGL peptide on the depression-like phenotype in NCAM–/– mice

The most intriguing finding in our study is that NCAM-mimetic FGL peptide, after systemic administration, demonstrated antidepressant-like activity in NCAM-deficient mice. The antidepressant-like effect of FGL was seen in both behavioural models and the neurogenesis assay. After acute treatment, FGL significantly reduced immobility time in TST and restored the preference for sucrose consumption in the SPT. Repeated administration of FGL increases the survival of the newly born cells and promotes their differentiation in the adult mouse dentate gyrus. Previous *in vitro* studies have demonstrated that the FGL peptide is able to bind and activate the FGF receptor thereby mimicking the actions of NCAM at FGFR (Kiselyov et al., 2003). As a result, FGL was able to stimulate neurite outgrowth and promote synapse formation *in vitro* (Kiselyov et al., 2003; Cambon et al., 2004). Administered subcutaneously, FGL readily penetrates the blood brain barrier and promotes sensorimotor development, memory retention and facilitates memory consolidation (Matus-Amat et al., 2004; Cambon et al., 2004; Secher et al., 2006).

Observations during this study of the antidepressant-like effects of FGL in NCAM-deficient mice open new avenues for the search of new molecules with antidepressant-like effects, particularly of molecules with neuroplasticity enhancing properties. The observed antidepressant-like effect of FGL might at least in part be explained by its action at FGFR. Most likely, FGL is able to activate the FGFR, whose activity is compromised in NCAM-deficient mice, thereby enhancing brain plasticity and ameliorating the signs of depression-like behaviour.

This proposal is supported by previous studies which demonstrated the role of the FGF system in the mechanisms of mood disorders. Indeed, it was shown that several members of the FGF family are dysregulated in individuals with major depression (Turner et al., 2006). Furthermore, the actions of anti-depressants are related to the enhancement of FGF signalling (Bachis et al., 2008; Turner et al., 2008). Also, it was also shown that reduced FGF signalling

induces impairment in neurogenesis and other signs of neuroplasticity (Reuss et al., 2003). Therefore, in light of this data, it is proposed that the FGFR signalling system is partially impaired in NCAM-deficient mice and this leads to the reduced neurogenesis. In contrast to other antidepressants, however, FGL did not affect the behaviour of wild-type mice. The lack of the efficacy of FGL in wild-type mice in the tests, which are commonly used for the screening of antidepressants, is not clear. The most likely proposal is that FGL has a lower affinity at the FGF receptor than NCAM itself. It is not excluded, therefore, that in the presence of NCAM, FGL is not able to activate FGFR and even antagonize NCAM-FGF receptor interactions.

The data of our study demonstrate that the FGL peptide is able to ameliorate the depression-like phenotype and counteract the reduced survival and differentiation of the newly born cells in the dentate gyrus in mice with constitutive deficiency in NCAM. These data establish new roles of the NCAM signalling mechanism through FGF receptor activation in the formation of depression-like phenotype.

2. Altered NCAM-dependent signalling pathways and the effects of FGL peptide in NCAM–/– mice

2.1. Altered NCAM-dependent signalling pathways in the brain of NCAM–/– mice

The results of the present study on NCAM-knockout mice show that deficiency of NCAM results in the decreased basal phosphorylation of FGFR1 and reduced phosphorylation of CaMKII and CaMKIV kinases, whereas the MAP kinase signalling pathway and the survival (PI3K-PKB/Akt) pathway remain largely unaffected.

Phosphorylated residues of the FGFR activate phospholipase Cγ (PLCγ) leading to the generation of secondary messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), and elevation of the intracellular Ca^{2+}. The increase of intracellular Ca^{2+}induces activation of CaMKII (Doherty and Walsh, 1996; Walmod et al., 2004) and CaMKIV (Wayman et al., 2008). Thus reduced phosphorylation of FGFR1 might account for the observed reduction in the phosphorylation of CaMKII and CaMKIV. Reduced phosphorylation of CaMKII and CaMKIV might also account for the observed phenotype in NCAM-deficient mice. Several recent studies have demonstrated the important roles of CaMKII and CaMKIV signalling pathways in the formation of memory (Silva et al., 1992; Lisman, 1994; Soderling, 2000; Frankland et al., 2001; Lisman et al., 2002), mood disorders and actions of antidepressants (Popoli et al., 2001; Tiraboschi et al., 2004; Du et al., 2004; Tardito et al., 2006).

Thus cognitive impairment and the depression-like phenotype seen in NCAM-deficient mice might be partly explained by impaired CaMKII and CaMKIV signalling. It should be kept in mind, however, that the observed changes in the phosphorylation levels of CaMKII and CaMKIV could be secondary to the morphological alterations observed in the hippocampus of NCAM-deficient mice, such as changes in the structure and connectivity of mossy fibres and alterations in neurogenesis, and not directly related to NCAM-deficiency (Cremer et al., 1994; 1998).

In vitro studies (Ditlevsen et al., 2008) have demonstrated that NCAM is also able to directly interact with Fyn kinase belonging to the Src family of nonreceptor tyrosine kinases (Beggs et al., 1994; He and Meiri, 2002; Walmod et al., 2004; Bodrikov et al., 2005) and Raf kinase (Kolkova et al., 2000). Similar data were found by Bodrikov et al. (2005) in a study on neuronal cultures obtained from NCAM-knockout mice where a reduced activity of Fyn was demonstrated by a decreased phosphorylation of Fyn at Tyr420 and increased expression of de-phosphorylated Fyn at Tyr531. The interaction of NCAM with Fyn kinase results in the activation of MAP kinase- and Akt kinase-mediated pathways (Ditlevsen et al., 2003; Ditlevsen et al., 2008). In contrast to *in vitro* studies, the data obtained in the present study show that phosphorylation levels of Fyn, Raf, MEK, ERK and Akt kinases are not disrupted in NCAM-knockout mice. It seems that under *in vivo* conditions the basal activity of these pathways is not largely affected by NCAM deficiency. The reason for the discrepancy between *in vitro* data and data obtained in this study on adult NCAM-knockout mice is not clear. The most obvious difference between our experiments and the experiments of Bodrkov et al. (2005) is that reduced Fyn activity was found in the brain tissues derived from 4-day-old mice (Bodrikov et al., 2005), whereas in our experiments adult (4-month-old) NCAM-deficient mice were used. It is possible that at the early stages of brain development NCAM deficiency results in reduced Fyn kinase signalling, which becomes compensated for in adulthood. Another explanation for this discrepancy is that in this study we measured the inactive form of pFyn(Tyr530), but Bodrikov et al. (2005) measured the active form of pFyn phosphorylated at Tyr420. It is also possible that constitutive deficiency of NCAM triggers some compensatory mechanisms. For example, another adhesion molecule L1, which shares common signalling pathways with NCAM and is able to stimulate and activate MAP and Akt kinase pathways (Schmid et al., 2000; Loers et al., 2005), can be considered. Indeed, we found an increased expression of L1 in NCAM-knockout mice and, therefore, it is reasonable to propose that over-expression of the L1 adhesion molecule might result in restoration of the phosphorylation of MAP and Akt kinases in NCAM-knockout mice independently of the FGFR1 signalling pathway. Signalling via the L1 adhesion molecule involves either the formation of a dimer in the cell membrane accompanied by the intracellular phosphorylation of L1 molecules at several sites, or heterophilic interaction of L1 with several molecules, and these

actions are essential for L1 functioning (Kamiguchi and Lemmon, 1997; Herron et al., 2009). The intracellular domain of L1 is also able to initiate intracellular signalling via interaction with other molecules such as the ankyrin family of adaptor proteins (Davis and Bennett, 1994), ezrin-radix-moesin family (Dickson et al., 2002), PI3K, Rac1 (Schmid and Maness 2008) and also Src family kinases (pp60-c-src) (Schmid et al., 2000). Thus, the observed increase in the L1 expression in the NCAM-knockout mice does not yet provide necessary information on the functional activity of this molecule. Further experiments are necessary to clarify the functional significance of L1 in NCAM-knockout mice. It should be also noted that in our experimental settings we measured only basal levels of the phosphorylated MAP and Akt kinases and it is possible that impairment in NCAM-mediated MAP kinase signalling pathway will appear when neuronal activity is induced, for example, by stress.

Our study demonstrates that NCAM–/– mice have reduced basal levels of phosphorylated transcription factor CREB. Immunohistochemical experiments revealed that the reduced number of cells expressing pCREB was specific to regions which are associated with emotional responses: PFC, FC and BLA. By contrast, DG, CA1, BMA and Pir of NCAM–/– and NCAM+/+ mice had similar basal numbers of pCREB positive cells. These experiments were confirmed by western blot assay where a reduction in the levels of pCREB in the cortical and hippocampal regions of NCAM-knockout mice was also found. These data confirm those obtained from *in vitro* experiments, where the activation of NCAM-induced CREB phosphorylation occurred at serine 133 (Schmid et al., 1999, Jessen et al., 2001). There are numerous studies demonstrating that impaired CREB activity is implicated in the mechanisms of mood disorders (Koch et al., 2009; Gass and Riva, 2007; D'Sa and Duman, 2002). CREB is known to regulate the expression of genes involved in neuronal plasticity, cell survival and cognition (Kandel et al., 2001; West et al., 2001; Lonze and Ginty, 2002; Tiraboschi et al., 2004), and phosphorylation of CREB at Ser^{133} is essential to the transcriptional activation of the CREB/CRE-mediated signalling pathway (Shaywitz and Greenberg, 1999) which plays a decisive role in the concept of altered neuroplasticity in major depression (Koch et al., 2009).

Previous studies have demonstrated that CREB activation (phosphorylation) is mediated by two major pathways: the cAMP signalling pathway and the calcium-calmodulin-dependent protein kinase pathway (Gonzalez and Montminy, 1989; Soderling, 2000), and both signalling pathways are important in the mechanisms of depression and in the actions of antidepressants (Tardito et al., 2006; Pittenger and Duman, 2008). One of these pathways involves direct interaction of NCAM molecules with Fyn kinase and FAK, which interacts with numerous proteins and may activate several other signalling molecules which eventually converge on the MAP kinase pathway (Beggs et al., 1997; Kolkova et al., 2000). Since these experiments did not find any changes in the Fyn kinase

and MAP kinase pathways this mechanism of the CREB regulation should be ruled out.

In vitro studies have demonstrated that CaMKII and CaMKIV also phosphorylate Ser^{133} of CREB, but in the case of CaMKII, this does not stimulate CREB-mediated transcription. The lack of activation has been attributed to negative regulation through phosphorylation of another site of CREB (Ser^{142}) by CaMKII (Sun et al., 1994). In contrast, *in vivo* studies have suggested involvement of CaMKII in the activation of CREB (Masson, 1993). In these experiments both CaMKII and CaMKIV were dysregulated in NCAM-knockout mice and this might result in the reduction of CREB phosphorylation. However, the exact roles of each CaMK in the regulation of CREB activity remains unknown and requires further elucidation.

2.2. The effects of FGL peptide on the altered NCAM-dependent signalling pathways in the brain of NCAM–/– mice

Another important finding of this study was the ability of the FGL peptide to restore the phosphorylation of FGFR1, CaMKII, CaMKIV and CREB upon its systemic administration. This effect of FGL was seen only in mice with the constitutive NCAM deficiency and no effects of FGL on the levels of phosphorylated CaMKII and CaMKIV were observed in the wild-type animals. The effect of FGL can be considered specific as it has been shown that FGL interacts with FGFR1 and FGFR2 and mimics the actions of NCAM at these receptors (Jacobsen et al., 2008; Neiiendam et al., 2004; Cambon et al., 2004). The ability of the FGL peptide to restore FGFR, CaMKII and CaMKIV phosphorylation might account for the previously described cognition enhancing (Cambon et al., 2004, Secher et al., 2006) and antidepressant–like activity of FGL peptide in NCAM-deficient mice. It is also supported by the data on important roles of FGF-, CaMKII and CaMKIV signalling cascades in the pathogenesis of depression (Turner et al., 2006), actions of antidepressants (Tiraboschi et al, 2004; Tardito et al., 2006) and formation of memory (Frankland et al., 2001; Soderling, 2000; Tardito et al., 2006). The lack of effects of FGL in NCAM+/+ mice on NCAM-mediated signalling was rather unexpected since numerous previous studies have demonstrated pharmacological activity of FGL in wild-type animals (Cambon et al., 2004; Secher et al., 2006). The observed discrepancies might be due to the differences in the treatment schedule, the route of administration of FGL and the animal species employed in the present study. In these experiments, FGL was repeatedly administered via subcutaneous injection for 21 days and the animals were sacrificed 24 h after the final FGL injection. In the experiments of Cambon et al. (2004) where the effects of FGL on spatial learning in wild-type animals were observed, the peptide was administered intracerebroventricularly and

thereby high concentrations of FGL in the brain tissue were achieved. In another study (Secher et al., 2006) FGL was administered only acutely and the rats were tested 1 and 24 h after drug treatment for memory enhancing effects. It cannot be excluded that repeated administration of FGL to wild-type animals might induce a tolerance-like effect upon repeated administration. Another explanation could be that the pharmacological effects of FGL peptide were found on rats and gerbils (Cambon et al., 2004; Downer et a., 2008; Klementiev et al., 2007; Secher et al., 2006; Skibo et al., 2005) whereas these experiments were performed on mice. In our studies performed on NCAM–/– mice and their wild-type littermates the antidepressant-like effect was found only in mice with NCAM-deficiency and not in wild-type mice.

These studies demonstrate that after acute or repeated administration, FGL increases the levels of pCREB in the hippocampus of NCAM–/– mice. These data support the idea that FGL has antidepressant-like activity and numerous previous studies have also demonstrated that administration of antidepressants induces CREB phosphorylation in the hippocampus (Mostert et al., 2008; Blendy, 2006).

These data demonstrate that constitutive deficiency in NCAM leads to the reduction in the activation of FGFR, CaMKII and CaMKIV concurrently, leaving Fyn and Raf1 interaction partners and MAP and Akt kinase signalling pathways unchanged. This could be due to the increased levels of another cell adhesion molecule, L1, which shares some signalling pathways with NCAM. Also, a reduction in the phosphorylation of transcription factor CREB in the brain regions involved in the emotional responses was observed. We propose that the reduction in pCREB levels was due to impairment in the CaMKII and CaMKIV pathways and this might account for the depressive-like phenotype in NCAM-deficient mice. FGL peptide is able to restore the activity of disrupted signalling pathways and restore the activity of CREB in the brains of NCAM-deficient mice. The ability of FGL to modulate the levels of phosphorylated FGFR, CaMKII and IV, and CREB might partly explain antidepressant-like and cognition-enhancing properties of the peptide.

CONCLUSIONS

I. The data of our study demonstrate that constitutive deficiency of NCAM results in the depression-like phenotype and was associated with altered adult hippocampal neurogenesis.

II. The signs of depression and altered neurogenesis in NCAM-knockout mice can be ameliorated by the peptide FGL mimicking NCAM interactions with the FGF receptor. The signs of depression-like behaviour in NCAM–/– mice were also eliminated by antidepressant treatment.

III. NCAM-deficient mice have reduced levels of phosphorylated FGFR, CaMKII, CaMKIV and CREB in the brain. We propose that the reduction in CREB phosphorylation is due to the impairment in NCAM-FGFR interaction and mediated via the calcium-calmodulin-dependent protein kinase (CaMKII and CaMKIV) pathways, and that this might account for the depressive-like phenotype in NCAM-deficient mice.

IV. FGL peptide restored the activation levels of FGFR, CaMKII, CaMKIV and CREB. The ability of FGL to modulate the levels of phosphorylated FGFR, CaMKII, CaMKIV and CREB might partly explain antidepressant-like properties of the peptide.

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SUMMARY IN ESTONIAN

Depressiooni-sarnane fenotüüp ja häiritud rakusisene signaaliülekanne närviraku adhesioonimolekuli (NCAM)-defitsiitsetel hiirtel

Üks viimaseid teooriaid depressiooni patogeneesis seob selle haiguse tekkemehhanismid vähenenud aju plastilisusega (Jacobs et al., 2000; Santarelli et al., 2003). Aju plastilisus kui selline, viitab aju võimele kohanduda erinevate stiimulitega ning lisada ja eemaldada rakkudevahelisi kontakte (Lledo et al., 2006; Butz et al., 2009). Kesknärvisüsteemis esinev molekul, närviraku adhesioonimolekul (NCAM) ja tema polüsiaalhappega seotud vorm (PSA-NCAM) on peamisteks plastilisuse kujundajateks (Walmod et al., 2004; Hoffman et al., 1982; Finne et al., 1983). Sellest lähtuvalt võiks olla NCAM seotud depressiooni tekkemehhanismidega. Eelnevalt on näidatud, et NCAM on võimeline seostuma nii teise NCAM molekuliga kui ka teiste rakupinna molekulidega ja sealtkaudu vahendama rakusisest signaaliülekannet. Üheks olulisemaks interaktsioonipartneriks NCAM'le on FGF retseptor (FGFR). Omavahelise seostumise kaudu käivitavad nad mitmeid signaalradu, mis kõik viivad transkriptsioonifaktor CREB aktiveerumiseni (Kiselyov et al., 2003; Ditlevsen et al., 2008).

Töö eesmärgid:

I. Selgitada, kas NCAM defitsiitsetel hiirtel esineb depressioonisarnane käitumine ja kuidas on muutunud täiskasvanuea neurogenees.
II. Millist toimet omab FGL peptiid, mis mimikeerib NCAM toimeid FGF retseptoril, esinevale depressioonisarnasele käitumisele ja neurogeneesile.
III. Täpsemalt selgitada NCAM interaktsioonipartnerite ja NCAM-vahendatud rakusiseste signaalradades esinevaid muutusi NCAM defitsiitsetel hiirtel.
IV. Kuidas mõjutab FGL peptiid muutusi signaalradases.

Töö tulemused ja järeldused:

Antud töö tulemused näitasid, et NCAM defitsiitsetel hiirtel esineb tõepoolest depressioonisarnane käitumine ja neil on ka langenud täiskasvanuea neurogenees, eriti uute tekkinud rakkude elulemus on vähenenud, kuid uute rakkude tekkimine ei ole suuresti muutunud. Manustades NCAM defitsiitsetele hiirtele tuntud antidepressanti, amitriptülliini ja FGL peptiidi vähenes depressioonisarnane käitumine ning ka rakkude elulemus suurenes.

NCAM interaktsioonipartneritest oli langenud FGF retseptori aktivatsioon ja tõenäoliselt selle tulemusena oli langenud ka CaMKII ja CaMKIV aktivatsioon. Samuti nägime langust CREB fosforüleerimise tasemes. FGL peptiid oli võimeline taastama esinenud häireid antud interaktsioonipartnerite ja signaalradade aktivatsioonis.

Selle töö tulemused lubavad järeldada, et NCAM defitsiitsus kutsub esile depressioonisarnase käitumise tekkimise ja langenud neurogeneesi esinemise hiirtel, mis võiks olla tingitud häiritud FGF retseptori, CaMKII, CaMKIV ja CREB aktivatsioonist. NCAM toimeid mimikeeriv peptiid FGL on võimeline kõrvaldama depressioonisarnase käitumise, taastama neurogeneesi ja interaktsioonipartnerite ning rakusiseste signaalkaskaadide aktivatsiooni.

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The scientific work has focused on the role of neural cell adhesion molecule (NCAM) in depressive-like phenotype, altered intracellular NCAM-dependent cell signalling and on the effects of NCAM-mimetic peptide FGL in NCAM-deficient mice. Three publications of the present study have been published, altogether four publications in peer-reviewed journals.

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Teadustöö

Peamiseks uuringusuunaks on olnud neuronaalse adhesioonimolekuli (NCAM) rolli uurimine depressioonisarnases-käitumises, rakusisestes ülekanderadades NCAM-defitsiitsetel hiirtel ning NCAM-mimeetikumi, FGL toime hindamine. Kolm artiklit on avaldatud selle töö raames, kokku on ilmunud neli artiklit eelretsenseeritavates rahvusvahelistes ajakirjades.

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