DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
16

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MONIKA JÜRGENSON

A complex phenotype in mice with partial or complete deficiency of the NCAM protein

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CONTENTS

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ABBREVIATIONS

AA	arachidonic acid
2-AG	2-arachidonylglycerol
BDNF	brain-derived neurotrophic factor
BLA	basolateral nucleus of amygdala
BMA	basomedial nucleus of amygdala
CAM	cell adhesion molecule
CaMK(II, IV)	Ca^{2+}-calmodulin-dependent protein kinase (II, IV)
CREB	cyclic-AMP response-element binding protein
DAG	diacylglycerol
DG	dentate gyrus of hippocampus
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EPM	elevated plus-maze
ERK1/2	mitogen-activated protein kinases
FAK	focal adhesion kinase
FC	frontal cortex
FGF-(1–23)	fibroblast growth factor-(1–23)
FGFR (1–4)	fibroblast growth factor receptor (1–4)
FYN	Src family tyrosine kinase
GAP-43	growth cone associated protein
GDNF	glial cell line-derived neurotrophic factor
GFRα	GDNF family receptor-α
GPI	glycophosphatidylinositol
5-HT	serotonin (5-hydroxytryptamine)
Ig	immunoglobulins
MAPK	mitogen activated protein kinase
NCAM	neural cell adhesion molecule
PFC	prefrontal cortex
pCaMK (II, IV)	phosphorylated CaMK(II, IV)
pCREB	phosphorylated CREB
PDGF	platelet-derived growth factor
pFGFR1	phosphorylated FGFR1
Pir	piriform cortex
PKC	protein kinase C
PLCγ	phospholipase Cγ
PSA	polysialic acid
PSA-NCAM	polysialylated form of NCAM
SERT	serotonin transporter
ST8Sia (II, IV)	polysialyltransferases (II, IV)
TST	tail suspension test

INTRODUCTION

Synaptic plasticity is one of the main factors underlying brain plasticity, and it refers to the ability of brain tissue to adequately react and adapt to continuous endogenous and environmental changes. For cognitive processes, such as learning, memory, sense and consciousness, continuous activity-induced remodelling of neuronal circuits is mandatory (Albright et al., 2000; Purves and Andrews, 1997). Neural cell adhesion molecule (NCAM) and the polysialylated form of NCAM (PSA-NCAM) are molecules that participate in a broad range of biological processes, such as cell adhesion, synaptic plasticity, neurite outgrowth or migration (Kiryushko et al., 2003; Rønn et al, 2000). Recent studies have demonstrated that brain plasticity plays a role in emotional behaviour and memory formation. Indeed, different behavioural tests have demonstrated that complete deficiency in the NCAM protein in mice results in impaired cognitive behaviour: deficits in spatial learning and exploratory behaviour and also cognitive deficits in contextual and cued fear conditioning have been described.

Several lines of evidence show that NCAM is the key-player in the regulation of brain plasticity and that dysfunction of NCAM may play an important role in the development of depression. Several molecules, including fibroblast growth factor receptor 1 (FGFR1), that have been shown to interact with NCAM may be involved in the development of mood-disorders.

The aim of the present study was to explore the effect of reduction of NCAM/PSA-NCAM expression levels on the behavioural phenotype of mice and to illuminate the intracellular pathway responsible for the cognitive dysfunction in these mice and those implicated in their reduced ability to cope with stress. By using mice partially or completely deficient in NCAM/PSA-NCAM, the main pathways and interaction partners of NCAM were studied. To clarify how alterations in the serotonergic system were implicated in the depression-like phenotype of these animals, the serotonin transporter (SERT), which is the main mechanism for the removal of serotonin from the synapse, was analysed. How disrupted cognitive abilities influence NCAM-deficient mice in the elevated plus-maze (EPM) test was also studied, as it has been shown that learning can occur during the EPM test session.

REVIEW OF THE LITERATURE

1. Cell adhesion molecules

The cell adhesion molecules (CAMs) are glycoproteins that are located on the external surface of the cell membrane. They are required for dynamic contact with other cells, i.e., to recognize and interact either with other CAMs or with proteins of the extracellular matrix (ECM). Cell adhesion is necessary for tissue formation, maintenance and functioning during development and adulthood. They have been grouped into four super-families consisting of the immunoglobulins (Ig), cadherins, integrins and the selectins that are further subdivided into smaller subfamilies. Subfamilies of Ig CAMs in the nervous system have been categorized according to the number of Ig-like domains, the presence and number of fibronectin type III-like repeats, the mode of attachment to the cell membrane (Cunningham, 1995), and the presence of catalytic cytoplasmic domain. This study is focused on the functions of the neural cell adhesion molecule (NCAM) from the Ig superfamily. All proteins in this superfamily are characterized by the presence of a specific number of Ig-like domains and fibronectin type III repeats (reviewed in Crossin and Krushel, 2000), whereas the intracellular parts show a more diverse structure.

2. Neural cell adhesion molecule (NCAM)

Neural cell adhesion molecule (NCAM; also referred to as N-CAM, CD56 and – originally – D2) was the first cell adhesion molecule from the Ig superfamily that was identified (Jørgensen and Bock, 1974; Rutishauser et al., 1976) and characterized in detail (Cunningham et al., 1987). It has been reported that as many as 27 distinct NCAM isoforms can be generated via alternative RNA splicing (Cunningham et al., 1987). Neural cell adhesion molecule mediates homophilic (i.e., between two or more NCAMs) and heterophilic (i.e., between NCAM and another molecule) cell–cell interactions. NCAM is expressed in neurons, glial cells, heart and skeletal muscles. The highest expression level is found in the central and peripheral nervous systems (Rutishauser, 1991, 1993; Walsh and Doherty, 1991, 1997).

3. Structure and localization of NCAM

NCAM is encoded by a single *Ncam1* gene that contains at least 19 exons, the primary transcript of which exhibits a complex pattern of alternative splicing (Jørgensen and Bock, 1974; Cunningham et al., 1987; Owens et al., 1987). The extracellular domain of all NCAM isoforms are composed of five regions at the amino terminus that are homologous to Ig domains (Ig I–V) and that are followed by two fibronectin type III repeats (Cunningham et al., 1987;

Figure 1A). The various isoforms of NCAM can be categorized, based on the size of their cytoplasmic tails and cell surface membrane association, into three main groups named for their approximate molecular weight (Figure 1B): NCAM-120 (120kD), NCAM-140 (140kD), and NCAM-180 (180kD) (Cunningham et al., 1983; Chuong and Edelman, 1984). NCAM-180 is a single-pass transmembrane protein generated from exons 0–19; NCAM-140 differs from NCAM-180 only in exon 18 and it is also a transmembrane protein but has a considerably shorter cytoplasmic domain; NCAM-120 is a glycophosphatidylinositol (GPI)-anchored protein resulting from the transcription of exons 0–15. NCAM also exists in a secreted form that is produced when one of the small exons located between exons 12 and 13 is included in the mRNA. This small exon contains a stop codon and gives rise to a truncated form of the extracellular part of NCAM (Walmod et al., 2004). Soluble forms of NCAM can also be generated via enzymatic excision of NCAM-120 from its GPI anchor (He et al., 1986) or via proteolytic cleavage of the extracellular domain of NCAM molecules (Hinkle et al., 2006). Soluble NCAM was also found in cerebrospinal fluid by Jørgensen and Bock (1975). During early phases of embryogenesis, these proteins are expressed in cells from all three germ layers. Within the synaptic environment, all three NCAM isoforms have distinct expression profiles; NCAM-120 is expressed predominantly on glia rather than on neurons (Goodman, 1996; Kiss and Muller, 2001), NCAM-180 appears to be expressed exclusively on neurons, particularly at the postsynaptic side of synapses, whereas NCAM-140 can be found on both glial cells and neurons (Schachner, 1997).

4. Polysialic acid (PSA) and polysialic acid linked NCAM (PSA-NCAM)

Polysialic acid (PSA) is a long (up to 200 residues), linear homopolymer of α-2,8-sialic acid that is found in large amounts in mammalian brains (Finne, 1982). The addition of PSA to NCAM is mediated by two Golgi-associated polysialyltransferases: ST8SiaII and ST8SiaIV (Eckhardt et al., 1995; Nakayama et al., 1995; Yoshida et al., 1995), of which ST8SiaII is dominant during development whereas ST8SiaIV persists at relatively high levels in the postnatal brain (Kojima et al., 1996; Ong et al., 1998). Both of these sialyltransferases can catalyse the transfer of α-2,3- or α-2,6-linked sialic acid onto NCAM without an initiator (Muhlenhoff et al., 1996; Kojima et al., 1996), and they are potentially involved in the biosynthesis of the PSA that is associated with NCAM in mammalian tissues. Furthermore, it has been reported that all three major NCAM isoforms (NCAM-120,-140, and, -180) can be modified by PSA, which attaches to two N-glycans located within the fifth immunoglobulin domain. Polysialic acid has a particularly large hydrated volume and a high density of negative charges and therefore occupies a much larger volume than NCAM

itself. Thus, the presence of PSA-NCAM reduces cell-to-cell and cell-to-extracellular matrix adhesion (Rutishauser, 1996; 2008), enabling NCAM and PSA-NCAM to modulate synaptic plasticity (Dityatev et al., 2004). Besides modulating cell adhesion, it has been proposed that PSA may be required to enhance the sensitivity of neurons to some growth factors, for example, brain-derived neurotrophic factor (BDNF, Vutskits et al., 2001). During embryonic development, the majority of NCAM exists in polysialylated form, thereby promoting cell migration, neurogenesis, axonal sprouting and synaptic plasticity (Brusés and Rutishauser, 2001). In the adult brain, cell–cell contacts are stabilized by weakly sialylated forms of NCAM and only areas that exhibit neurogenic capacity or high levels of plasticity contain PSA-NCAM (Bonfanti, 2006). Recent data indicate that PSA-NCAM expression is important for the connectivity of interneurons, and it has been suggested that NCAM is the only carrier of PSA in cortical interneurons (Gómez-Climent et al., 2011). However, there is evidence that although NCAM is the preferred substrate for polysialyltransferases, NCAM is not the exclusive carrier of PSA. Other cell-surface molecules, such as sodium channel alpha subunit (Zuber et al., 1992), neurolipin 2 (Curreli el al., 2007) and SynCAM (Galuska et al., 2010), also show detectable levels of polysialylation. However, the function of PSA in these molecules remains largely unclear.

5. Homophilic and heterophilic interaction of NCAM

5.1. Homophilic interactions

NCAM has long been evidenced to interact in a homophilic trans fashion; NCAM molecules on one cell surface interact with NCAM molecules on the opposing cell surface (Rutishauser et al., 1982). NCAM has been found to be involved in cell surface recognition and can promote cell adhesion via a homophilic Ca^{2+}-independent binding mechanism (Edelman, 1986). The first measurements of binding between NCAM Ig modules demonstrated binding between IgI and IgII modules to each other (Kiselyov et al. 1997; Atkins et al. 1999). X-ray crystallography revealed homophilic binding between the IgI and IgIII domains (Soroka et al., 2003). It has also been shown that combined IgI-IgII modules interact with the same IgI-IgII modules of another NCAM molecule (Jensen et al., 1999). It has been demonstrated that interactions between IgI-IgII double modules is approximately 30 times more prevalent than between IgI-IgII single modules, and the interaction is not affected by the IgIII domain (Atkins et al., 1999; 2001). The IgI-IgII double structure model has been also further supported by crystallographic analysis (Kasper et al., 2000). Based on these structural and functional studies, which were performed by Soroka et al. (2003), a “double zipper model” has been proposed. It is speculated that homophilic NCAM-mediated adhesion is a three-step process. The first step is cis-dimerization, the second step is formation of the “compact

zipper" (because this zipper is formed at a larger distance between opposing cell membranes than the "flat zipper" conformation), and the third step is the formation of a "compact, flat double zipper" resulting in a homophilic NCAM adhesion complex involving numerous NCAM molecules (Walmod et al., 2004; Figure 1C).

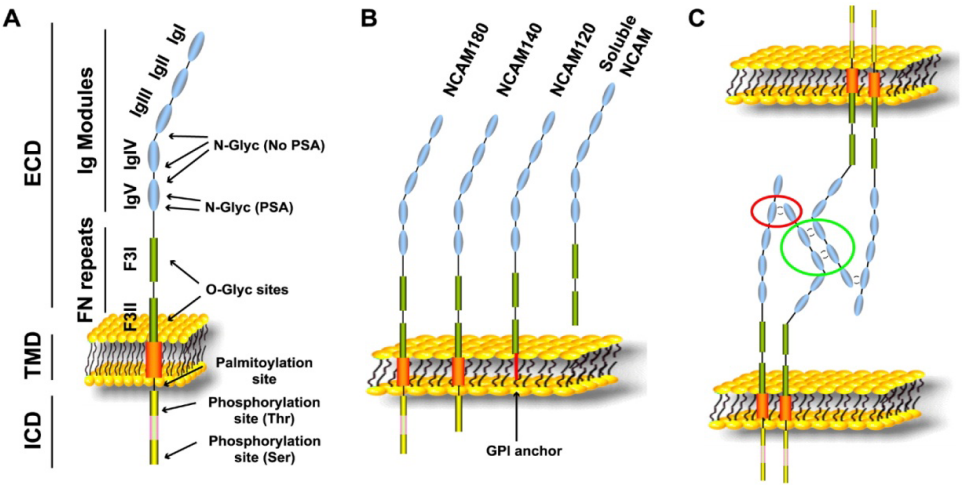

Figure 1. Molecular features of NCAM. (A) Schema illustrating the identifiable domains (left) and the post-translational modifications (right) found on the NCAM protein core. (B) The molecular structure of different NCAM isoforms. (C) The model for NCAM interactions. NCAM cis-dimers involve the interaction between IgI and IgII (red circle). NCAM trans-interactions require an initial formation of cis-dimers. Two kinds of interactions between NCAM molecules on opposing cell membranes are then possible (Soroka et al., 2003). The "flat zipper" interaction, illustrated in the picture, involves IgII and IgIII domains (green circle).

ECD: extracellular domain; TMD: transmembrane domain; ICD: intracellular domain: IgI–V: Ig-like domain I–V; F3I/II: fibronectin type 3 homology domain I/II (from review Gascon et al., 2007).

5.2. Heterophilic interactions

NCAM is capable of binding to a number of proteins on the cell surface including CAMs and extracellular matrix molecules. For example, it has been shown that NCAM may interact with the L1 molecule in a cis-fashion, which triggers phosphorylation of tyrosine and serine residues in L1 (Heiland et al., 1998). Additionally NCAM can also bind to another Ig-CAM: TAG-1/axonin-1, which was reported to bind to L1 in a cis-fashion (Malhotra et al., 1998), or to the adhesion molecule on glia, AMOG (Horstkorte et al., 1993). Furthermore, Dzhandzhugazyan and Bock (1993) have shown that the ATPase is tightly associated with NCAM and that ATP can bind directly to NCAM (Dzhand-

zhugazyan and Bock, 1997). NCAM may also interact with some components of the extracellular matrix. Cole and Akeson (1989) characterized the NCAM heparin-binding domain and showed that it consisted of two basic regions between residues 150 and 166 within the Ig2 module (Cole et al., 1986; Herndon et al., 1999). It is reported that NCAM can also bind to collagen, most likely via heparin sulphate (Kiselyov et al., 1997). In chicken brains, it was demonstrated that NCAM colocalizes and copurifies with an abundant heparan sulfate proteoglycan (Burg et al., 1995), which is presumably bound to NCAM via heparin-binding sites localized to the first and second Ig modules (Cole and Akeson, 1989; Kiselyov et al., 1997; Burg et al., 1995). Several growth factor interactions with NCAM have also been demonstrated. The glial cell line-derived neurotrophic factor (GDNF) and its receptor, the GPI-linked GDNF family receptor-α (GFRα) can both bind directly to NCAM (Paratcha et al., 2003). These studies on GDNF–NCAM interactions demonstrated that NCAM-mediated GDNF signalling leads to neurite outgrowth from embryonic hippocampal and cortical neurons (Paratcha et al., 2003). Interestingly, the binding of GDNF is independent of the presence of PSA on NCAM and does not interfere with homophilic NCAM-NCAM interactions. Binding of GFRα to NCAM inhibits homophilic NCAM-NCAM trans-interactions but may concurrently potentiate the binding of GDNF to NCAM (Paratcha et al., 2003; Nielsen et al., 2009). Besides GDNF, NCAM also interacts with brain-derived neurotrophic factor (BDNF; Vutskits et al., 2001), platelet-derived growth factor (PDGF; Zhang et al., 2004), epidermal growth factor receptor (EGFR; Povlsen et al., 2008) and growth cone associated protein (GAP-43) (Meiri et al., 1998). One of major interaction partners of NCAM is the fibroblast growth factor receptor 1 (FGFR1).

6. NCAM/PSA-NCAM interactions with FGFR1

Fibroblast growth factor receptors (FGFR1–FGFR4) are a family of trans-membrane tyrosine kinases that signal via interactions with the family of fibroblast growth factors (FGF-1–FGF-23) (Itoh and Ornitz, 2004). The first evidence of an interaction between NCAM and FGFR was obtained by Williams et al. (1994a) and confirmed by other studies (Povlsen et al. 2003; Hinsby et al. 2004). It has been demonstrated that NCAM may bind directly to FGFR, which bears tyrosine kinase activity. Furthermore, NCAM-NCAM binding leads to the phosphorylation of FGFR (Saffell et al., 1997), which suggests that NCAM binds to FGFR and can directly or indirectly stimulate it. It was proposed on basis of surface plasmon resonance analysis that F3-I and F3-II modules of NCAM can bind the FGFR Ig-modules D2 and D3 (Kiselyov et al., 2003) directly. NCAM and FGFR1 have been studied extensively and the interaction between FGFR1 and NCAM is well characterized (Kiselyov, 2010). NCAM and FGFR1 has been demonstrated to be associated in several cell types

(Cavallaro et al., 2001; Francavilla et al., 2007) and to modulate various FGFR-mediated neuronal functions (Hansen et al., 2010). Previous findings have shown that polysialylated NCAM is preferred for the activation of FGFR1 (Kiselyov et al., 2005) and that ATP inhibits the interaction between NCAM and FGFR1 (Kiselyov et al., 2003). It has been shown that NCAM stimulation leads to the phosphorylation of FGFR1 with subsequent neurite outgrowth (via activation of the Ras-MAPK pathway), a process by which differentiating neurons extend axons and dendrites (Kolkova et al. 2000). Recent studies have shown a close association between NCAM and FGFR2 (Vesterlund et al., 2011) but the mechanism underlying NCAM-FGFR2 interactions and the role of this interaction remain unclear. Basic fibroblast growth factor (FGF-2) is important in the activation of FGFR1. FGF-2 is a heparin-binding basic protein that is the prototypic member of a family of 22 related proteins (Ornitz and Itoh, 2001). It has been demonstrated that FGF-2 exhibits high affinity with FGFR1 and FGFR2 (Frantl et al., 1993; Ibrahimi et al., 2004), and furthermore, it has been shown that FGF-2, as with NCAM, can activate the FGFR1 (Plotnikov et al., 2000; Frinchi et al., 2008); however, NCAM activates FGFR signalling in a manner distinct from FGF-2 stimulation (Hinsby et al., 2004).

7. NCAM/PSA-NCAM mediated intracellular signalling

Soon after the discovery of NCAM, it was demonstrated that NCAM/PSA-NCAM participates in a number of direct or indirect interactions with many intra- or extracellular molecules, and many downstream signal pathways are dependent on NCAM/PSA-NCAM-mediated cellular signalling. The neurite promotion properties demonstrated for NCAM were shown to be dependent upon homophilic interactions between corresponding molecules (Doherty and Walsh, 1996). This idea is supported by experiments that show that homophilic NCAM interactions are dependent on the mitogen activated protein kinase (MAPK) pathway (Kolkova et al. 2000). Furthermore, NCAM induces intracellular signalling that leads to the activation of MAPK and cyclic-AMP response-element binding protein (CREB), which are activated either by Rsk kinase or via MSK1 kinase (mitogen and stress-activated kinases), both of which are downstream of mitogen-activated protein kinases ERK1/2 (Schmid et al., 1999). Immunoprecipitation studies revealed an association between NCAM and non-receptor tyrosine kinases Fyn and focal adhesion kinase (FAK) (Beggs et al., 1997). The mechanism underlying the association of NCAM with Fyn likely depends on the binding of receptor protein tyrosine phosphatase-α (RPTPα) (Roskoski R, 2005).

The interplay between NCAM and fibroblast growth factor receptor (FGFR) in neuronal cells mediates NCAM-dependent neurite outgrowth (Walsh and Doherty, 1997) and plays an important role in cell adhesion, cell migration,

neural differentiation, synapse formation, and synaptic plasticity (Kiryushko et al., 2004; Sytnyk et al., 2004; Maness and Schachner, 2007). It was shown that NCAM can directly interact with FGFRs thereby inducing their auto-phosphorylation (Kiselyov et al., 2003). The phosphorylated residues on the cytoplasmic part of FGFR dock with several proteins including the enzyme phospholipase Cγ (PLCγ), which becomes activated upon binding. The substrate of PLCγ is phosphatidylinositol 4,5-bisphosphate (PIP_2), which is cleaved to generate the two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). 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 intracellular calcium concentrations $[Ca^{2+}]_i$. DAG remains a part of the plasma membrane and can either activate protein kinase C (PKC) or is converted by DAG lipase to 2-arachidonylglycerol (2-AG) and arachidonic acid (AA), the latter of which can induce various downstream signalling events (Walmod et al., 2004). It has also been demonstrated that AA may modulate specific Ca^{2+} channels located in the plasma membrane, thus triggering Ca^{2+} influx into the cytosol and subsequent induction of neurite out-growth (Williams et al., 1994b). FGF-2, another FGFR1 activator, was shown to be mediated by 2-AG. It is thought that the addition of AA to cells may lead to an increased level of 2-AG and that addition of AA therefore indirectly stimulates 2-AG mediated signalling (Williams et al., 2003). However, the relative importance of AA and 2-AG for NCAM-mediated neuritogenesis remains unclear. Nevertheless, because NCAM can interact with GDNF and GFRα and thereby activate downstream signalling components including Fyn, FAK, and ERK1/2, it can also activate neurite outgrowth and cell migration in an FGFR independent manner (Paratcha et al., 2003).

Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) is another serine/ threonine protein kinase that associates with NCAM (Sytnyk et al., 2006). It has been shown that clustering of NCAM at the cell surface induces lipid raft–dependent activation of CaMKIIα, which then phosphorylates RPTPα at two serine residues, which in turn leads to the activation of Fyn (Bodrikov et al., 2005; 2008), and thus, this concurrence indicates an important role in the trifunctional interaction between NCAM, CaMKIIα, and RPTPα in lipid rafts. NCAM can also trigger phosphorylation of the transcription factor CREB, which is activated either by Rsk kinase or via MSK1 kinase, which are down-stream of ERK1/2 kinases (Schmid et al., 1999). Activation of CREB has been proposed to also regulate synaptic plasticity, neuronal survival, neuronal maturation, and developmental plasticity (West et al., 2002; Deisseroth et al., 2003). CREB can be phosphorylated on serine 133 by multiple kinases including protein kinase A (PKA), calcium/calmodulin dependent kinases (CaMKs), and MAPK. It has been proposed that PLCγ-mediated release of intracellular Ca^{2+} triggers CREB phosphorylation via activation of calcium/calmodulin-dependent kinase IV (CaMKIV) (Finkbeiner et al., 1997; Minichiello et al., 2002). The NCAM-mediated increase in intracellular Ca^{2+} can also lead to an

activation of CREB-phosphorylation via CaMKII because it has been shown that NCAM-FGFRs-mediated neuritogenesis can be blocked by CaMK-inhibitors (Williams et al., 1995).

NCAM-induced neuritogenesis can also be regulated via the cAMP/PKA pathway. This pathway may be involved in NCAM-mediated signal transduction, leading to the activation of two transcription factors, CREB and c-Fos, which are downstream of PKA, because it has been shown that inhibitors of cAMP and PKA can selectively abolish NCAM-mediated axonal outgrowth (Jessen et al., 2001).

One of the molecules reported to increase phosphorylation of Akt and CREB in adult hippocampal neural progenitor cells was FGF-2 (Peltier et al., 2007). Ditlevsen et al. (2008) supported this finding by showing that FGF-2 induces phosphorylation of Akt, ERK, and CREB but they also found that FGFR1 must be present during this processes.

8. The roles of NCAM/PSA-NCAM in brain plasticity and cognition

Brain plasticity or neuroplasticity refers to the ability of brain tissue to adequately react and adapt to continuous endogenous and environmental changes. Synaptic plasticity is one of the main drivers of brain plasticity, and adhesion molecules are the most significant key players, providing stable connections between the cells and at the same time, remodelling synaptic networks. It has been proposed that for cognitive processes, such as learning, memory, sense and consciousness, continuous activity-induced remodelling of neuronal circuits is required (Albright et al., 2000; Purves and Andrews, 1997). NCAM participates in a broad range of these biological processes including cell adhesion, synaptic plasticity, neurite outgrowth or migration (Kiryushko et al., 2003; Rønn et al, 2000; Muller et al., 1996; Rutishauser et al., 1976). The adhesive properties of NCAM are regulated by the addition of PSA (Rutishauser, 1996), which attenuates NCAM-mediated cell interactions and thereby creates plasticity in the position and movement of cells and/or their processes (Rutishauser, 1996 and Brusés and Rutishauser, 2001). Indeed, removal of PSA by endosialidase-N, an enzyme that specifically cleaves PSA, disturbs neuronal migration, axonal sprouting, branching and fasciculation (Durbec and Cremer, 2001; Yamamoto et al., 2000) and also synaptogenesis (Dityatev et al., 2004), synaptic plasticity (Becker et al., 1996; Muller et al., 1996) and spatial memory (Becker et al., 1996). Previous studies suggested functions for NCAM in the fasciculation of axons to form bundles, such as the retinotectal mossy fiber projections (Rutishauser, 1985; Cremer et al., 1997). Some data suggest that NCAM is involved in both early synaptogenesis and subsequent synaptic maturation (Sytnyk et al., 2002). PSA-NCAM-mediated sensitization of neurons to BDNF is an important mechanism that has been shown to be important in neuronal network plasticity

and could contribute to cortical reorganization (Kiss et al., 2001; Vutskits et al., 2001). It is assumed that the most important function of NCAM/PSA-NCAM is in the regulation of the dynamic balance between stability and plasticity of synaptic contacts. Brusés and Rutishauser (2001) showed that PSA-NCAM is primarily expressed on axons and dendrites prior to contact formation but that after the contacts are formed, the expression is rapidly down-regulated. This theory is supported by findings that expression of PSA and PSA-NCAM is highest during development (Seki and Arai, 1991; Cox et al., 2009) and that it is up-regulated in the adult brain by neuronal activity and during learning-induced neuroplasticity in the hippocampus (Murphy et al., 1996).

There are several data that demonstrate that brain plasticity plays a role in emotional behaviour and memory formation. Learning and memory impairments are associated with a number of psychiatric and neurological disorders, such as Alzheimer's disease, schizophrenia, depression, Parkinson's disease, learning disabilities, age-related cognitive decline and mental retardation. Several lines of evidence also link dysregulation of NCAM signalling with psychiatric disorders including schizophrenia, depression and Alzheimer's disease (reviewed in Brennaman and Maness, 2008). It has been shown that disrupting NCAM function by ablation of NCAM impairs synaptic plasticity in the hippocampus in vitro and in vivo (Cremer et al., 1994, Arami et al., 1996; Bukalo et al., 2004; Stoenica et al., 2006), which can lead to several memory and learning deficits (Cremer et al., 1994, 1998; Arami et al., 1996; Bukalo et al., 2004). NCAM and PSA-NCAM have been proposed to be important regulators of the effects of acute intrinsic stress on memory consolidation (Bisaz et al., 2009) and diminished NCAM expression may be a critical vulnerability factor for the development of behavioural alterations induced by stress (Bisaz and Sandi, 2012).

9. NCAM involvement in stress and mood disorders

Accumulating evidence indicates that chronic stress or continuous elevated levels of glucocorticoids affects the functioning of NCAM and its polysialylated form, PSA-NCAM (Sandi, 2004). Exposure to stressful situations can reduce NCAM levels in the hippocampus and prefrontal cortex (Sandi et al., 2005). Several stress-based depression models, such as chronic restraint stress (Venero et al., 2002), social stress (Touyarot et al., 2004), early life stress (Tsoory et al., 2008; Chocyk et al., 2010) and contextual fear conditioning (Merino et al., 2000), induce downregulation of hippocampal NCAM. In contrast to the reduction in NCAM, chronic restrained stress is associated with increased corticosteroids and increased levels of the polysialylated form of NCAM in the hippocampus (Pham et al., 2003; Sandi, 2004). These changes correlate with decreased levels of BDNF, whereas treatment with antidepressants can increase the levels of both proteins (Varea et al., 2007a; Koponen et al., 2005). It is

thought that PSA-NCAM may bind to BDNF, resulting in the phosphorylation of its major receptor, tropomyosin-related kinase B (TrkB). Thus, the increased levels of PSA-NCAM may be a compensatory response to the lower levels of BDNF in the depressed hippocampus (Brennamann and Maness, 2008). This hypothesis may be supported by findings that show that the expression of BDNF and TrkB is decreased in the hippocampus of depressed individuals and this reduction can be reversed by antidepressants (Nibuya et al., 1995). Moreover, it has been demonstrated that BDNF can interact with serotonin (5-hydroxytryptamine, 5-HT) at several levels (Martinowich and Lu, 2008), and serotonin influences neuronal circuits relevant for anxiety and depression (Lucki, 1998). Defects in BDNF can thus alter serotonergic function (Guiard et al., 2008), and a key role in serotonergic function is played by the 5-HT transporter (5-HTT or SERT), which terminates the action of 5-HT by facilitating its reuptake into the presynaptic terminal (Blakely et al., 1994). Remarkably, polymorphisms in the SERT gene have been associated with depression and other mood disorders (Heils et al., 1996; Caspi et al., 2003).

Major depression is believed to originate from an interaction of deficits in neuronal resiliency and in neuroprotective responses or from exacerbated functioning of deleterious pathways that eventually lead to neuronal dysfunction and psychopathology (Duman, 2009). Several studies have described opposing actions of chronic stress and antidepressant treatment on neuronal structural plasticity (Magarinos et al, 1999; Varea et al., 2007a), and a significant level of data implicate the hippocampus in the development of depression (Bremner 2002; MacQueen et al., 2003; Janssen et al., 2004). It should be noted that patients diagnosed with major depression demonstrate morphological and functional alterations in the hippocampus (Bremner 2002; Cole et al., 2010) and impaired executive function (Frodl et al., 2006). Furthermore, an enhanced degradation of NCAM has been demonstrated in depressive patients (Tochigi et al., 2008), and a reduction in PSA-NCAM expression was found in the brains of patients diagnosed with major depression (Varea et al., 2007b; Varea et al., 2012). Moreover, it has been demonstrated that the major interaction partner of NCAM, FGFR1, also plays a role in the development of mood disorders (Gaughran et al., 2006; Turner et al., 2011). Furthermore, administration of antidepressants increases the levels of FGFs and FGF-2 in hippocampal and cortical areas (Mallei et al., 2002; Bachis et al., 2008). It was also shown that acute or chronic administration of FGF-2 resulted in antidepressant-like effects, which was accompanied by an increase in FGFR1 levels, specifically in the dentate gyrus (Turner et al., 2008).

Thus, several molecules that have been shown to interact with NCAM may be involved in the development of mood-disorders. NCAM is also a key-player in the regulation of brain plasticity, a dysfunction of which may play an important role in the development of depression. Thus, further study is required to determine how reduced NCAM/PSA-NCAM expression influences the development of mood disorders.

10. Mice with reduced expression of NCAM

NCAM-deficient mice were originally generated by Cremer et al. (1994) using gene targeting in the C57B/6 strain. It was demonstrated that complete NCAM knock-out resulted in almost complete loss of PSA, reduced brain weight, reduced olfactory bulb size and a reduced density of mossy fibres in the hippocampus (Cremer et al., 1994; Cremer et al., 1997). Homozygous mutants appeared fertile and healthy but their average weight was approximately 10% less than wild-type and heterozygous littermates (Cremer et al., 1994). However, the reduced size of the olfactory bulb in mutant mice did not affect their odour distinction, and their motor abilities were similar to controls (Cremer et al., 1994). It has been proposed that the reduction in size of the olfactory bulb is caused by the smaller size of the granular cell layer whereas all other structures in the olfactory bulb appear normal (Cremer et al., 1994). Morphological changes caused by NCAM deficiency have also been found in other structures in the brain. A slightly laminated organisation of the CA3 region (Cremer et al., 1998), an enlarged rostal migratory pathway and hippocampal gliosis (Chazal et al., 2000) have been described. Constitutive loss of NCAM expression (gene knock-out) affects LTP in the CA3 region of the hippocampus, which may be related to the abnormal development of mossy fibre projections (Cremer et al., 1998). Synaptic plasticity in the dentate gyrus is NCAM glycoprotein- but not PSA-dependent (Stoenica et al., 2006), and studies have demonstrated that a deficiency in NCAM but not PSA-NCAM induces an impairment in LTP in the dentate gyrus (Eckhardt et al., 2000; Stoenica et al., 2006). It has also been described that although neuromuscular maturation occurred normally in NCAM-deficient mice (see also Moscoso et al., 1998), their neuromuscular junctions were smaller (Rafuse et al., 2000).

Recent study has demonstrated that complete NCAM knock-out in mice leads to the reduced levels in CREB phosphorylation in brain regions that play important roles in spatial and aversive learning (in prefrontal/frontal cortex, hippocampus and basolateral nucleus of amygdala). This reduction was accompanied by reduced levels of phosphorylated CaMKII and CaMKIV in the prefrontal/frontal cortex and hippocampus (Aonurm-Helm et al., 2008). Furthermore, NCAM–/– mice showed reduced phosphorylation of the major NCAM interaction partner FGFR1 (Aonurm-Helm et al., 2010). Different behavioural tests showed that complete knock-out of the NCAM gene in mice resulted in impaired cognitive behaviour, specifically, deficits in spatial learning and exploratory behaviour (Cremer, 1994; Stork et al., 2000), and cognitive deficits in contextual and cued fear conditioning (Stork et al., 2000; Senkov et al., 2006) are also described. Additionally, these mice demonstrate increased inter-male aggression (Stork et al., 1997) and anxiety-like behaviour (Stork et al., 1999). However, the impaired intracellular pathways underlying the cognitive dysfunction and those involved in the reduced ability to cope with stress remain unclear.

In the experiments of Stork et al. (1999), the anxiety-like behaviour was evidenced by an increased preference for the dark compartment in the light/dark avoidance test. This behaviour was accompanied with altered 5-HT1A response of NCAM-deficient mice, which supports the idea of a functional disturbance in the serotonergic system involved in their abnormal anxiety-like and aggressive behaviour (Stork et al., 1999). However, NCAM-deficient mice show normal synthesis and metabolism of 5-HT, and no significant alteration in the expression of 5-HT1A binding sites was detected in any brain area of NCAM–/– mice studied including in the dorsal raphe nucleus, frontal cortex, hippocampus, and amygdala (Stork et al., 1999).

It has been shown that the major mechanism of terminating 5-HT neurotransmission is the reuptake of 5-HT by SERT, which absorbs extracellular 5-HT into the cytoplasm (Blakely et al., 1994). Therefore, the activity of SERT has an important influence on the extracellular 5-HT concentration. Hence, more studies must be conducted to clarify the role of NCAM in 5-HT receptor signalling and to define the molecular and neural mechanisms involved in this process.

Contrary to the results of the light/dark avoidance test, in the elevated plus-maze test (EPM), NCAM-deficient mice demonstrated an increased preference for open arms, which is indicative of anxiolytic-like behaviour (Stork, et al., 1999, 2000). Thus, it appeared that the performance of NCAM-deficient mice in tests of anxiety was dependent on the test employed. As accumulating data has shown that learning can occur during the EPM test session (Calzavara et al., 2004; Calzavara, et al., 2005; Carobrez and Bertoglio, 2005; Bertoglio et al., 2006), it is likely that the impaired learning and memory of NCAM-deficient mice could influence EPM test results.

Interestingly, it has been shown that mice with a partial reduction in NCAM expression (NCAM+/–) display increased anxiety and inter-male aggression and a post-aggression test increase in corticosterone plasma concentration, similar to NCAM–/– mice (Stork et al., 1997, 1999). These data suggest that even a partial reduction in NCAM protein may cause alterations in behavioural phenotype.

THE AIMS OF THE STUDY

The aims of the present study were:

1. To assess how partial or complete deficiency of NCAM in mice affects their phenotype;
2. To detect and measure how complete deficiency of NCAM influences the neuronal activity in the brain regions that are involved in memory functions;
3. To determine how impaired cognition due to complete deficiency of NCAM affected behaviour of mice in the elevated plus-maze task;
4. To determine how partial or complete deficiency of NCAM affected NCAM-related pathways;
5. To determine whether deficiency of NCAM affected an important component of the serotonergic system – the serotonin transporter (SERT).

MATERIALS AND METHODS

1. Subjects and drug treatment

All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (Directive 86/609/EEC), and procedures were conducted in compliance with Estonian Law of Animal Protection and upon approval of the protocol nr. 117 by the Licensing Committee of Animal Experiments at the Estonian Ministry of Agriculture. All efforts were made to minimize the number of animals and their suffering. All experiments were performed by individuals who held an appropriate licence. The NCAM-deficient mice were originally generated by Cremer et al. (1994). The wild-type, NCAM+/– and NCAM–/– mice used for this study were obtained by crossing C57BL/6-Ncam tm1Cgn+/– heterozygous mice, which were purchased from Jackson Laboratories (Maine, USA). F5-generation NCAM+/– mice, NCAM–/– mice and their wild-type littermates, aged 2–4 months and with an average weight of 23.5 g, were used. All animals were housed under standard housing conditions as follows: the mice were group-housed (five or six mice per cage) using a 12 h light-dark cycle (lights on at 9.00/lights off at 21.00). All mice had free access to food and water. The anti-depressant used in the experiments was amitriptyline (Tocris Bioscience, UK), which was dissolved in 0.9% NaCl solution (vehicle) and administered intraperitoneally (i.p.).

2. Behavioural testing

2.1. General locomotor activity

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

2.2. The elevated plus-maze test

The EPM was made of metal and black plastic and the apparatus consisted of two opposite open arms (45 cm × 10 cm) without sidewalls and two enclosed arms (45 cm × 10 cm × 30 cm) with sides and end walls, all extending from a central square (10 cm × 10 cm). The maze was elevated to a height of 60 cm above the floor and placed in a dimly lit room (8 lux as measured at the centre of the maze). At the onset of the test, the animals (n=10 in each group) were

placed in the centre of the EPM facing towards an open arm and the experimenter recorded the number of open- and closed-arm entries and the time spent in either type of the arms, during a 5-min test period. An entry was counted when an animal placed all four paws in an arm. The maze was cleaned using 5% ethanol solution and water after each trial. All experiments were also video-recorded and analysed later for activity per minute. In addition to minute-by-minute scoring, a test/retest protocol was used. Twenty-four hours after the first trial (Trial 1), a second trial was performed (Trial 2). All tests were performed at the same time of the day, between 11.00 and 14.00, and with the same experimenter. The percentage of entries into open arms out of total arm entries and the percentage of time spent in the open arms were used as measures of anxiety. In addition, the total number of entries was scored and used as a measure of locomotor activity.

2.3. Object-recognition test

The object-recognition task took place in a 50 cm × 50 cm × 50 cm (L × W × H) open field, made of brown wood, located in a testing room that was dimly (approximately 60 lux in the test arena) lit. The objects chosen were porcelain cups of different shapes and sizes; these objects were sufficiently heavy that the mice did not move them. All objects had similar textures and colours but distinctive shapes. For testing, each animal (n=7 per group) was first habituated by allowing it to freely explore the open field for 5 min. No objects were placed in the box during the habituation session. Twenty-four hours after habituation, individual mice were trained by placing them into the field for 5 min, into which two identical objects (2 × A) were positioned in two adjacent corners, 10 cm from the walls. The amount of time each mouse spent exploring both objects was recorded. Short-term memory (STM) and long-term memory (LTM) tests were performed at 2 h and 24 h after training, respectively. In both tests, the mice explored the open field for 5 min in the presence of one familiar (A) and one novel (B for STM or C for LTM) object and the time the mice spent exploring the objects was recorded. A preference ratio for each mouse was expressed as a ratio of the amount of time spent exploring the new (B or C) object (Tnew ×100)/(TA + Tnew), where TA and Tnew are the times spent exploring the familiar object A and the novel object B or C, respectively. Between trials, the objects were cleaned using 5% ethanol solution. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. The time spent exploring each object was recorded manually with a stop-watch by a trained observer who was blinded to the genotypes of the animals.

2.4. Fear conditioning and extinction

The contextual fear-memory test and contextual fear-extinction test in wild-type, NCAM+/– and NCAM–/– mice were performed. For fear conditioning, an experimental chamber of 22 cm × 22 cm × 35 cm (L × W × H) was used. The box was made of transparent plastic. The floor was made of stainless steel rods that were designed for mice and that were connected to a scrambled shock generator (TSE Systems, Germany). The chamber contained a speaker that emitted audible tones.

The chamber was housed inside a larger noise-attenuating box, and a built-in ventilation fan provided background noise. After each trial, the chamber was cleaned using 5% ethanol solution. On the training day, each mouse (n=8 per group) was allowed to freely explore the conditioning chamber during a 3-min contextual pre-exposure session. Following habituation, three conditioned stimulus (CS)/unconditioned stimulus (US) pairings were performed, with 1 min intervals between pairs. The CS was an 85 db, 2800 Hz, 20 s tone, and the unconditioned stimulus was a scrambled foot shock of 0.70 mA, presented during the last 2 s of the CS. Freezing was defined as the absence of any movement other than that due to respiration. A contextual fear-retention session was performed 24 h after the training session (day 1). Animals were placed into the conditioning context for 3 min in the absence of tone and shock and the duration of freezing was measured. Extinction tests began on the second day and consisted of daily 3-min re-exposures of mice to the conditioning context in the absence of shock over 6 consecutive days. Each session served as the contextual memory test. Twenty-four hours after the end of the extinction sessions (day 8), a tone-dependent memory recall test was performed by exposing mice to tone for 3 min in a novel context (Tronson et al., 2008). This test was performed to exclude nonspecific effects of the manipulations on freezing to a nonextinguished conditioned stimulus (tone). Freezing, characterised by a lack of movement other than respiration and heart-beat, was used as an indicator of learning. Total freezing time was scored manually and converted to a percentage (calculated as the percentage of time the mice spent freezing during the 3-min context). All incidences of freezing behaviour were timed with a stopwatch by an experimenter blinded to the genotypes of the animals.

A separate group of animals from the wild-type and NCAM–/– group was used to measure and map neuronal activity in the brain regions that appeared to be involved in fear-conditioned memory functions. On the training day, these mice (wild-type, NCAM–/–; n=5 per group) received CS with US (shock) and 24 h later were re-exposed to the CS and the duration (s) of freezing behaviour was measured. Two hours later each mouse was tested for their freezing response to the conditioned tone in a modified chamber. Six hours after re-exposure to CS the brain tissue was harvested for immunohistochemical detection of FosB/ΔFosB. For both genotypes, animals unexposed to tone fear conditioning were used as naïve controls.

2.5. Tail-suspension test (TST)

The TST is similar to the forced-swim test and is based on the fact that mice suspended by the tail alternate between periods of struggle and immobility (reviewed in Cryan and Mombereau, 2004). Mice (n=8–10 per group) were suspended by the tail from a wooden beam, using adhesive tape at 1 cm from the tip of the tail. The total duration of immobility during the 6-min test period was measured. Immobility was defined as a complete lack of movement other than respiration. The immobility times were timed, using a stop-watch, by a trained observer who was blinded to the genotypes of the animals.

To test the effect of antidepressant treatment on the behaviour of wild-type, NCAM+/– and NCAM–/– mice in the TST, separate groups of animals were divided into two subgroups (n=8–10 per group) and one of the following was administered i.p.: vehicle or amitriptyline (10 mg ⁄kg). Two hours later, the animals were subjected to the TST.

2.6. Novelty-suppressed feeding test

Mice were weighed and the chow was removed from their cage. Twenty-four hours after the removal of chow, mice were transferred to the testing room and placed in a clean holding cage where they were allowed to habituate for at least 30 min. The testing apparatus consisted of a square wooden box (50 cm × 50 cm × 50 cm). The floor was covered with 2 cm of wooden bedding. A small piece of mouse chow was placed in the centre of the arena. At the start of the experiment, each mouse was placed in the corner of the testing area, and the time to the first feeding event was recorded. Animals that did not feed within this 5-min period were removed from all analyses. The latency to feed was timed, using a stop-watch, by a trained observer who was blinded to the genotypes of the animals.

2.7. Sucrose-preference test

To test for anhedonic behaviour, wild-type, NCAM+/– and NCAM–/– mice (n=6–7 per group) were subjected to a sucrose-preference test, which was conducted over 19 consecutive days. Each mouse was placed in a separate cage where it was given a free choice between two graduated bottles – one contained 0.8% sucrose solution and the other tap water. To prevent place preference for drinking, the position of the bottles was alternated every 24 h. No food or water deprivation was applied prior to testing. The consumption of both liquids was estimated at the same time each day by measuring the level of liquid in each bottle. The sucrose preference was calculated as the amount of sucrose solution consumed as a percentage of the total liquid consumed.

2.8. Taste-aversion test

Mice from all genotypes (n=6) were given a free choice between two graduated bottles, one containing a taste solution (100 mM HCl) and the other tap water. To prevent place preference for drinking, the position of the bottles was alternated every 24 h. The consumption of both liquids was estimated at the same time each day by measuring the level of liquid in each bottle. The taste aversion was calculated as the amount of taste solution consumed as a percentage of total liquid consumed.

3. Protein quantitation

3.1. Western blotting

Adult (3 months old) wild-type, NCAM+/– and NCAM–/– mice (n=6 per group) were weighted and euthanized by decapitation. The brains were removed from the skulls, weighted and placed on ice. The hippocampi and prefrontal/ frontal cortex were quickly removed on ice in a +4°C room and immediately placed into liquid nitrogen and stored at –80°C until further processing. Tissues were lysed in 10 vol RIPA 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, homogenised manually, incubated for 20 min on ice and then centrifuged (13,000 rpm for 20 min at +4°C).

The following mono-or polyclonal primary antibodies were used: mouse anti-β-actin (clone AC-74, 1:10000, monoclonal antibody 107K4791; Sigma-Aldrich Inc., MO, USA), mouse anti-SERT (1:1000; Chemicon International Inc., US), goat anti-pCaMKIIα (Thr286, 1:800; sc-12886; lot: 63007), rabbit anti-CaMKII (M-176, 1:800; sc-9035; lot: H1407), rabbit anti-pCaMKIV (Thr196, 1:800; sc-28443-R; lot: H1806), goat anti-CaMKIV (M-20, 1:800; sc-1546; lot: B1605), rabbit anti-pCREB-1 (1:1000; sc-101663; lot: D229), rabbit anti-CREB-1 (1:1000; sc-186; lot: 83374), mouse anti-FGF-2 (1:1000; sc-136255; lot: I1309) and mouse anti-NCAM (1:1000; 556324; lot: 83374), goat anti-PSA-NCAM (1:1000; c0019; lot: 1107). The secondary antibodies used were: anti-goat IgG (HRP-conjugated, 1:400; cat no: PI-9500, Vector Laboratories, UK), anti-mouse IgG (HRP-conjugated), anti-mouse IgM (HRP-conjugated) and anti-rabbit IgG (HRP-conjugated, 1:400; cat no: 32530, 31440 and 32460, respectively, Pierce, US). All proteins of interest were measured and after stripping with buffer (10× stock; Millipore, CA, US), β-actin was measured on the same membrane. The membranes were incubated with ECL detection reagent (ECL, Amersham, UK) for 5 min to visualise the proteins and were then exposed to an autoradiography X-ray film (Amersham hyperfilm ECL, UK). The blots were probed for the proteins of interest and the density of each band was then analysed and compared using the QuantityOne 710 System (BioRad).

The optical densities of the bands from the wild-type group were set to 100% and the data were expressed as a percentage of the wild-type ±SEM.

3.2. Immunoprecipitation

To determine the expression of the FGF receptor-1 (FGFR1) and its phosphorylated form (pFGFR1) in the hippocampus of wild-type, NCAM+/– and NCAM–/– mice, initially, the total protein concentration of hippocampal lysates was determined using the Lowry protein assay (BioRad DC Protein Assay Kit, BioRad, USA; bovine serum albumin was used as the standard control). Equal amounts of total protein (0.5 mg) from the lysate were incubated overnight at +4°C with 1.5 µg of rabbit anti-FGFR1 antibody (1.5 µg/µl; ab10646; lot: 622611), and the antigen-antibody complex was coupled with 25 µl of 50% solution of ProteinG Sepharose beads (GE Healthcare, USA) for 3 h at +4°C. The beads were centrifuged at 13,000 rpm for 5 min and washed three times using NP/T++ buffer (20 mM Tris-HCl (pH 6.8), 120 mM NaCl, 10% glycerol, 1% NP40, 0.5% TritonX-100, 0.3% Na-dodecylsulphate) containing phosphatase and protease inhibitors. Following the final centrifugation step, the washing buffer was removed and the pellet was resuspended in equal volumes of Western blotting loading buffer (20 mM Tris-HCl (pH 6.8), 4% Na-dodecylsulphate, 0.2% glycerol, 100 mM 1,4-dithiothreitol and bromophenol blue). The complexes were resolved by electrophoresis on 8% SDS-polyacrylamide gels. The proteins were transferred onto Hybond™-P PVDF transfer membranes (Amersham Biosciences, UK) in 0.1 M Tris-base, 0.192 M glycine and 20% (v/v) methanol using an electrophoretic transfer system. The membranes were blocked with 0.1% (w/w) Tween-20/TBS containing 5% (w/v) non-fat dried milk powder for 1 h at room temperature. After blocking, the membranes were incubated overnight at +4°C with rabbit anti-pFGFR1 (Y645, 1:5000; ab59194; lot: 464139) or rabbit anti-FGFR1 (1:5000; ab10646; lot: 622611, both antibodies purchased from AbCam, USA), followed by an incubation with a secondary antibody (anti-rabbit HRP-conjugated antibody, 1:2000, cat no: 32460, Pierce, USA) for 1 h at room temperature. The membranes were briefly incubated with ECL detection reagent (ECL, Amersham, RPN-2135, UK) to visualise the proteins, and they were then exposed to X-ray film. The blots were then analysed for optical density of each band using the QuantityOne 710 System (BioRad). The optical density ratios of the proteins were calculated and the ratio of phosphorylated protein to total protein was used for analysis. The ratio from the wild-type group was set to 100%, and the data were expressed as a percentage of the wild-type ±SEM.

4. Immunohistochemistry

4.1. FosB/ΔFosB immunohistochemistry

To determine whether conditioned fear-related neuronal activity was induced in NCAM-deficient mice, a separate group of animals of both genotypes (wild-type, NCAM–/–; n=5 each), which had been exposed to CS in the tone-induced fear-conditioning task, were used for FosB/ΔFosB immunohistochemistry. In addition, naïve wild-type (n=5) and NCAM-deficient mice (n=5) were used for immunohistochemical determination of FosB/ΔFosB and served as controls. Mice were deeply anaesthetized with chloral hydrate (300 mg/kg, i.p.) and transcardially perfused with 0.9% saline and then with 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, 40-µm-thick sections were cut on a vibromicrotome (Leica VT1000S, Germany), placed in PBS and stored at +4°C until further processing.

For FosB immunohistochemistry, free-floating sections were incubated in 0.3% H_2O_2 in PBS for 30 min followed by incubation in a blocking solution for 1 h. Blocking was followed by 24 h incubation at room temperature with rabbit polyclonal anti-FosB/ΔFosB (Santa Cruz Biotechnology Inc., Germany; 1:100 dilution) diluted in blocking solution. Following washes in PBS, sections were incubated in biotinylated goat anti-rabbit antibody (Vector Laboratories, UK; 1:200 dilution) diluted in blocking solution for 1 h. FosB-positive cells were visualized using the peroxidase method (ABC system and diaminobenzidine as the chromogen, Vector Laboratories, UK). The sections were dried, cleared with xylol and coverslipped with mounting medium (Vector Laboratories, UK).

4.2. Quantification of FosB/ΔFosB-positive cells

FosB/ΔFosB-positive nuclei were counted in the following brain areas according to demarcations provided by the Brain Mouse Atlas (Paxinos and Franklin, 2001): prefrontal cortex (PFC) and frontal cortex (FC), dentate gyrus of hippocampus (DG), basolateral nucleus of amygdala (BLA), basomedial nucleus of amygdala (BMA) and piriform cortex (Pir). For each structure, four random sections per animal were selected and positively stained nuclei were counted manually according to the optical fractionation method (West, 1993), and the number of counting frames in the delineated region was applied randomly by the 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. The experimenter was blinded to the experimental groupings at all stages of the assessment.

5. Data analysis and statistics

All behavioural data are presented as the mean ±SEM. The immuno-precipitation and western blotting data are expressed as a percentage of the wild-type ±SEM. Data were analysed using Prism software version 5.0 (GraphPad Software Inc., San Diego¸CA). Statistical analyses were performed using Student's t-tests and one-way ANOVAs or two-way ANOVAs followed by the Bonferroni post-hoc test, where appropriate. A p-value of less than 0.05 was considered significant.

RESULTS

1. Phenotype in mice with a partial or complete deficiency of NCAM protein

1.1. Expression of PSA-NCAM and NCAM (180, 140, 120 kD) proteins in hippocampi of wild-type, NCAM+/– and NCAM–/– mice

Western blotting was performed to measure the quantity of NCAM isoforms and PSA-NCAM in hippocampal lysates from wild-type, NCAM+/– and NCAM–/– mice. There was a 50% reduction in the immunoreactivity of all NCAM isoforms and PSA-NCAM in the hippocampus of NCAM+/– mice compared with wild-type mice. The tissue from NCAM–/– mice did not demonstrate any NCAM or PSA-NCAM immunoreactivity (Figure 1).

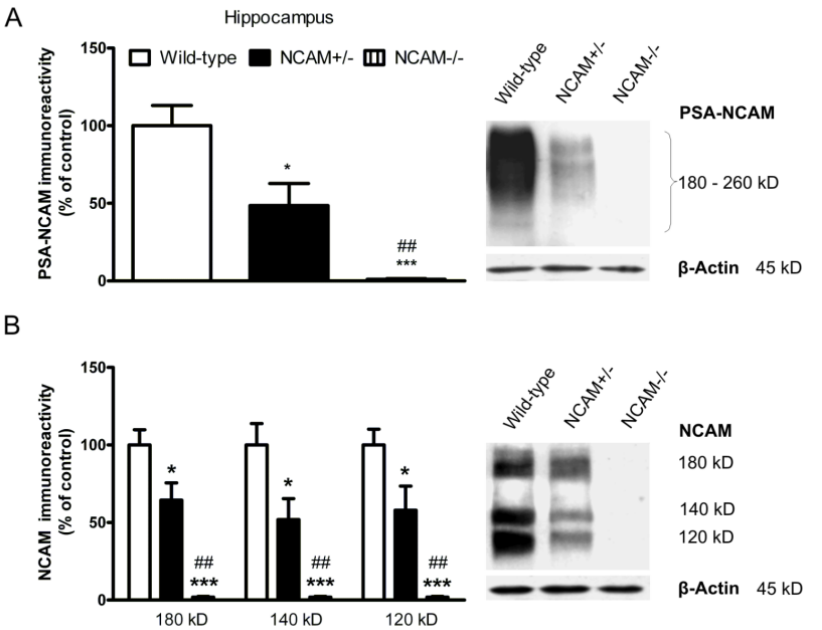

Figure 1. Expression of PSA-NCAM (panel A) and NCAM (180, 140 and 120 kD) proteins (panel B) in the hippocampi of wild-type, NCAM+/– and NCAM–/– mice. The data are expressed as a percentage of the wild-type ±SEM (n=6). *p<0.05; ***p<0.001 compared with wild-type mice; ##p<0.01 compared with NCAM+/– mice (one-way ANOVA followed by Bonferroni post-hoc tests).

1.2. Behaviour in mice with a partial or complete deficiency of NCAM protein

1.2.1. Tail-suspension, novelty-suppressed feeding and sucrose-preference tests

To evaluate the ability to cope with stress and determine anhedonic behaviour in animals with a partial or complete deficiency of NCAM protein, we employed the tail-suspension test (TST), the novelty-suppressed feeding test and the sucrose-preference test. As shown in Figure 2A, the NCAM+/– and NCAM–/– mice spent a significantly longer time immobile in the tail-suspension test than their wild-type littermates. A one-way ANOVA demonstrated a highly significant genotype effect: $p<0.0001$, $F=10.57$, d.f.=27 (n=8–10). Post-hoc analyses showed a significant increase in immobility time in both NCAM+/– ($p<0.01$) and NCAM–/– ($p<0.001$) mice (Figure 2A). To rule out the possibility that the longer period of immobility in the TST was due to impaired locomotion, we measured general locomotor activity. A one-way ANOVA revealed an effect of genotype: $p<0.05$, $F=4.63$, d.f.=28 (n=9–10). Post-hoc analyses revealed that NCAM–/– mice were more active ($p<0.01$) whereas NCAM+/– mice showed similar locomotor activity compared with wild-type animals (Figure 2B).

Animals were next treated with the nonselective monoamine re-uptake inhibitor amitriptyline (10 mg/kg, i.p.) and tested for immobility in the TST. Two hours after administering the agent, the TST was performed. Amitriptyline significally reduced immobility time in all groups of animals (Table 1). However, a two-way ANOVA revealed an effect of genotype: ($F_{2,50} = 15.80$, $p <0.0001$), effect of amitriptyline ($F_{1,50} = 51.62$, $p<0.0001$) and an interaction between genotype and treatment ($F_{2,50} = 3.75$, $p= 0.03$). These data suggest that the effect of amitriptyline is more pronounced in mice with a deficiency in NCAM.

The novelty-suppressed feeding test (NSF) is thought to assess emotional reactivity toward a new environment and induces competition between motivational states (drive to eat vs. fear of venturing into the centre of the test arena). As shown in Figure 2C, the NCAM+/– and NCAM–/– mice demonstrated a significantly longer latency to feed than their wild-type littermates (one-way ANOVA, $p<0.05$, $F=3.90$, d.f.=19, n=6–7). The enhanced effect of novelty on the suppression of feeding behaviour in NCAM+/– and NCAM–/– mice appears to be unrelated to food consumption as the mutant mice had similar body weights to their wild-type littermates (Figure 2D) and consumed similar amounts of food in their home cages (data not shown).

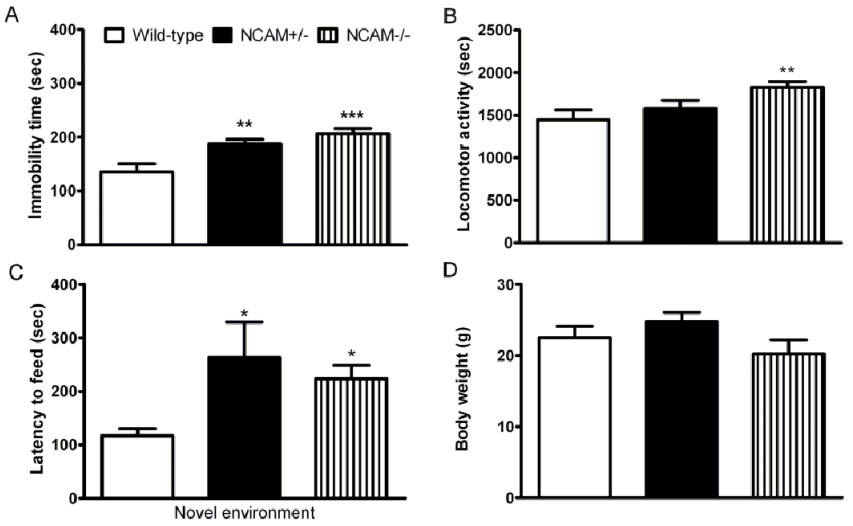

Figure 2. Length of immobility in the tail suspension test (panel A), locomotor activity (panel B), latency to feed in novelty suppressed feeding test (panel C) and body weight (panel D) of wild-type, NCAM+/– and NCAM–/– mice. The data are expressed as mean ±SEM (n=8–10). *p<0.05; **p<0.01; ***p<0.001 compared with wild-type mice (one-way ANOVA, followed by Bonferroni post-hoc test).

Table 1. The effect of acute administration of amitriptyline (10 mg/kg, i.p.) on the immobility time in the TST in wild-type, NCAM+/– and NCAM–/– mice.

Group	Vehicle	Amitriptyline	Reduction of immobility time (%)
Wild-type	132.80 ± 10.60	100.50 ± 6.74$^{\#}$	24.32 ± 7.34
NCAM +/–	168.70 ± 3.78**	123.00 ± 4.92$^{\#\#}$	27.09 ± 3.12
NCAM–/–	206.90 ± 8.81***	126.90 ± 16.47$^{\#\#\#}$	38.66 ± 8.62

The data are expressed as the mean ±SEM (n=8–10). **p<0.01 and ***p<0.001 compared with wild-type animals; #p<0.05, ##p<0.01 and ###p<0.001 compared with vehicle controls (two-way ANOVA followed by the Bonferroni post-hoc test).

To test whether the ability to experience pleasure (anhedonia) was reduced in the mutant animals, wild-type, NCAM+/– and NCAM–/– mice were subjected to a sucrose-preference test, which is commonly used to detect motivational deficits (Strekalova et al., 2004; Rygula et al., 2005). The sucrose-preference test was performed over a period of 19 days. Control mice demonstrated a clear preference for the sucrose solution as it comprised ~75–80% of the total liquid consumed. In contrast, the NCAM+/– and NCAM–/– mice showed decreased preference for sucrose (Figure 3). A two-way ANOVA with repeated measures

revealed a significant effect of genotype in NCAM +/– and NCAM–/– mice ($F_{1.216}=16.33$, $p<0.01$ and $F_{1.144}=58.13$, $p<0.0001$, respectively) but no significant effect of time ($F_{1.216}=0.88$ and $F_{1.144}=0.68$, respectively) and no interaction ($F_{1.216}=1.27$ and $F_{1.144}=0.16$, respectively). Total fluid consumption did not differ between the wild-type and mutant mice (Figure 3).

As the taste receptors in taste buds develop in the microenvironment supported by NCAM (Miura et al., 2005), the reduced preference for sucrose consumption may have resulted from a global impairment in taste cell and taste receptor development due to the reduced level of NCAM. To exclude this possibility, we performed a taste aversion test. The animals were given a free choice between water and 100 mM HCl. In preliminary experiments, we determined that wild-type animals began to avoid consuming bitter solutions containing HCl at concentrations of 100 mM, and therefore, we used this concentration of HCl to assess taste aversion in NCAM+/– or NCAM–/– mice. All groups demonstrated clear avoidance of the 100 mM HCl solution and consumed 9.4±0.9%, 10.1±1.2% or 8.9±1.8% HCl solution of total, respectively.

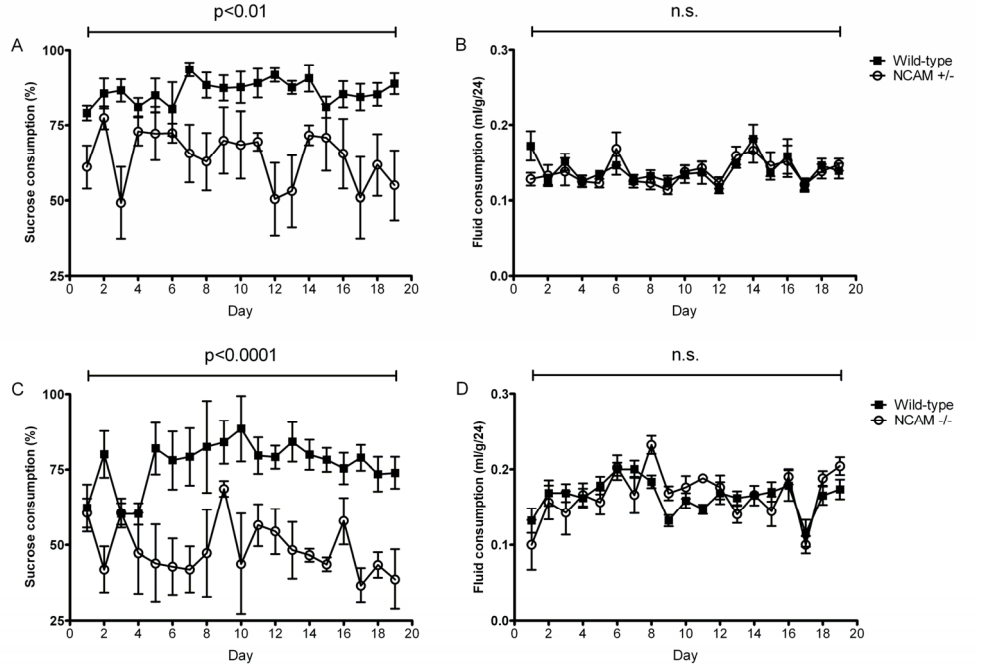

Figure 3. Preference for sucrose and the total fluid consumption of NCAM+/– (panel A and B) and NCAM–/– (panel C and D) mice compared with wild-type mice in the sucrose-preference test. The data are expressed as the mean ±SEM (n=7). $p<0.01$, $p<0.0001$ (derived from repeated measures two-way ANOVAs followed by Bonferroni post-hoc tests).

1.2.2. Object-recognition test

To evaluate learning and memory functions in NCAM+/– and NCAM–/– mice, the object-recognition test was performed. In the object-recognition test, no group (NCAM+/–, NCAM–/– mice or their wild-type littermates) showed any significant preference for either of the two identical objects during the training phase (data not shown). During the test phase, for animals from all test-groups were presented with novel objects at 2 h (short-term memory, STM) and at 24 h (long-term memory, LTM) after the training phase. During STM and LTM phases, wild-type and NCAM+/– animals spent significantly more time exploring the new objects as shown by their preference ratio (Figure 4). No preferences for the novel object was observed for homozygote NCAM-deficient mice in either the STM or LTM test (Figure 4).

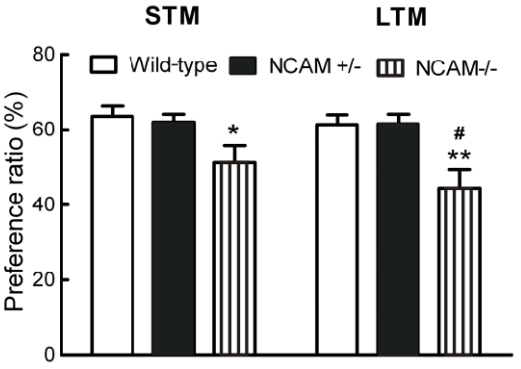

Figure 4. Short-term memory- (STM) or long-term memory (LTM)-mediated exploratory preference (%) in the novel object-recognition test. The data are expressed as mean ±SEM (n=7). *p<0.05; **p<0.01 compared with wild-type animals; #p<0.05 compared with NCAM+/– mice (one-way ANOVA followed by Bonferroni post-hoc tests).

1.2.3. Fear conditioning and extinction

To further explore cognitive functions and the ability to extinguish negative memories in NCAM+/– and NCAM–/– mice, the animals were subjected to tone and contextual fear-conditioning and contextual fear-extinction tests. In the tone and contextual fear-conditioning test, the time spent freezing at baseline prior to the test was similar between all genotypes (data not shown). Twenty-four hours after fear conditioning (day 1), the animals were placed in the same context without tone or foot shock and the freezing behaviour was measured. A one-way ANOVA revealed a significant effect of genotype: $p<0.05$, $F=4.41$, d.f.=23 (n=8) and post-hoc analyses revealed that NCAM–/– mice showed significantly less freezing ($p<0.05$) compared with wild-type animals. The freezing time of NCAM+/– mice did not differ from that of wild-type mice

indicating that a partial deficiency in NCAM level does not affect contextual fear learning (Figure 5A).

On the following day after testing animals for the retention of contextual fear memory, the extinctions sessions were initiated and they continued for 6 consecutive days. Wild-type mice efficiently extinguished fear memory as demonstrated by the reduction in freezing time over the 6-day-testing period, whereas mice with a partial or full deficiency in NCAM protein showed significantly reduced extinction of contextual fear as revealed by the continued increased level of freezing behaviour during the extinction sessions (Figure 5A). A two-way ANOVA with repeated measures revealed a significant effect of genotype ($F1.105=3.96$, $p<0.05$), a significant effect of time ($F1.105= 3.52$, $p<0.01$) and no interactions ($F1.105= 0.84$, $p<0.6$). Following extinction training (on the 8th day), we performed a tone-induced fear recall test. Freezing behaviour was measured after exposing the mice to the tone in a novel context. The freezing time in the novel context prior to the recall test was similar between groups (Figure 5B). A one-way ANOVA revealed a significant effect of genotype ($p<0.05$, $F=3.99$, d.f.=23 (n=8)) in the tone-induced fear-recall test, and post-hoc analyses revealed that NCAM–/– mice showed significantly less freezing ($p<0.05$) compared with wild-type animals whereas NCAM+/– mice froze for similar periods of time compared with wild-type mice (Figure 5B).

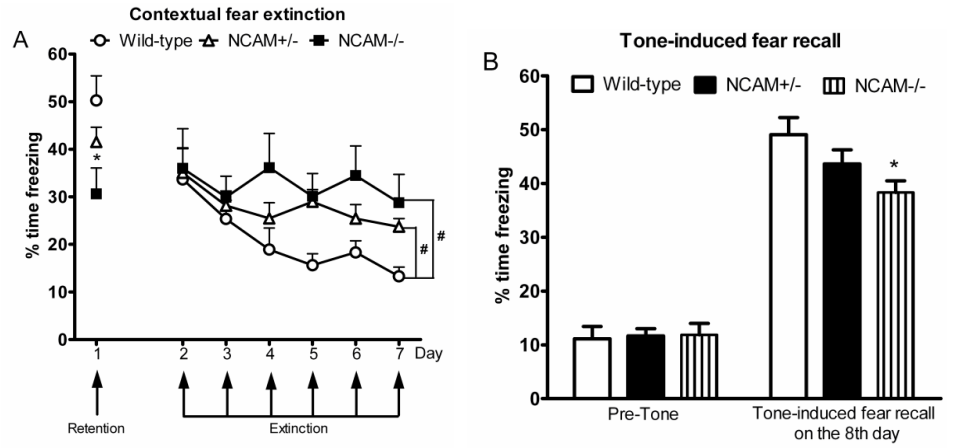

Figure 5. Contextual fear retention, contextual fear extinction (panel A) and tone-induced fear recall (panel B) in wild-type, NCAM+/– and NCAM–/– mice. The data are expressed as the mean ±SEM (n=8). *$p<0.05$ compared with wild-type animals (one-way ANOVA followed by a Bonferroni post-hoc test); #$p<0.05$ compared with wild-type animals (repeated measures two-way ANOVA followed by Bonferroni post-hoc tests).

2. Influence of impaired cognition in NCAM–/– mice on their behaviour

2.1. Behaviour of NCAM-deficient mice in the EPM test

We tested NCAM–/– mice for levels of anxiety using the plus-maze test. In this test, NCAM–/– mice demonstrated an increased percentage of entries into open arms and an increased percentage of time spent in the open arms of the plus-maze during the 5 min test compared with wild-type animals (Figure 6). They also showed a higher number of total entries into the arms of the EPM. These data suggest that NCAM–/– mice have lower innate anxiety than their wild-type littermates.

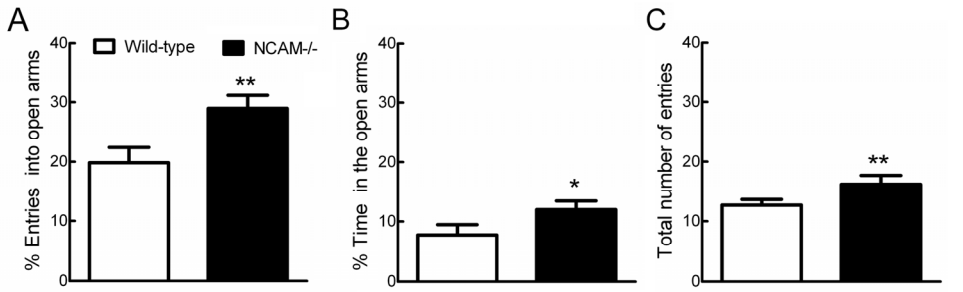

Figure 6. Behaviour of NCAM-deficient mice and their wild-type littermates in the elevated plus-maze test. Percentage of entries into open arms (A); percentage of time spent in the open arms (B) and total number of entries (as a measure of locomotion, C) were scored. Data are expressed as the mean ±SEM (n=10).*$p<0.05$; **$p<0.01$; (Student's t-test) compared with wild-type mice.

2.2. Behaviour of NCAM-deficient mice in the EPM over time and following retesting

Previous studies have suggested that a rapid learning of the aversive properties of the open arms in the EPM occurs during a 5 min test period that may affect the performance of animals (Carobrez and Bertoglio, 2005). To test such a possibility, we compared the percentage of entries into the open arms of the plus-maze on a minute-by-minute basis and found that in wild-type mice, the percentage of entries into the open arms was high during the first minute of the test but then dropped dramatically throughout the following four minutes of the 5-min test (Figure 7). Thus, in wild-type mice, the test situation led to a gradual increase in avoidance behaviour and the decision to remain in the safer part of the maze, the enclosed arms, and suggests that some learning may have occurred during the testing session. In contrast, NCAM–/– mice demonstrated a stable and high percentage of entries throughout the 5 min test period (Figure 7). No significant effect of the genotype on the total number of entries at each

time point during the 5-min session was found. Previous studies have demonstrated that re-exposure of rats to the EPM results in a dramatic reduction in percentage of entries into the open arms, which represents an avoidance-learning response to open arms (Bertoglio et al., 2000). Therefore, in our experiments, we re-exposed NCAM–/– mice and their wild-type littermates to the EPM. The results of this study are shown in Figure 8. A two-way ANOVA of the percentage of entries revealed a significant effect of genotype ($F_{1.36}=280.7$, $p<0.001$) and a significant effect of trial ($F_{1.36}=10.0$, $p<0.01$) but no significant interaction between genotype and trial ($F_{1.36}=3.8$, $p=0.059$). Post-hoc analyses revealed that the re-exposure to the EPM reduced the percentage of entries only in wild-type mice and not in NCAM–/– mice (Figure 8). An analysis of the percentage of time spent in the open arms also demonstrated a significant effect of genotype ($F_{1.36}=280.7$, $p<0.001$) and a significant effect of trial ($F_{1.36}=9.9$, $p<0.05$) but no significant interaction between genotype and trial ($F_{1.36}=3.8$, $p=0.06$). Re-exposure of the animals to the EPM did not affect the total number of entries to both open and closed arms compared with the first trial (Figure 8). However, there was a significant effect of genotype ($F_{1.36}=146.4$, $p<0.001$) suggesting that NCAM–/– mice had higher locomotor activity compared with their wild-type littermates.

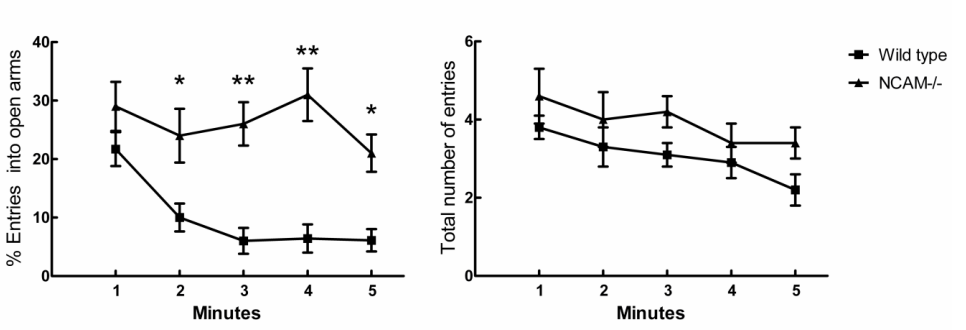

Figure 7. Behaviour of NCAM-deficient mice and their wild-type littermates on the elevated plus-maze test on a minute-by-minute basis. The figure shows the percentage of entries into the open arms (left) and the number of total entries (right). Data are expressed as the mean ±SEM (n=10) *p<0.05; **p<0.01; (Student's t-test) compared with wild-type mice.

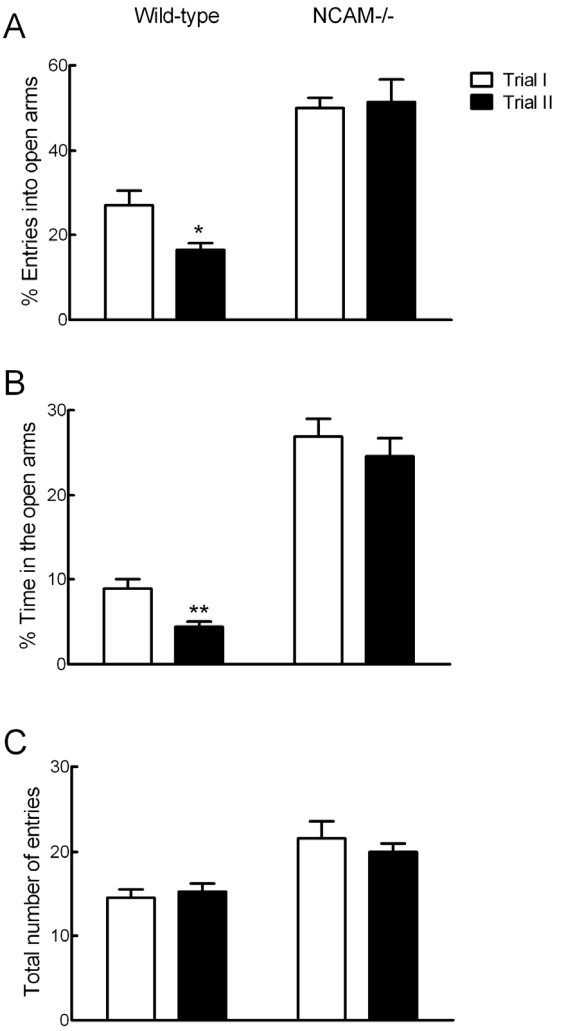

Figure 8. Behaviour in the elevated plus-maze following re-testing of NCAM-deficient mice and their wild-type littermates. Percentage of entries into open arms (A); percentage of time spent in the open arms (B) and total number of entries (as a measure of locomotion, C) in test (Trial I) and re-test (Trial II) are shown. Data are expressed as the mean ±SEM (n=10).*p<0.05; **p <0.01; (two-way ANOVA followed by Bonferroni post-hoc tests) comparing performances from Trial I and Trial II.

2.3. FosB/ΔFosB expression in wild-type and NCAM-deficient mice

We next evaluated the distribution of FosB/ΔFosB-positive cells in different brain regions of control mice and mice that had been exposed to tone fear-conditioning. Mice with a complete deficiency of NCAM showed a signi-

ficantly higher number of cells expressing FosB/ΔFosB in the PFC, FC and BLA. A two way-ANOVA revealed a significant effect of genotype (PFC: $p<0.01$, $F1.16=57.03$; FC: $p<0.01$, $F1.16=61.45$; BLA: $p<0.05$, $F1,16=7.336$), a significant effect of tone fear conditioning (PFC: $p<0.01$, $F1.16=124.1$; FC: $p<0.01$, $F1.16=110.0$; BLA: $p<0.05$, $F1,16=407.3$) and a significant interaction between genotype and tone fear conditioning (PFC: $p<0.01$, $F1.16=121.4$; FC: $p<0.01$, $F1.16=107.6$; BLA: $p<0.05$, $F1,16=473.3$). Bonferroni post-hoc tests revealed an effect of tone fear conditioning on the number of FosB/ΔFosB-positive nuclei only in control mice and not in NCAM–/– mice (Figure 9, Table 2). There were no differences in the number of FosB/ΔFosB-positive cells between wild-type and NCAM–/– mice in the BMA, DG or Pir (Figure 9, Table 2). Re-exposure of wild-type mice that had been conditioned for fear to the CS induced an increase in the number of FosB/ΔFosB-positive cells in the PFC ($p<0.001$), FC ($p<0.01$) and BLA ($p<0.001$). Re-exposure of NCAM–/– mice to the conditioned stimulus did not affect FosB/ΔFosB cell counts in any structure studied (Figure 9, Table 2).

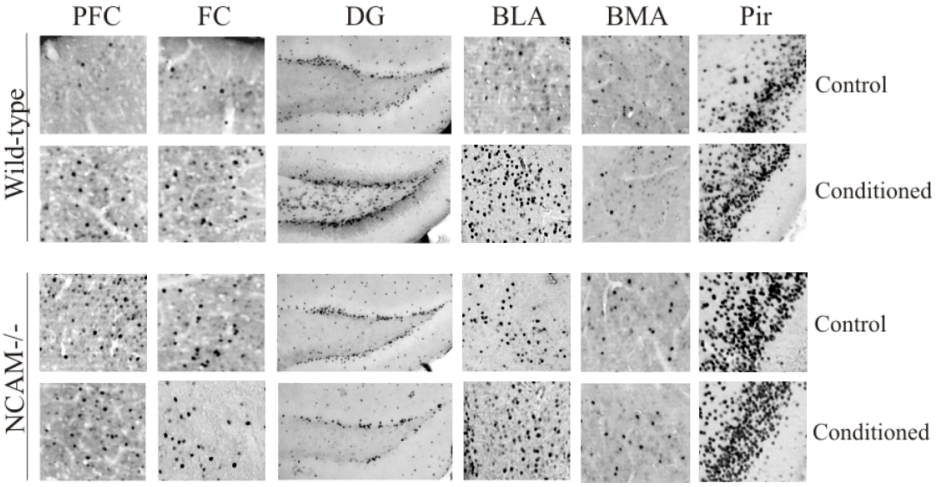

Figure 9. Representative microphotographs demonstrating FosB/ΔFosB-positive cells in wild-type and NCAM-deficient mice 6 h after the presentation of CS in the tone fear-conditioning task in the following brain regions: prefrontal cortex (PFC), frontal cortex (FC), dentate gyrus of hippocampus (DG), basolateral nucleus of amygdala (BLA), basomedial nucleus of amygdala (BMA) and piriform cortex (Pir). During training, mice received CS (tone) with US (shock) and 24 h later, they were tested for freezing behaviour, and six hours after re-exposure to CS, the brain tissue was harvested for immunohistochemical detection of FosB/ΔFosB. Animals unexposed to fear conditioning were used as naïve controls for both genotypes (magnification 100× for PFC, FC, BLA BMA and Pir; magnification 40× for DG).

Table 2. Number of FosB/ΔFosB-positive cells in brain regions of NCAM-deficient and wild-type mice that had been exposed to the conditioned stimulus (CS) in the tone fear-conditioning task.

	Wild-type		NCAM –/–	
Brain region	Control	Conditioned	Control	Conditioned
PFC	2.2 ± 0.2	20.1 ± 1.2 ***	5.0 ± 0.7 ###	5.1 ± 0.8
FC	2.5 ± 0.8	20.6 ± 1.4 ***	4.7 ± 0.4 ###	4.8 ± 0.5
DG	15.5 ± 2.6	15.2 ± 0.5	14.2 ± 2.2	15.2 ± 0.2
BLA	7.5 ± 0.5	28.3 ± 0.4 ***	17.5 ± 0.7 #	17.4 ± 0.9
BMA	18.9 ± 1.8	18.6 ± 0.2	17.9 ± 2.8	18.9 ± 0.2
Pir	15.6 ± 1.4	15.4 ± 0.5	18.6 ± 3.2	19.1 ± 0.7

The numbers of FosB/ΔFosB-positive nuclei were counted in the following brain areas: prefrontal cortex (PFC) and frontal cortex (FC), dentate gyrus of hippocampus (DG), basolateral nucleus of amygdala (BLA), basomedial nucleus of amygdala (BMA) and piriform cortex (Pir). The data are expressed as the mean ±SEM of the number of positive cells per 0.1 mm^2.
Each group consisted of 5 mice. ***$p<0.001$ compared with corresponding control; #$p<0.05$, ###$p<0.001$ compared with wild-type mice from the control group (two-way ANOVA, Bonferroni post-hoc test).

3. The effect of partial or complete deficiency of NCAM on NCAM-related pathways

3.1. Reduced FGFR 1 phosphorylation and FGF-2 levels

The FGFR1 is the major interaction partner for NCAM and PSA-NCAM (Doherty and Walsh, 1996; Kiselyov et al., 2003; reviewed in Kiselyov et al., 2005). Therefore, it was of interest to determine whether a partial deficiency in NCAM proteins could lead to an alteration in FGFR1 phosphorylation. We measured the levels of total FGFR1 and its phosphorylated (pFGFR1) form in the hippocampus of NCAM+/– mice. NCAM–/– mice were again used for comparison in these experiments. Both the NCAM+/– and NCAM–/– mice demonstrated reduced levels of pFGFR1 in hippocampal tissue (one-way ANOVA, $p<0.0001$, $F=22.96$, d.f.=17, Figure 10). Homozygous NCAM–/– mice showed a much greater reduction (65–70%) in the basal phosphorylation level of FGFR1 than heterozygous NCAM+/– mice, which showed approximately 30% reduction from control levels (100%). No change in total FGFR1 protein level was found in either NCAM+/– or NCAM–/– mice compared with control animals (Figure 10).

As basic fibroblast growth factor 2 (FGF-2) is an important activator of FGFR1 phosphorylation (Plotnikov et al., 1999; Frinchi et al., 2008), we also measured the levels of FGF-2 in the hippocampus of NCAM+/– and NCAM–/–

mice and their wild-type littermates. The results revealed a significant increase in the levels of FGF-2 in both NCAM+/– and NCAM–/– mice compared with wild-type mice (Figure 10).

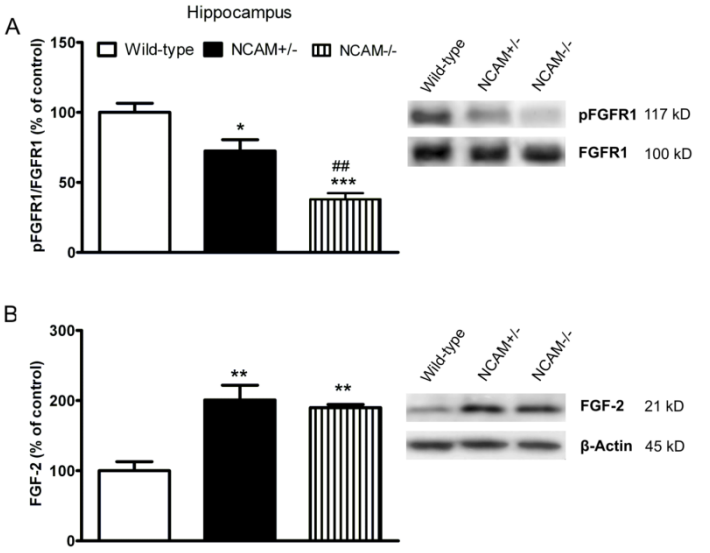

Figure 10. The levels of phosphorylated and total FGFR1 proteins (panel A) and the levels of FGF-2 (panel B) in the hippocampi of wild-type, NCAM+/– and NCAM–/– mice. The data are expressed as a percentage of the wild-type ±SEM (n=6). *p<0.05; **p<0.01; ***p<0.001 compared with wild-type animals; ##p<0.01 compared with NCAM+/– mice (one-way ANOVA followed by Bonferroni post-hoc tests).

3.2. The expression of phosphorylated CaMKII, CaMKIV and CREB

A previous study (Aonurm-Helm et al., 2008) showed reduced levels of phosphorylated CREB and pCaMKII and pCaMKIV in the hippocampus of NCAM–/– mice, suggesting that this dysregulation may underlie the observed behavioural phenotype in NCAM–/– mice. Therefore, the levels of phosphorylated CaMKII, CaMKIV and CREB proteins in NCAM+/– mice were measured. NCAM–/– mice were again used for comparison in these experiments. No differences in the immunoreactivity for total and phosphorylated CaMKII, CaMKIV or CREB were found in the hippocampi of NCAM+/– mice compared with wild-type mice (Figure 11).

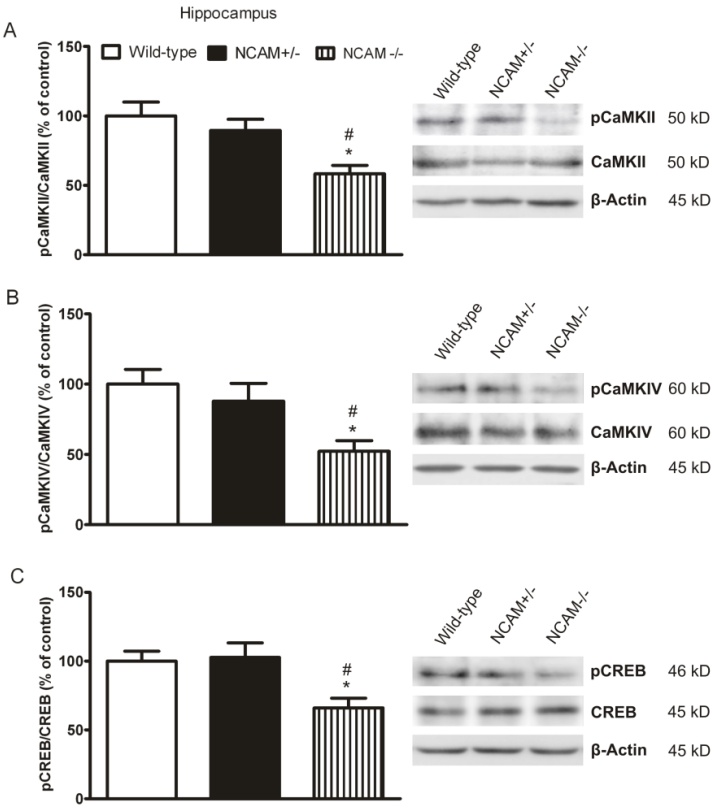

Figure 11. The levels of phosphorylated and total CaMKII (panel A), CaMKIV (panel B) and CREB (panel C) in the hippocampi of wild-type, NCAM+/– and NCAM–/– mice. The data are expressed as a percentage of the wild-type ±SEM (n=6). *p<0.05 compared with wild-type animals; #p<0.01 compared with NCAM+/– mice (one-way ANOVA followed by Bonferroni post-hoc tests).

4. The effect of partial or complete deficiency in NCAM on the serotonergic system

The serotonergic system in the brain may be involved at several levels in depression pathways and depression-related behaviour (Lucki, 1998). The 5-HT transporter (SERT), which terminates the action of 5-HT by facilitating its reuptake into the presynaptic terminals (Blakely et al., 1994) plays a key role in serotonergic function. Therefore, it was of interest to determine whether SERT expression was affected by the reduced expression of NCAM.

4.1. The expression of SERT

Western blotting of SERT revealed two bands of 70 kD and 63–65 kD. Both bands were associated with SERT in the mouse brain, and both were significantly lower in the hippocampal and in prefrontal/frontal tissues of NCAM+/– and NCAM–/– mice compared to those in the wild-type littermates (in hippocampus: 70 kDa one-way ANOVA, $p<0.0001$, $F=52.06$, d.f.=18; 63–65 kD one-way ANOVA, $p<0.001$, $F=7.48$, d.f.=18; in prefrontal/frontal cortex 70 kDa one-way ANOVA, $p<0.0001$, $F=107.4$, d.f.=18; 63–65 kD one-way ANOVA, $p<0.0001$, $F=26.71$, d.f.=18; Figure 12).

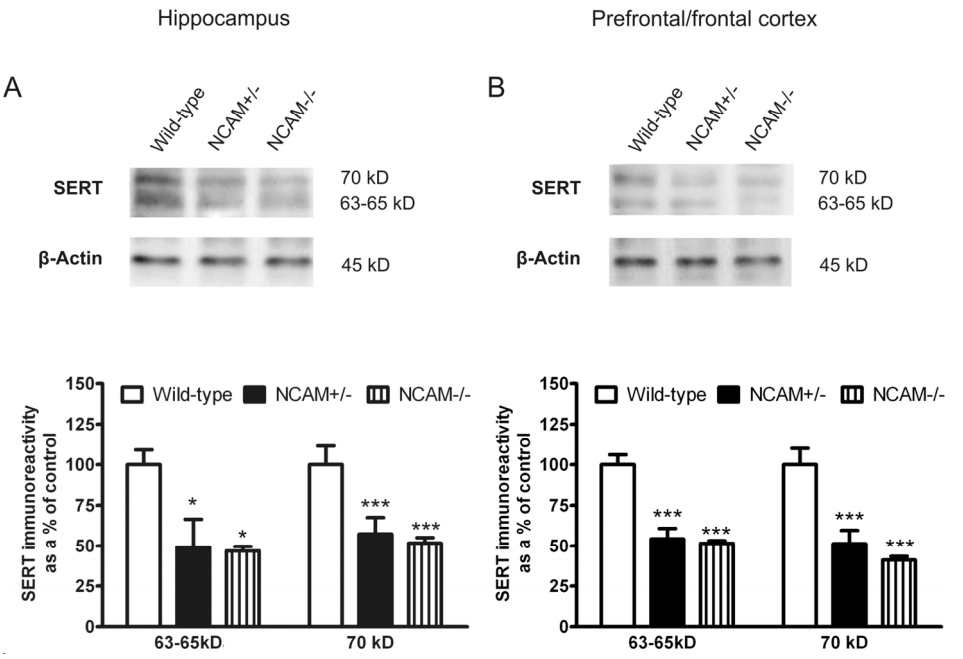

Figure 12. The expression of SERT protein in the hippocampi (panel A) and prefrontal/frontal cortex (panel B) of wild-type, NCAM+/– and NCAM–/– mice. The data are expressed as a percentage of the wild-type ±SEM (n=6). *p<0.05; ***p<0.001 compared with wild-type animals (one-way ANOVA followed by Bonferroni post-hoc tests).

DISCUSSION

1. Phenotype in mice with a partial or complete deficiency of NCAM protein

1.1. The behavioural phenotype in mice with a partial or complete deficiency of NCAM protein

The results of the present study show that mice with a partial deficiency of NCAM demonstrate increased immobility time in the TST, reduced sucrose preference in the sucrose preference test, increased latency in the novelty-suppressed feeding test, and reduced extinction of contextual fear conditioning. The data shown here demonstrate that similar behavioural changes occur in homozygous knockout (NCAM–/–) mice. Our experiments also demonstrated that the known antidepressant amitriptyline decreased TST immobility times in NCAM +/– and NCAM–/– mice similar to wild-type mice. On the basis of the observed phenotype, we propose that these animals have increased stress-induced anxiety, reduced ability to cope with stress, exhibit anhedonia and retain aversive emotional memories. Therefore, this behavioural phenotype could be described as a depression-related phenotype.

In the TST test, immobility behaviour represents "behavioural despair", which is based on observations that after initial escape-oriented movements, animals develop immobile postures when they are in an inescapable and stressful situation. Recent analysis has demonstrated that interventions that are known to cause susceptibility to depression or induce depression in humans also induce a depression-like effect (increased immobility) in the TST (reviewed in Cryan et al., 2005). Similarly, novelty-suppressed feeding behaviour reflects the reaction of animals to stress, and the face validity of this test has been demonstrated in several behavioural models of depression (Santarelli et al., 2003; Uchida et al., 2011). In our experiments, all genotypes showed similar body weights and consumed the same amount of food in their home cages. Thus, we propose that the increased latency to consume food in the novel environment is related to levels of anxiety rather than changes in appetite. The sucrose-preference test has also been used extensively to measure anhedonia, which is a core symptom of depression. It should be noted that the reduced preference for sucrose consumption may have resulted from a global impairment in the development of taste cells and receptors due to the deficiency in NCAM (Miura et al., 2005). We tested this latter possibility using bitter solutions containing HCl and demonstrated clear aversion in all wild-type, NCAM+/– and NCAM–/– animals. However, the possibility that taste receptor functions were affected by the reduction in NCAM and PSA-NCAM cannot be fully excluded.

Another important finding in our study is that NCAM+/– mice were not impaired in the novel object-recognition task and fear conditioning task. In

contrast, NCAM–/– mice demonstrated impaired memory retention in both tasks, which confirms previous studies (Stork et al., 2000; Senkov et al., 2006). Previous studies have demonstrated that acquisition of the context during contextual fear conditioning reflects associative memory functions (Wellman et al., 2007; Tronson et al., 2008). Rats with depression-like behaviour induced by prenatal stress (Green et al., 2011) or rats predisposed to learned helplessness (Shumake et al., 2005) showed reduced extinction of contextual fear. In contrast, animals with an antidepressant-like phenotype due to deficiency of ERK-1 kinase demonstrated enhanced extinction of contextual fear (Tronson et al., 2008). It appears that impaired fear extinction rather than the formation of fear memory is related to the depression-like state (Heldt et al., 2007; Wellman et al., 2007). In our experiments, both NCAM+/– and NCAM–/– demonstrated a reduced ability to extinguish contextual fear memory, and these data support our proposal that even partial reduction in NCAM proteins induces depression-related behaviour without an impairment in memory acquisition and recall, at least in the cognitive tests that were employed in our study. Our data are in accordance with previous studies that demonstrated greater inter-male aggression with a post-test increase in corticosterone plasma concentrations in NCAM+/– mice (Stork et al., 1997, 1999).

As NCAM–/– mice demonstrated impaired memory retention in the fear conditioning task, we used a separate group of animals to measure and map neuronal activity in the brain regions that are involved in memory functions. Abundant evidence suggests that the expression of families of immediate early genes, such as fos, fra, jun, krox and zif, is critical in changing the expression of genes important for memory formation (Dragunow et al., 1989; Hall et al., 2001; Moller et al., 1994, Tischmayer and Grimm, 1999). In contrast to c-fos, which is transiently induced upon stimulation, FosB and its truncated form ΔFosB, which is generated by alternative splicing of the FosB transcript, have prolonged induction characteristics and may be markers of chronic neuronal activation (Chen et al., 1997). Our experiments showed that re-exposure of wild-type mice to the CS (tone) induced expression of FosB/ΔFosB in discrete neuronal populations within the PFC, FC and BLA and confirmed that FosB/ΔFosB is also induced by memory retrieval in regions that are related to the formation of fear memories (Hall et al., 2001). In contrast, fear conditioning in NCAM–/– mice did not induce FosB/ΔFosB expression in the BLA, PFC or FC. Furthermore, NCAM–/– mice, under basal conditions, demonstrated significantly higher numbers of FosB/ΔFosB-positive cells in the BLA, PFC and FC compared with wild-type littermates. Assuming that FosB/ΔFosB serve as relatively stable indicators of neuronal activity (Chen et al., 1997), our data suggest that in NCAM-deficient mice, structures involved in fear memory formation are constantly activated and this activation interferes with their ability to form new fear-related memories.

Taken together, our data shows that homozygous NCAM knock-out mice demonstrate depression-like behaviour and altered cognitive functions.

Structures involved in fear memory formation seem to be hyperactive under basal conditions and this hyperactive state might interfere with ability of mice to form new memories. Mice with a partial reduction in NCAM protein levels demonstrate a depression-like behaviour without alterations in cognitive functions.

2. Anxiety and cognition of NCAM–/– mice

2.1. Learning and anxiety in NCAM–/– mice on the EPM task over time and in a test/retest paradigm

Previous studies have shown that NCAM is required for the establishment of durable memories (Roullet et al., 1997) and that NCAM–/– mice show abnormalities in cell migration and synaptic plasticity, which leads to memory deficits and impaired learning ability (Cremer et al., 1994). Our data also demonstrates that NCAM–/– mice have impaired cognitive functions, as evidenced by the object-recognition and contextual and tone fear-conditioning tests. These results corroborate earlier studies in which NCAM–/– mice showed impaired learning (Cremer et al., 1994; Bukalo et al., 2004).

Previous studies also demonstrated that deficiency in the NCAM gene results in increased inter-male aggression (Stork et al., 1997), depression-like behaviour (Aonurm-Helm et al., 2008) and anxiety-like behaviour (Stork et al., 1999). In the experiments of Stork et al. (1999), the anxiety-like behaviour was evidenced by an increased preference for the dark compartment in the light/dark avoidance test (Stork et al., 1999). When the NCAM-deficient mice were exposed to the EPM, they demonstrated an increased preference for the open arms, which is suggestive of anxiolytic-like behaviour (Stork, et al., 1999, 2000). Thus, it appeared that the performance of the NCAM-deficient mice in anxiety tests was dependent on the test employed. Our study also demonstrated some discrepancies when we measured anxiety-like behaviour in the novelty-suppressed feeding test and the EPM. Novelty-suppressed feeding behaviour reflects the reaction of animals to stress, and it has been shown that the open-field may induce moderate anxiety because the mice are placed in an open space without the possibility of escape (Prut and Belzug, 2003). In contrast, when NCAM-deficient mice were exposed to the EPM task they demonstrated an increased percentage of entries into and an increased percentage of time spent on the open arms of the EPM, which indicates anxiolytic-like behaviour. Because we used relative measures expressed as a percentage of entries and time, an increased exploratory behaviour, which indeed was observed in the NCAM-deficient mice, should have little if any impact on these relative measures. Previous studies using precise spatiotemporal analysis employing minute-by-minute analysis of the rodent's behaviour in the EPM task demonstrated a progressive increase in open arm avoidance during a 5 min session. These data demonstrated that during exposure to the EPM task, animals quickly

learn the aversive properties of the open arms, implying that learning can occur during the EPM session (Carobrez and Bertoglio, 2005). Therefore, we performed a minute-by-minute analysis of the behaviour of NCAM-deficient and wild-type littermates in the EPM task. Indeed, the analysis showed that the wild-type animals demonstrated a high percentage of entries onto the open arms only during the first minute of the EPM task. During the last four minutes, the percentage of entries onto the open arms declined considerably, suggesting that learning of the aversive properties of the open arms occurred. In contrast, the NCAM-deficient animals did not demonstrate any decline in the percentage of entries during the 5 min session. These findings show that the NCAM-deficient mice were unable to learn or to recognize the aversive properties of the open arms and that the observed anxiolytic-like behaviour was largely due to cognitive dysfunction. This hypothesis was further confirmed when the test/re-test protocol of the EPM task was employed. In the second EPM trial, wild-type animals demonstrated clear avoidance of the open arms. This avoidance indicates that the mice acquired information during their exploration of the potentially dangerous areas of the maze during the first trial and that they consolidated this information and retrieved it during the second session. These data are consistent with those previously reported by others (File et al., 1990; Lister, 1990; Treit et al., 1993; Bertoglio and Carobrez, 2000). In contrast, when the NCAM-deficient mice were exposed to the test/re-test paradigm of the plus-maze, no evidence of habituation was observed.

In conclusion, the data obtained in this study show that the anxiolytic-like behaviour of NCAM-deficient mice in the EPM is largely due to their inability to recognize and learn the danger associated with the open arms.

3. Altered NCAM-dependent signalling pathways in the brains of mice with partial or complete deficiency of the NCAM protein

Previous in vitro and in vivo studies have shown that the major interaction partner of NCAM is FGFR1 and that a deficiency in NCAM causes reduced FGF receptor activation (Cavallaro et al., 2001; Kiselyov et al., 2003; reviewed in Kiselyov et al., 2005, Aonurm-Helm et al., 2010). We therefore measured the levels of FGFR1 protein and its phosphorylated form in the hippocampus of NCAM+/– mice and compared the results with those of NCAM–/– animals. In addition, we measured the levels of FGF-2 protein in these tissues. Our results demonstrated reduced levels of FGFR1 phosphorylation in both mutant genotypes without any changes in the total level of the FGF receptor protein. However, it should be noted that the decrease in phosphorylated FGFR1 in NCAM+/– mice was less pronounced when compared with NCAM–/– mice. In the NCAM+/– mice, the level of phosphorylated FGFR1 was 65–70% of

control, whereas in NCAM–/– mice, the level was only 30–35% of control levels (100%).

In both mutant genotypes, the reduction in phosphorylation of the FGF receptor was accompanied by an increase in FGF-2 protein in hippocampal tissues. Recently, it was shown that alteration in hippocampal FGF-2 expression may represent a key mechanism in the genesis of anxiety disorders (Turner et al., 2011). To date, there is no evidence showing that NCAM directly affects the expression of FGF-2. The observed increase in growth factor protein in our data is likely a compensatory reaction due to the reduced FGF-mediated signalling. These data suggest important permissive roles of NCAM in FGF-mediated signalling because in NCAM deficient mice, an increase in FGF-2 protein was not capable of activating FGFR1. Our data also show that the behavioural phenotype due to NCAM deficiency may be dependent on the magnitude of FGFR1 phosphorylation. Indeed, the FGF signalling system has diverse biological functions during brain development and in adulthood (Powers et al., 2000). Several recent studies have proposed a link between the FGF system and depression (Evans et al., 2004; Gaughran et al., 2006).

A previous study (Aonurm-Helm et al., 2008) showed reduced levels of phosphorylated CREB and pCaMKII and pCaMKIV in the hippocampus of NCAM–/– mice and suggested that this dysregulation may underlie the observed behavioural phenotype of NCAM–/– mice. In contrast to the NCAM–/– mice, the levels of phosphorylated CaMKII and CaMKIV were unchanged in NCAM+/– mice. No difference in the phosphorylation level of the transcription factor CREB was observed between the NCAM+/– and wild-type animals either. It appears that a partial reduction in NCAM proteins does not affect the basal activity of CaMKII or CaMKIV signalling pathways.

There is no doubt that CaMKII and CaMKIV and CREB play critical roles in the regulation of neural plasticity and the formation of memory (Lisman, 1994; Frankland et al., 2001; reviewed in Lisman et al., 2002 and Carlezon et al., 2005; Restivo et al., 2009). The roles of these intracellular signalling pathways in the mechanisms of mood disorders are less obvious. Some preliminary studies have demonstrated decreased levels of CaMKII mRNA in the prefrontal cortex of patients with bipolar disorder (Xing et al., 2002). Reduced CREB phosphorylation has been found in the brains of patients with major depressive disorder (Yamada et al., 2003). A number of studies have shown that the activation of CREB and CaMKII is implicated in the actions of antidepressants (Popoli et al., 2001; Du et al., 2004; Tiraboschi et al., 2004; Blendy, 2006; reviewed in Tardito et al., 2006).

Thus, our data show that contrary to NCAM–/– mice, the observed behavioural phenotype in mice with a partial deficiency in NCAM is not associated with alterations in the basal phosphorylation levels of CaMKII and CaMKIV or CREB. This behavioural phenotype may be dependent on a partial reduction in phosphorylation of the FGF receptor, which might account in part for the observed depression-like behaviour in these animals.

4. Altered levels of the serotonin-transporter in the brains of mice with a partial or complete deficiency of the NCAM protein

Alterations in the serotonergic system have been implicated in depression and other mood and anxiety disorders (Caspi et al. 2003; Murphy and Lesch 2008; Owens and Nemeroff, 1998). Serotonin is a widespread neurotransmitter in the central nervous system. It has been shown that serotonergic cell bodies are located in the raphé nuclei of the brain stem, and from there, they project numerous axonal fibers to many brain regions, such as cortical areas, the hippocampus, and the basal ganglia (Pineyro and Blier, 1999). The most widely reported serotonergic abnormality in major depression involves the serotonin transporter (Owens and Nemeroff 1998; Mann et al., 2000). The serotonin transporter is the main mechanism of removal of serotonin from the synapse, thereby maintaining homeostatic levels of serotonin in the extracellular space (Blakely et al. 1991; Hariri and Holmes, 2006). Interestingly, calcium and calmodulin have likewise been identified as regulators of SERT function by altering the affinity of 5HT binding to the SERT (Jayanthi et al., 1994). Recently, mutations of the SERT were found to induce alterations in the expression of BDNF (Molteni et al., 2010). BDNF is a neurotrophin that is involved in the etiopathology of mood disorders and in the mechanism of action of antidepressant drugs (Nibuya et al., 1995; Calabrese et al., 2007), and a close link between serotonin and BDNF has been suggested (Martinowich and Lu, 2008). Furthermore, it has been shown that alterations in BDNF can also alter serotonergic function (Guiard et al., 2008). There is also a close link between BDNF and PSA-NCAM as PSA-NCAM may sensitize neurons to BDNF (Kiss et al., 2001; Vutskits et al., 2001). It is also believed that PSA-NCAM binds to BDNF resulting in the phosphorylation of its major receptor, tropomyosin-related kinase B (TrkB) (Brennamann and Maness, 2008). This hypothesis is supported by findings that expression of BDNF and TrkB are reduced in the hippocampus of depressed individuals and this reduction can be reversed with antidepressant treatment (Nibuya et al., 1995).

Thus, reduced levels of NCAM/PSA-NCAM can lead to alterations in the expression of SERT. Future investigations are required to determine the link between SERT, BDNF and TrkB in these animals.

In summary, the observed depression-like phenotype in NCAM+/– or NCAM–/– mice may be caused by a reduction in SERT expression.

CONCLUSIONS

1. Our data show that animals with a constitutive complete deficiency of NCAM protein demonstrate depression-like behaviour and altered cognitive functions. Heterozygous mice with a partial reduction in NCAM protein levels demonstrate a depression-like behaviour without alterations in cognitive functions. We conclude that impaired plasticity due to NCAM deficiency might induce both memory dysfunction and depression-like state, depending on the extent of plasticity impairment.

2. NCAM-deficient mice, under basal conditions, demonstrated significantly higher numbers of FosB/ΔFosB-positive cells in the BLA, PFC and FC compared with wild-type littermates. Fear conditioning in NCAM–/– mice did not induce FosB/ΔFosB expression in these brain structures.
   These data suggest that in NCAM-deficient mice, structures involved in fear memory formation are constantly activated and this activation interferes with their ability to form new fear-related memories.

3. Our study shows that the anxiolytic-like behaviour of NCAM-deficient mice in the EPM is largely related to their inability to recognize and learn the danger associated with the open arms.

4. We found that both NCAM+/– and NCAM–/– mice showed reduced phosphorylation of the FGFR1 but in contrast to NCAM–/– mice, the observed behavioural phenotype in NCAM+/– mice is not associated with alterations in the basal phosphorylation levels of CaMKII and CaMKIV or CREB.

5. Partial or complete deficiency of NCAM affects the expression of SERT in frontal cortex and hippocampus. We propose that the reduction in SERT may cause the observed depression-like phenotype in NCAM+/– and NCAM–/– mice.

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SUMMARY IN ESTONIAN

Osalise või täieliku NCAM valgu puudulikkusega hiirte fenotüüp

Aju plastilisust saab kirjeldada kui aju omadust adapteerida ning reageerida adekvaatselt erinevatele endogeensetele ja välistekkelistele stiimulitele. Sünaptiline plastilisus on aju plastilisuse üks oluline osa, mis võimaldab ajul pidevalt luua ja eemaldada rakkudevahelisi kontakte. Kognitiivsetele võimetele nagu õppimine, mälu, aistingud ja teadvus on oluline pidev aju neuronaalsete võrkude muutumine (Purves and Andrews, 1997; Albright et al., 2000). Lisaks on leitud, et aju plastilisus, mis hõlmab ka närvikoe erinevaid morfoloogilisi muutusi, on oluline faktor depressiooni välja kujunemisel (Duman, 2009). Neuronaalsete rakkude adhesioonimolekul (NCAM) ja tema polüsialüülitud vorm (PSA-NCAM) on valgud, mis on üheks peamisteks sünaptilise plastilisuse kujundajateks (Kiryushko et al., 2003; Rønn et al, 2000). Eelnevad uuringud kinnitavad, et NCAM molekulid on võimelised seostuma nii omavahel (Gascon et al., 2007) kui ka teiste erinevate rakupinna valkudega (Heiland et al., 1998; Vutskits et al., 2001; Povlsen et al., 2008). Sellisel moel osaleb NCAM/PSA-NCAM erinevate signaalradade käivitamises ja regulatsioonis. NCAM/PSA-NCAM'i üks olulisemaid interakstsioonipartnereid on fibroblastide kasvufaktori retseptor 1 (FGFR1, Kiselyov et al., 2003; 2005). NCAM/PSA-NCAM ja FGFR1 vastastikuse mõju tulemusena aktiveeritakse signaalrajad, mis omakorda mõjutavad differentseeruvate neuronite arengut (Kolkova et al. 2000) ja erinevaid FGFR1 vahendatud neuronaalseid funktsioone (Hansen et al., 2010). Varasemates töödes on näidatud, et hiirtel, kellel oli täielikult välja lülitatud *Ncam1* geen täheldati suurenenud ärevust ja vähenenud õppimisvõimet. Sellega kaasnes oluliselt langenud FGFR1 aktivatsioon ja ka kaltsium-kalmoduliinist sõltuvate kinaaside II ja IV (CaMKII ja IV) ning transkriptsioonifaktori CREB aktivatsiooni langus (Aonurm-Helm et al., 2008).

Käesoleva töö eesmärgid

1. Teha kindlaks, kuidas osaline (NCAM+/–) või täielik (NCAM–/–) NCAM/PSA-NCAM'i puudumine hiirtel mõjutab nende fenotüüpi;
2. Selgitada, kuidas täielik NCAM'i puudumine mõjutab neuronaalset aktiivsust mälu formeerumisega seotud ajupiirkondades;
3. Selgitada, kas halvenenud kognitiivsed võimed NCAM–/– hiirtel võivad mõjutada nende käitumist pluss-puuris;
4. Selgitada, kuidas osaline või täielik NCAM'i puudumine mõjutab NCAM-vahendatud signaalradu;
5. Uurida, kas NCAM'i puudulikkus mõjutab serotonergilise süsteemi üht olulist komponenti – serotoniini transporterit.

Töö tulemused ja järeldused

Antud töö tulemused kinnitasid, et NCAM–/– hiirtel esines depressioonisarnane käitumine ning olid vähenenud kognitiivsed võimed. NCAM+/– hiirtel esines samuti depressioonisarnane käitumine, kuid nende kognitiivsed võimed ei olnud kontrollhiirtega võrreldes muutunud. Ühtlasi ilmnes, et ajupiirkonnad, mis on olulised hirmumälu tekkeks, olid NCAM–/– hiirtel püsivalt aktiveeritud ning see hüperaktiivsus võib olla ka mäluhäirete tekke üheks põhjuseks. Selgus, et vähenenud ärevuskäitumine pluss-puuris oli seotud NCAM–/– hiirte halvenenud kognitiivsete võimetega kuna nad ei olnud võimelised õppima ja adekvaatselt hindama avatud haarale minemisega seotud ohtu. NCAM+/– hiirtel leidsime, et ka nendel oli hipokampuse piirkonnas langenud FGFR1 aktivatsioon, mis võibki olla nende loomade käitumusliku fenotüübi põhjus. Kuid erinevalt NCAM–/– hiirtest ei kaasnenud NCAM+/– hiirtel FGFR1 aktivatsiooni langusega CaMKII ja CaMKIV ning CREB aktivatsiooni muutust. Ühtlasi leidsime, et NCAM puudulikkus mõjutab serotoniini transporteri ekspressiooni. Võib oletada, et just vähenenud serotoniini transporteri ekspressioon võib olla NCAM puudulikkusega loomadel ilmnenud depressioonisarnase käitumise põhjuseks.

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PUBLICATIONS

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