Aus dem Institut für Biologie
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der Otto-von-Guericke-Universität Magdeburg

Rolle des *Neural Cell Adhesion Molecule* (NCAM) in amygdalo-hippokampaler Interaktion und der Salienzkodierung des kontextuellen Furchtgedächtnisses

Dissertation

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Was dem Herzen widerstrebt lässt der Kopf nicht ein

- Arthur Schopenhauer

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Kurzreferat

In der vorliegenden Studie untersuchte ich den Beitrag der Expression des Neural Cell Adhesion Molecule (NCAM) in Amygdala und Hippokampus zur Bildung eines kontextuellen Furchtgedächtnisses. Mit Laser-gestützter Mikrodissektion und quantitativer Polymerasekettenreaktion bestimmte ich zunächst Veränderungen der NCAM mRNA Expression in Subarealen von Amygdala und Hippokampus nach Furchtkonditionierung. Dabei zeigte sich ein generell hohes NCAM mRNA-Expressionsniveau in den lateralen und basolateralen Nuclei der Amygdala, sowie deren trainingsintensitäts- und kontextabhängige Regulierung nach Furchtkonditionierung. Darüber hinaus konnte ich zeigen, dass Mäuse mit einer gezielten Ausschaltung des NCAM Gens ($NCAM^{-/-}$ Mäuse) ihr bereits bekanntes Defizit in kontextueller Furchtkonditionierung überwinden können, wenn eine Prä-Exposition an den Trainingskontext erfolgt, bei welcher der modulatorische Einfluss der Amygdala vermindert wird. Bei Training mit gesteigerter Stimulussalienz und erhöhter Amygdalaaktivierung zeigten $NCAM^{-/-}$ Mäuse im Unterschied zu Wildtyp-Kontrolltieren keine Verstärkung des kontextuellen Furchtgedächtnisses. Jedoch zeigten $NCAM^{-/-}$ Mäuse auch eine gesteigerte Furchtantwort auf den konditionierten Ton unter diesen Bedingungen. Meine Ergebnisse werden ergänzt durch die Beobachtung einer verminderten Synchronisierung oszillatorischer Aktivität im Theta-Frequenz-Bereich zwischen Amygdala und Hippokampus furchtkonditionierter $NCAM^{-/-}$ Mäuse. Zusammen weisen diese Befunde auf eine Beteiligung NCAM-vermittelter Zellerkennung an der Informationsverarbeitung im amygdalo-hippokampalen System und an der Amygdala-vermittelten Salienzkodierung kontextuellen Furchtgedächtnisses hin.

Schlüsselwörter: Amygdala, Furchtkonditionierung, Hippokampus, Neural Cell Adhesion Molecule, Salienzkodierung

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List of abbreviations

A	Adenine
αCaMKII	α-calcium/ calmodulin-dependent kinase II
ANOVA	Analysis of Variance
AP-1	Activator protein-1
AutoLPC	Automated laser pressure catapulting
BLA	Basolateral amygdala
bp	Base pairs
C	Cytosine
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
cDNA	copy DNA
CeA	Central amygdala
CNS	Central nervous system
CR	Conditioned response
CREB	cAMP response element- binding protein
CS	Conditioned stimulus
CT	Cycle threshold
dB	Decibel
dCT	delta-CT
ddCT	delta-delta-CT
DG	Dentate Gyrus
DMDC	Dimethyl dicarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Desoxy-nucleotide
e.g.	for example (from latin: *exempli gratia*)
endoN	Endoneuramidase N
ERK	Extracellular signal-regulated kinase
F3	Fibronectin type III domains
FAK	Focal adhesion kinase
FAM	6-carboxy-fluorescine
FGL	FG loop peptide

FGFR	Fibroblast growth factor receptor
Fig.	Figure
FRET	Förster Resonance Energy Transfer
G	Guanine
GAP-43	Growth-associated protein-43
GPI	Glycosylphosphatidylinositol
H_2O	destilled water
i.e.	that is (from latin *id est*)
IgI-IV	Immuneglobuline homology modules I-IV
IP_3	Inositol 1,4,5-trisphosphate
ISI	Inter-stimulus-interval
kb	Kilobase
LA	Lateral amygdala
LCM	Laser capture microdissection
LTP	Long term potentiation
MANOVA	Multiple Analysis of Variance
MAPK	Mitogen-activated protein kinase
MGB	Minor groove binder
$MgCl_2$	Magnesium chloride
mRNA	messenger RNA
n	number of animals/ samples used
NCAM	Neural Cell Adhesion Molecule
$NCAM^{-/-}$	NCAM deficient mice
$NCAM^{+/+}$	NCAM wildtype mice
NeuNAc	N-acetylneuraminic acid
NFκB	Nuclear factor κB
NFQ	non-fluorescent quencher
NMDA	N-methyl-D-aspartate
PCR	Polymerase chain reaction
PEN	Polyethylene naphthalate
PGK	Phosphoglycerate kinase
$PKC\beta_2$	Protein kinase $C\beta_2$
PLCγ	Phospholipase Cγ
PLL	Poly-L-Lysine

PLSD	Protected Least Significant Difference
PSA	Polysialylic acid
PTSD	Posttraumatic stress disorder
REM	Rapid eye movement
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	rounds per minute
RQ	Relative quantification value
RT	Reverse transcription
SEM	Standard error of mean
SPL	Sound pressure level
T	Thymine
Tab.	Table
T_m	Melting temperature
UNG	Uracil-N-glycosylase
UR	Unconditioned response
US	Unconditioned stimulus
UTP	Uraciltriphosphat

1. Introduction

The Neural Cell Adhesion Molecule (NCAM) is thought to be an important molecular component of memory formation and its modulation by stress. Conceivably, this makes NCAM a candidate molecule for the salience determination of emotional memories. A widely used model for studying such emotional memory formation is classical fear conditioning, an associative learning paradigm that involves the amygdala and hippocampus. In fact both limbic regions display an interaction during memory formation that is believed to be modulated dependent on salience of events occurring in a learning task.

1.1 NCAM

NCAM was first identified as a cell surface protein in chick embryo neural cells (Rutishauser et al., 1976) and was later also isolated from mouse and rat neuronal tissue (Chuong et al., 1982).

1.1.1 The NCAM gene

NCAM is transcribed from a single gene composed of 20 major and six minor exons in the mouse. The three major isoforms of NCAM are derived by alternative splicing: NCAM-180, named after its molecular weight, is the largest of these isoforms. It is a transmembrane protein derived from exon 0-19. In NCAM-140, exon 18 is skipped and the cytoplasmatic domain is shorter. However, NCAM-120 is a glycosylphosphatidylinositol (GPI) anchored protein that originates from exon 0 to 15. A secreted form of NCAM occurs when a small exon between the major exons 12 and 13 is included, which contains a stop codon and thus produces a truncated NCAM molecule (Kiselyov et al., 2005). In addition, a soluble form of NCAM can originate from proteolytic cleavage of the extracellular NCAM part as well (Fig. 1.1b).

1.1.2 NCAM protein structure

In general, the extracellular part of the different NCAM isoforms is composed in a similar manner. Being a member of the immunoglobulin superfamily, the extracellular NCAM portion consists of five immunoglobulin homology modules (IgI-V) followed by two fibronectin-type III domains (F3I and F3II) close to the cell membrane (Fig. 1.1a) (Kiselyov et al., 2005; Gascon et al., 2007). As mentioned above, in contrast to NCAM-120, the NCAM-140 and NCAM-180 isoforms are transmembrane proteins that possess distinct intracellular parts. The NCAM-120 isoform is thought to be constantly associated to lipid rafts via its GPI anchor, whereas the assembly of NCAM-140 and NCAM-180 to such plasma membrane microdomains appears to be dynamically regulated. The intracellular portion of the molecule can form signaling complexes and lipid rafts that are thought to provide a platform for gathering certain scaffolding and signaling molecules, thereby mediating the first steps of a cellular response upon activation (Povlsen and Ditlevsen, 2010).

1.1.3 Homo- and heterophilic interactions of NCAM

For activation of intracellular pathways interaction of NCAM with either other NCAMs or with other cell surface molecules is required. Binding between NCAMs of opposing cell membranes is thought to mediate the classical action of this molecule – cell adhesion. For this so-called homophilic interaction, two different forms of NCAM-NCAM-binding are necessary. First, certain subdomains of the extracellular parts of parallelly orientated NCAMs on the same cell membrane bind to each other (*cis*-interaction). The conformational change induced thereby allows then the formation of a *trans*-homophilic interaction, i.e. the binding between certain subdomains of NCAMs from opposing cell membranes (Fig. 1.1c) (Kiselyov et al., 2005). Besides homophilic NCAM-NCAM-binding, numerous interactions with other molecules have been described also. Among those so-called heterophilic interactions NCAM is able to bind to other cell surface molecules like L1 as well as to molecules of the extracellular matrix like heparin sulfate proteoglycans or the chondroitin sulfate proteoglycans phosphocan and neurocan (Kiselyov et al., 2005; Gascon et al., 2007). NCAM is also capable of activating growth factor receptors. The fibroblast growth factor receptor (FGFR) is the best studied example.

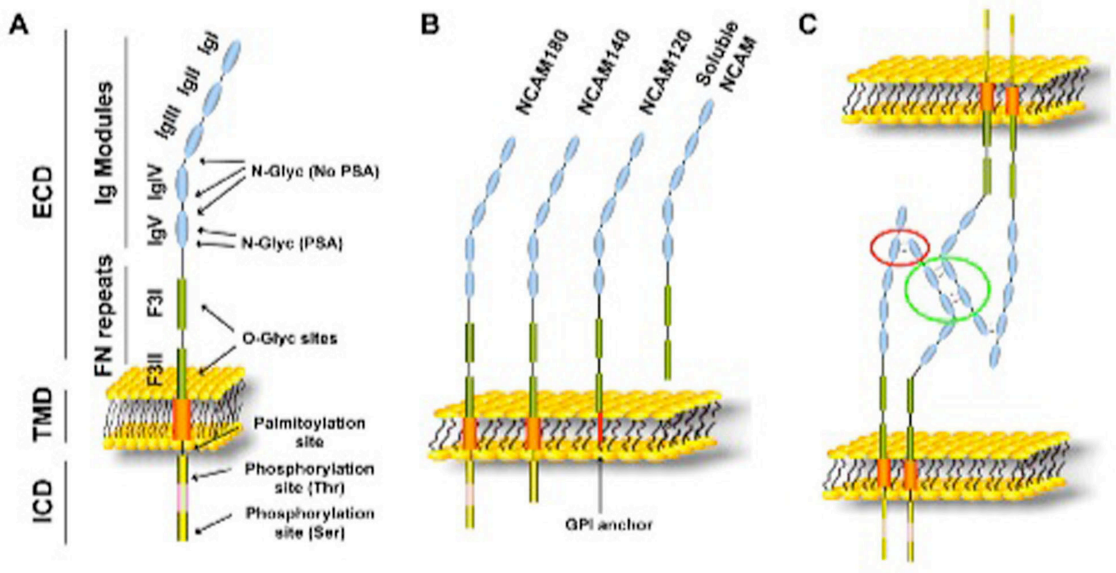

Fig. 1.1. The Neural Cell Adhesion Molecule (NCAM), a member of the immunoglobulin superfamily, (a) composed of two fibronectin-like repeats and five immune-globulin-like (Ig) modules. (b) Three major isoforms exist as well as soluble forms of NCAM. (c) NCAM mediates cell adhesion through homophilic binding in a *trans*-conformation (green elipse) that requires *cis*-interaction (red elipse) of parallel oriented NCAMs. The Figure is retrieved from Gascon et al., 2007.

1.1.4 Polysialylation of NCAM

To all three major NCAM isoforms Polysialic acid (PSA), a homopolymer of α2-8-linked N-acetylneuraminic acid (NeuNAc), can be added by posttranslational modification (Bonfanti, 2006). PSA can be linked to NCAM exclusively through two Golgi-associated polysialyltransferases, ST8SiaIV and ST8SiaII. The expression of these enzymes is strictly regulated during development and in a cell-specific manner. PSA-NCAM is abundantly expressed in the developing CNS and is therefore suggested as the functionally immature form of NCAM. In adult rodents its expression is restricted to specific brain areas like the hypothalamus, the olfactory system, the hippocampus including newly generated neurons in the dentate gyrus, but also in the amygdala (Nacher et al., 2002; Bonfanti, 2006; Gascon et al., 2007). Adding PSA to NCAM will decrease homophilic NCAM-NCAM-binding by steric interactions. Moreover, due to the high hydrated volume of PSA-NCAM, the extracellular space is enlarged, affecting cellular interactions mediated by heterophilic NCAM-binding to other cell surface molecules as well. Through these effects PSA-NCAM is involved in events

of structural plasticity like neurite outgrowth, synapse formation, but also adult neurogenesis and activity-induced synaptic plasticity (Bonfanti, 2006).

1.1.5 NCAM signaling

As mentioned above, NCAM is capable of triggering important intracellular signaling pathways. Upon homophilic NCAM interaction, NCAM is assembled in lipid rafts and builds complexes with the scaffolding protein spectrin at the postsynaptic density. In an isoform specific manner, other signaling molecules are recruited to this complex (Povlsen and Ditlevsen, 2010). So NCAM-140 is further associated with the non-receptor tyrosine kinase Fyn and the focal adhesion kinase (FAK), which are then activated by phosphorylation. Thereby Fyn and FAK are able to activate elements of the mitogen-activated protein kinase (MAPK) pathway like Ras, Raf and extracellular signal-regulated kinase (ERK) (Gascon et al., 2007; Ditlevsen et al., 2008). The complex of NCAM-180 and spectrin then recruits growth-associated protein-43 (GAP-43) and protein kinase $C\beta_2$ ($PKC\beta_2$), whereby GAP-43 is associated with cytoskeletal proteins and is therefore capable of mediating axonal growth and neuritogenesis (Povlsen and Ditlevsen, 2010). Such neurite outgrowth promoting functions of NCAM are important features of neuronal development. Nevertheless, signaling cascades like the MAPK-pathway play an important role in learning and memory of adult individuals as well (Trifilieff et al., 2007).

Interestingly, at least in the case of FGFR, heterophilic NCAM interaction requires *trans*-homophilic NCAM binding before. Only after that, the F3 module of NCAM is able to activate FGFR, causing its autophosphorylation (Ditlevsen et al., 2008). This results in an activation of phospholipase Cγ (PLCγ) that produces the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. Remarkably, for the NCAM-FGFR-interaction polysialylated NCAM seems to be required (Dityatev et al., 2004; Kiselyov et al., 2005).

Since most research focused on molecular events triggered by homo- and heterophilic NCAM interactions and their downstream effects, little is still known about the transcriptional regulation and induction of NCAM itself. So far, it has been reported that PSA-NCAM expression is regulated via the transcription factors activator protein-1 (AP-1) and nuclear factor κB (NFκB; Singh and Kaur, 2007) and the immediate early gene c-Jun (Feng et al., 2002) in cell culture systems. Interestingly, these three factors are also induced during learning tasks (Alberini, 2009).

1.1.6 The role of NCAM in the adult central nervous system

While early studies of NCAM function demonstrated its important role in neurite outgrowth and other features of neuronal development, NCAM mediates also plasticity events in the adult central nervous system, i.e. synapse formation and remodeling as well as adult neurogenesis and recruitment of such new neurons to functional networks (Gascon et al., 2007). In addition to this cellular level of function, it is strongly suggested that NCAM plays a role in memory consolidation and its modulation by stress (Sandi, 2004).

1.1.6.1 NCAM in synaptic plasticity

As a consequence of neuronal activity long lasting changes occur in the mature CNS on a molecular and cellular level induced by gene transcription and protein synthesis as well as structural changes, e.g. spinogenesis. These events can be modeled by so-called long term potentiation (LTP) *in vitro* and *in vivo*. Here, high frequency stimulation of afferent fiber tracks can induce a long lasting enhancement of synaptic transmission accompanied by molecular and structural changes. Thereby, LTP serves as a cellular model of memory formation and can be observed in the amygdala during fear memory formation (Schafe et al., 2001). Like in memory, LTP appears in a temporal pattern with an early phase that is independent of mRNA synthesis and lasts for minutes and a late phase, requiring synthesis of new proteins and resulting in structural remodeling like spine formation. On a molecular level, strong activation at an excitatory synapse leads to the opening of N-methyl-D-asparate (NMDA) receptors at the postsynaptic membrane, thus resulting in an increased intracellular Ca^{2+} concentration that then activates the MAPK pathway and induces gene transcription via transcription factors like the cAMP response element- binding protein (CREB; Schafe et al., 2001). As stated above, NCAM signaling is also associated with these intracellular pathways, therefore LTP is also a valuable model to investigate the role of NCAM in plasticity related events. So it could be demonstrated that administration of antibodies against NCAM interfere with the induction of LTP in the Cornu Ammonis 1 (CA1) region of the hippocampus (Lüthi et al., 1994). This function is thought to dependent on PSA, since removal of PSA by endoneuramidase N (endoN) prevents the formation of LTP in the CA1 region (Muller et al., 1996). Accordingly, in mutant mice deficient for NCAM LTP is decreased in CA1, but it can be rescued when PSA-NCAM or PSA is applied to the CA1 region *in vitro* (Senkov et al., 2006). However, LTP in excitatory granule cells of the Dentate Gyrus (DG) depends on the NCAM protein itself rather than its polysialylated form (Stoenica et al., 2006).

Moreover, NMDA-dependent synaptic remodeling and spine formation induced by LTP is accompanied by increased expression of the NCAM-180 isoform (Schuster et al., 1998). In this line, another approach using co-cultures of neurons derived from wildtype and NCAM-deficient mice revealed, that the synaptic strength at excitatory synapses depends on postsynaptic NCAM expression (Dityatev et al., 2000).

Nevertheless, bearing in mind the developmental shift of the PSA-NCAM/NCAM-ratio, a dynamic shift of this ratio could contribute also to activity-induced synaptic plasticity. In this line, it is thought that PSA-NCAM is increased in synapses after neuronal activation, allowing structural remodeling, whereas NCAM occurs predominantly in stable synapses. PSA-NCAM would result in reduced cell adhesion and would therefore enable structural changes. Additionally, NCAM can trigger intracellular signaling cascades via changes of the postsynaptic density, activation of FGFR and neurotrophin receptors and modulation of glutamate channel activity. Finally, expression of non-polysialylated NCAM may consolidate and stabilize the previous changes again (Ronn et al., 2000; Gascon et al., 2007).

1.1.6.2 NCAM in learning and memory

As stated above NCAM appears to bear a central role in synapse modulation in response to neuronal activity. Besides playing a role in synaptic plasticity as a cellular model for memory formation, NCAM contributes to learning and memory related events in the behaving animal. This issue is also well addressed by applying NCAM-modulating peptides or evaluating learning-induced NCAM expression changes. As discussed in the section below, these studies are further accomplished by the analysis of mice deficient for NCAM.

The first evidence for an involvement of NCAM in learning-related events comes from findings in a simple organism performing a simple form of associative learning. During long-term sensitization of the gill- and siphon-withdrawal reflex in the mollusk *Aplysia californica* an increase in synaptic connections between the siphon's sensory neurons and the gill motor neurons occur. This is accompanied by a downregulation of the *aplysia* homologue of NCAM, apCAM, on the sensory neuron, allowing remodeling of synaptic connections (Mayford et al., 1992). Additionally, also in the fruit fly *Drosophila melanogaster* a form of NCAM, Fasciclin II, can be found (Kristiansen and Hortsch, 2008). Thus, NCAM appears to be an ancient and evolutionary highly conserved protein, thus suggesting an important function of this protein in development, but also in plasticity-related events of the mature CNS. Accordingly, early studies of NCAM function showed that administration of antibodies against NCAM interfere with the consolidation of previously acquired associative and spatial

memory (Doyle et al., 1992; Arami et al., 1996). On the other hand, application of NCAM-mimicking peptides could increase such memory (Cambon et al., 2004) and also Pr2, a PSA-mimicking peptide, was capable of facilitating hippocampus-dependent spatial memory (Florian et al., 2006).

Learning events can alter also the expression of NCAM or PSA-NCAM. 24 h after water maze training expression of NCAM and PSA-NCAM is increased in the hippocampus (Venero et al., 2006). Subsequently to contextual fear conditioning, also a hippocampus-dependent learning paradigm, PSA-NCAM levels are increased 24 h later as well (Lopez-Fernandez et al., 2007). Interestingly, enzymatic removal of PSA by endoN affected only hippocampus-dependent contextual fear conditioning, but not the more amygdala-related auditory cued fear conditioning (Lopez-Fernandez et al., 2007; Markram et al., 2007b). However, PSA-NCAM is upregulated in the BLA 24 h after auditory cued fear conditioning, but only when a strong stimulus is applied, suggesting a stress-dependent regulation of NCAM expression (Markram et al., 2007b).

1.1.6.3 NCAM-mediated stress effects on memory formation

Evidence suggests that the expression regulation of NCAM and PSA-NCAM due to memory formation is dependent on the stress level the individual experiences either chronically before or acute during the learning task in an intrinsic (i.e. stress by the cognitive task itself) or extrinsic (i.e. stress by other, not task related events) manner (Sandi, 2004; Bisaz et al., 2009a). Fear conditioning with different training intensities can account as a model for acute, intrinsic stress and its effects on NCAM expression were mostly evaluated on the protein level in preparations of the hippocampus or hippocampal subregions. Here, either increases or decreases of NCAM or PSA-NCAM levels have been reported, dependent on the hippocampal region and the posttraining time point analyzed (Merino et al., 2000; Sandi et al., 2003; Lopez-Fernandez et al., 2007). As suggested by Bisaz and Sandi (2009a), in summary the results point towards a transient upregulation of NCAM and PSA-NCAM expression following stressful training, thus allowing remodeling of neural circuits. Thereby, NCAM most likely contributes to the memory enhancing effects of stressful, intensive training. On the other hand, acute extrinsic stress like predator exposure after a spatial learning task impaires memory formation and is accompanied by decreased NCAM-180 levels (Sandi et al., 2005). Chronic stress, e.g. daily restraining sessions for 21 days, also result in cognitive impairments and a reduced expression of NCAM (Venero et al., 2002; Sandi, 2004). However, administration of a NCAM-mimicking peptide, the FG loop peptide (FGL), is

capable of rescuing the devastating effects of chronic stress on spatial memory (Bisaz et al., 2009b).

In contrast to hippocampus-dependent learning forms like spatial learning in the Morris Water Maze or contextual fear conditioning, learning-induced alterations of NCAM-expression in the amygdala are less well investigated. Markram et al. (2007b) reported an increase in PSA-NCAM in the basolateral and central subnucleus of the amygdala upon intensive training in an auditory cued fear conditioning paradigm; enzymatic removal of PSA by endoN did not affect fear memory consolidation, but its extinction.

Interestingly, applying stress in a juvenile age, a procedure that impairs learning in adulthood, shifts NCAM to PSA-NCAM ratio in key regions for emotional memory formation of the adult, suggesting a stress-dependent maturation deficit of the limbic system (Tsoory et al., 2008).

Together, analysis of NCAM in memory formation highlights the involvement of this molecule in memory consolidation processes and in the mediation of adaptive responses during fear memory formation.

1.1.7 NCAM mutant mice

An elegant tool to further analyze the contribution of NCAM to cognitive and emotional processes is provided by genetically altered mice. In conventional NCAM knock out mice exons three and four of the NCAM gene are ablated by gene targeting, resulting in an abolished expression of NCAM and PSA-NCAM (Cremer et al., 1994). NCAM deficiency causes a decreased size of the olfactory bulb, disturbance of mossy fibers fasciculation in the hippocampus along with decreased mossy fiber LTP and impairment of spatial learning in the Morris Water Maze (Cremer et al., 1994; Cremer et al., 1997). Moreover, emotional behavior is disturbed in NCAM knock out mice ($NCAM^{-/-}$). $NCAM^{-/-}$ mice display increased aggression in a male intruder paradigm, accompanied by elevated post-trial corticosterone levels (Stork et al., 1997), as well as increased anxiety-like behavior in a light-dark-test (Stork et al., 1999). The disturbed emotional behavior due to NCAM deficiency can be rescued by transgenic expression of the NCAM-180 isoform in $NCAM^{-/-}$ mice (Stork et al., 2000). In these $NCAM^{-/-180+}$ animals aggression and anxiety-like behavior are normalized to wild type levels ($NCAM^{+/+180-}$). In addition, depression-like behavior in the Forced Swim Test is recovered by transgenic re-expression of NCAM-180 in $NCAM^{-/-180+}$ mice (Stork et al.,

2000). These effects appear to be mediated by an effect of NCAM on the serotonergic system that is hypersensitized in $NCAM^{-/-}$ and normalized in $NCAM^{-/-180+}$ mice.

On the other hand expression of the NCAM-180 transgene has no effect on size of the olfactory bulb or mossy fiber density in the $NCAM^{-/-}$ or $NCAM^{+/+}$ background. Memory deficits observed in $NCAM^{-/-}$ mice also cannot be restored by NCAM-180 transgene as both $NCAM^{-/-180+}$ and $NCAM^{-/-180-}$ mice display reduced spatial learning in the Morris Water Maze and a decreased conditioned fear response towards a tone and the shock context in an auditory cued fear conditioning paradigm (Stork et al., 2000).

Taken together, the findings from $NCAM^{-/-}$ mice further highlight the role of NCAM in emotional memory formation. However, since conventional $NCAM^{-/-}$ mice are not able to express the NCAM molecule in their whole lifespan, developmental defects may be responsible for the observed phenotype. To resolve this issue, mice with a forebrain-specific conditional ablation of NCAM have been generated. In these animals, the Cre-loxP recombination system under the control of the promoter of the α subunit of the calcium/ calmodulin-dependent kinase II (αCaMKII) is used to reduce NCAM expression at postnatal stages (Bukalo et al., 2004). In the conditional NCAM deficient mice no disturbance of mossy fiber fasciculation and no change in mossy fiber LTP is apparent. Moreover, spatial learning deficits are also milder than after constitutive NCAM knock out, with a retardation of spatial orientation in the Morris Water Maze that can be overcome by training (Bukalo et al., 2004). Likely, such a less severe phenotype could be also related to the fact that NCAM levels in the conditional mutant mice are rather reduced instead of completely abolished like in the constitutive knock out mice. However, in a recent study it could be demonstrated that conditional NCAM-deficient mice display deficits in auditory cued fear conditioning (Bisaz and Sandi, 2010), underlining a development-independent contribution of NCAM to the consolidation of emotional memory.

Since ablation of NCAM in a knock out animal is accompanied by deficiency for PSA as well, the use of mice deficient for the polysialylation mediating enzymes allows to dissect the contribution of PSA-NCAM from that of the NCAM core protein. Interestingly, mice deficient for ST8SiaIV, the predominant polysialyltransferase in adulthood, show deficits in spatial learning in the water maze and also in contextual fear memory, whereas cued fear memory is not altered (Senkov et al., 2006; Markram et al., 2007a). Moreover, contextual fear memory can be partially restored by injections of PSA into the hippocampus of NCAM-/- mice (Senkov et al., 2006). These results suggest a pronounced involvement of PSA-NCAM

in hippocampus-dependent memory formation, whereas the NCAM core protein may be required for the formation of amygdala-dependent memory.

1.2. Fear Conditioning

Pavlovian fear conditioning is a well established paradigm for studying processes underlying the storage of emotional memories. Depending on the parameters of conditioning, amygdala and hippocampus are differentially involved in these processes. Thereby, modulating fear conditioning provides a valuable tool to gain insights into the role of NCAM in the consolidation of emotional memory in hippocampus and its modulation by the amygdala.

1.2.1 Classical fear conditioning

Classical fear conditioning is a form of associative memory emerged from the basic principles of Pavlovian conditioning, in that events which a priori do not relate to each other become associated by a subject upon their mere coincidence. In terms of classical "Pavlovian" fear conditioning, an individual learns a relationship between a previously neutral stimulus and an aversive event. Building up such an association is indispensable for survival, since it allows prediction of threatening events and therefore their avoidance. Hence, classical fear conditioning is a widely used paradigm to investigate emotion and memory in various species like rodents and humans. Subjects quickly learn to associate the threatening with the previous neutral stimuli and build a robust and long lasting memory for this (Maren, 2001; Schwartz et al., 2002). During fear conditioning training in rodents, a neutral stimulus like a tone is presented contingently with a mild electric foot shock serving as the unconditioned stimulus (US). The paired presentation of tone and foot shock establishes an association between the previously independent stimuli and so the tone becomes a conditioned stimulus (CS). When the animal is re-exposed to this tone in a retrieval session, it will show typical fear behavior (Conditioned Response, CR; Fig. 1.2). Memory for the CS-US-association acquired during the training allows the animal to anticipate a threatening situation whenever the CS occurs. To maintain this memory for a long-term period a cascade of molecular and cellular events is initiated that among others requires mRNA and protein synthesis. These events occur for several hours after the training, during the so-called consolidation phase. Therefore, the recall

of the consolidated long-term fear memory during retrieval is scheduled to a later time point like 24 or 48 hours in most protocols (Schafe et al., 2001; Stork et al., 2001; Stoppel et al., 2006).

Rodents express typical defensive behavior in a fearful situation corresponding to their fear level and quantification of the frequency and duration of defensive elements in behavior is commonly used for an estimation of fear memory. In fact, fear conditioning training thought to induce a strong and long lasting memory results in the expression of high levels of defensive behavior (Laxmi et al., 2003). The typical defensive behavior of rodents like mice and rats comprises of different components that occur dependent on the salience of the stimulus and the stress level experienced during training. Risk assessment (orientation towards the stimulus, alert watching with head movements and stretched attend), freezing (immobility expect for respiratory movements) and flight responses can be observed in a retrieval session, whereas flight reactions occur only occasionally in highly stressful situations (Laxmi et al., 2003).

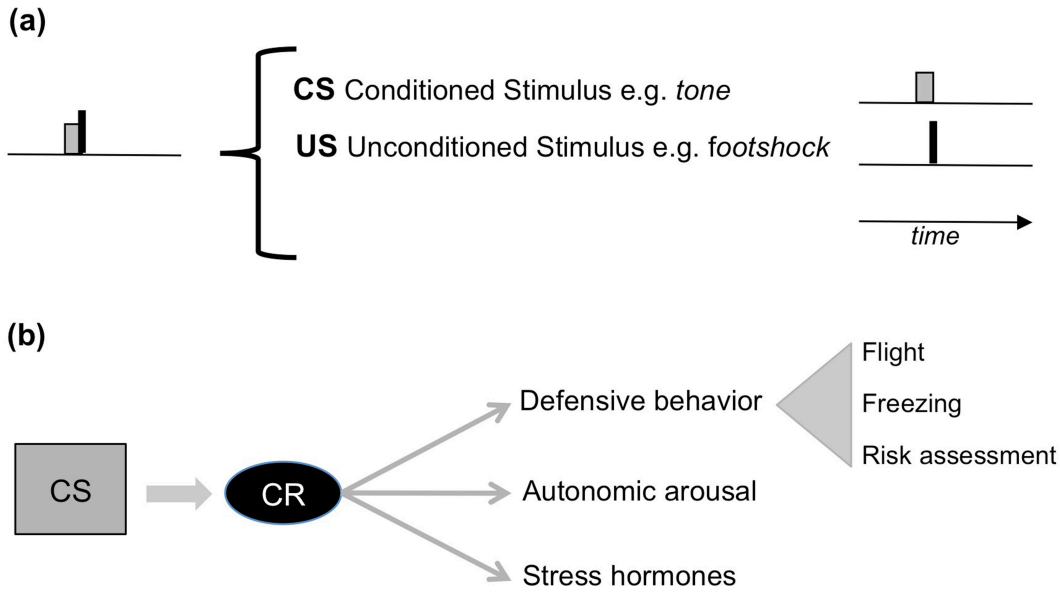

Fig. 1.2. Classical Fear Conditioning. (a) In a classical fear conditioning paradigm a threatening stimulus like a foot shock is presented as the unconditioned stimulus (US) together with a neutral stimulus like a tone. Thereby, the animal learns to associate both stimuli and the tone will function as the conditioned stimulus (CS). (b) In a subsequent retrieval session the CS will be sufficient to elicit a fear response, then called the conditioned response (CR), consisting of different components. Adapted from LeDoux, 2000, and Laxmi et al., 2003.

In the paradigm described above a tone is used as a stimulus bearing only a single sensory modality. Of course, also stimuli of other modality can be employed as a cue, such as a light

or an odor, but so-called auditory cued fear conditioning is probably the most commonly applied paradigm. Fear conditioning towards the environment in which the individual experiences the US is referred to as contextual fear conditioning. In a classical foreground context conditioning experiment, the animal learns to build an association between the training environment and the US. Re-exposure to the training environment alone, i.e. the conditioning chamber, will evoke a defensive response. Here, the animal has learned to associates multiple cues of different sensory modalities form the training environment, e.g. the odor in the conditioning chamber, the texture of the floor, noises from the ventilation fan etc., with the foot shock (Maren, 2001).

Importantly, even when the animal undergoes an auditory cued fear conditioning paradigm, it is exposed to the training environment and its multimodal stimuli. Therefore, a cued conditioned animal will also exhibit a conditioned fear response towards the context, although likely to a lesser extend than towards the CS. Since in the auditory cued fear conditioning paradigm the stimulus with the greatest salience is the CS, the training environment provides only "background" information. Therefore, it is referred to as the "background context". This nomenclature allows also to distinguish the fear responses to the environment according to the paradigms engaged, i.e. to the "background context" in an auditory cued fear conditioning paradigm and to the "foreground context", i.e. in an contextual fear conditioning paradigm without presentation of a discrete cue (Phillips and LeDoux, 1994; Gerlai, 1998; Calandreau et al., 2005).

1.2.2 The role of amygdala and hippocampus in fear memory formation

Extensive studies were conducted to determine the neural circuitries required for fear conditioning. Over years, two key regions emerged that contribute distinctly to different features of fear conditioning – the amygdala and the hippocampus (Fig. 1.3).

The amygdala, named according to its almond-shaped appearance, is located in the medial region of the temporal lobe and comprises various subnuclei differing in their cytoarchitecture, molecular composition and anatomical connectivity (Pitkänen et al., 2000). Among these, the lateral (LA), basolateral (BLA) and central (CeA) subnuclei are highly relevant for fear conditioning (LeDoux, 2000). According to the vast body of evidence regarding information processing during auditory cued fear conditioning, the LA appears to be the main input site for sensory information about the CS from thalamic and cortical areas.

At the same time nociceptive information concerning the US is directed to the LA. That allows emotional stimulus association between CS and US information in the LA (Maren and Quirk, 2004). Such associative content is then relayed to the BLA and the CeA. Since the CeA is intensively interconnected with the LA and also receives nociceptive information directly, the CeA is able to modulate LA function. Moreover, the CeA is the output site of the amygdala with projections to various brain regions responsible for expression of fear behavior, hence generating the fear response (LeDoux, 2000).

The hippocampus appears to be involved whenever a more complex level of information analysis is required, i.e. in contextual fear conditioning where multimodal information is processed or in trace fear conditioning, when a temporal separation between CS and US occurs (Phillips and LeDoux, 1992; Maren, 2001; Rudy et al., 2004). The hippocampal formation is composed of the cornu ammonis (CA) part and the dentate gyrus (DG), although the DG is cytoarchitecturally different from the hippocampus proper. The CA can be further divided into subunits numbered from one to three with each part divided into different cell layers that reach a high degree of lamination. The DG, CA3 and CA1 are connected by three major pathways and are thought to mediate the information flow within the hippocampus (Kandel et al., 2000). Recently lesion studies of DG, CA1 and CA3 in a contextual fear conditioning paradigm provide evidence for an involvement of CA3 in the acquisition and CA1 as well as DG in the retrieval of contextual fear memory (Lee and Kesner, 2004). This is further underlined by another study that demonstrates the participation of the CA3 area in building a context representation during the acquisition phase, although here CA1 was involved in consolidation rather than retrieval of contextual fear memory (Daumas et al., 2005). Although the specific role of the subregions in contextual fear memory formation remains elusive, its general contribution to these processes is rather striking. It is thought that the hippocampus relays contextual information to the BLA (Maren and Fanselow, 1995). There, contextual and US-related information can convergence. The BLA is connected with the CeA as well and again the CeA is believed to generate the fear response and may further modulate the US processing (LeDoux, 2000). As there exist intensive connectivity between amygdala and hippocampus that is reciprocal and in part also direct (Pitkänen et al., 2000), the amygdala itself is also capable of modulating hippocampal function. Interestingly, this happens in a stress-dependent manner, presumably via the stress hormones corticosterone and norepinephrine (Akirav and Richter-Levin, 2002; McGaugh, 2004).

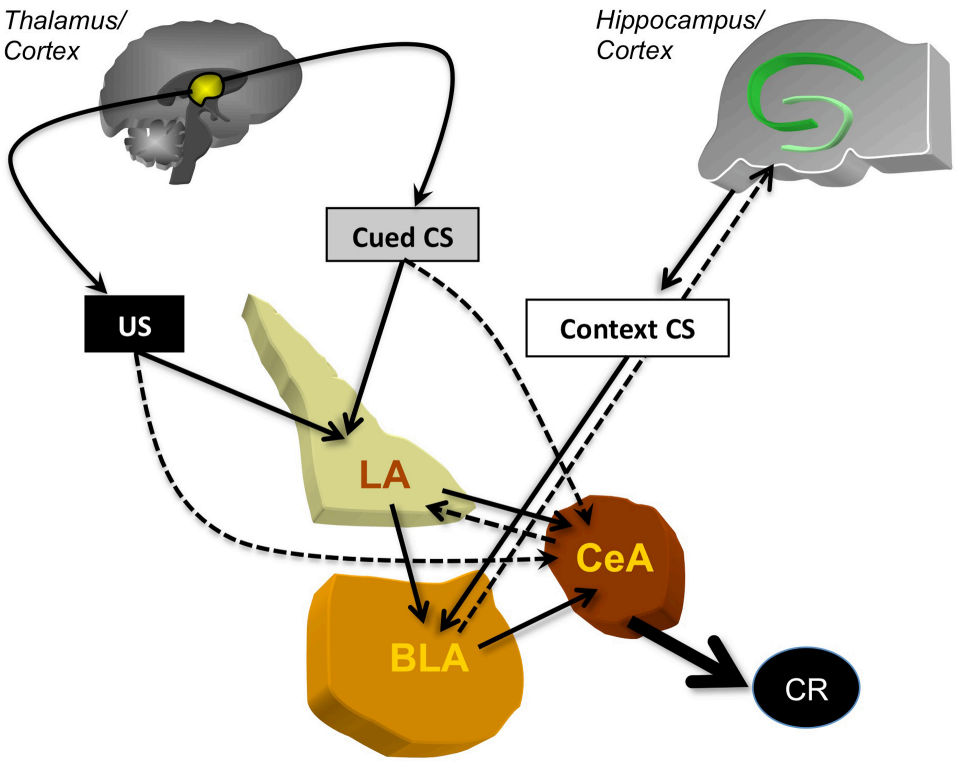

Fig. 1.3. Fear Conditioning Circuits. Model for stimulus processing in cued and contextual fear conditioning in amygdala subregions LA, BLA and CeA and their interplay with hippocampus as well as thalamical and cortical areas. Dashed lines display functional interactions. Adapted from Stoppel et al., 2006.

One tool to determine amygdalo-hippocampal interaction is the analysis of the neuronal activity within this pathway. Rhythmic oscillation at the theta frequency range (4-12 Hz) occurs in amygdala neurons during emotional arousal (Pelletier and Paré, 2004). In the hippocampus, neuronal activity at the theta frequency can be recorded during locomotion and exploratory behavior, but also during rapid eye movement (REM) sleep (Buzsáki, 2002). Interestingly, hippocampus and amygdala start to synchronizes their activity at the theta frequency range after fear conditioning; during retrieval, the theta synchronization is maximal in response to the conditioned stimulus (Seidenbecher et al., 2003). Further evaluation of this phenomenon revealed that amygdalo-hippocampal theta synchronization occurs in response to a conditioned cue as well as towards the conditioned context. Moreover, theta synchronization is not observed during short term fear memory or during retrieval of remotely conditioned fear, suggesting a dynamical contribution of this process to the consolidation of long-term fear memory (Narayanan et al., 2007).

1.2.3 Modulating stimulus salience: effects on fear memory

Unrevealing the functional contribution of amygdalar and hippocampal subregions it becomes evident that the BLA plays a dual role in fear conditioning (1) being the entry site for contextual information into the amygdala and (2) modulating hippocampal function (Maren and Fanselow, 1995; Akirav and Richter-Levin, 2002; Maren, 2008).

Although it is a widely accepted view that the amygdala is necessary for both cued and contextual fear conditioning whereas the hippocampus is only involved in contextual fear memory formation (Phillips and LeDoux, 1992), a careful analysis of fear conditioning using different paradigms suggests that this functional distinction cannot be upheld.

A systematical analysis of fear memory generalization in mice by Laxmi and colleagues (Laxmi et al., 2003) revealed that using an auditory cued overtraining with ten tone-foot shock pairings and intensive foot shocks (0.6 mA) resulted in increased defensive behavior towards the background context, whereas in training with moderate intensities the response to the background context was minimal. This generalization of the fear response towards the background context due to intensive training was also obvious in rats (Baldi et al., 2004).

In Pavlovian Fear Conditioning the term generalization describes this shift of the CR to a non-reinforced cue (CS-) such as a tone with another frequency than the CS+ (Schwartz et al., 2002), or an enhanced fear response to the training environment.

During auditory cued fear conditioning, both the cue and the background context are present and can be viewed as compound stimuli. The Rescorla-Wagner theory elegantly describes that the associative strength formed towards a CS depends on its salience. For compound stimuli it means that only stimuli with equal salience form an equal associative strength (Rescorla, 1976; Schwartz et al., 2002). As here the read-out for the associative strength is the defensive response expressed during fear memory retrieval, an increased response to the background context after overtraining suggests an increase in the salience of the background context.

On a functional aspect, when the balance between cued and contextual fear memory is altered due to overtraining, a shift in the degree of amygdalo-hippocampal interaction is conceivable and the hippocampus seems to become more involved. In this line, damage to the dorsal hippocampus decreased background context conditioning dramatically (Phillips and LeDoux, 1994). This is also true for lesioning the BLA, whereas damage to the LA increased the background context response (Calandreau et al., 2005).

Overtraining very likely elicits a state of emotional arousal and such emotionally charged memories are thought to be better remembered than neutral ones due to their increased salience. Over the last years, evidence indicates that stress hormones acting on the BLA and the hippocampus are attributable for mediating such effects (Paré, 2003; McGaugh, 2004). For various tasks it could be demonstrated that an emotional event elicits activation of the BLA via glucocorticoids and norepinephrine, thus fascilitating plasticity-related events in the hippocampus and formation of memory (LaLumiere et al., 2003; Roozendaal et al., 2006b; Roozendaal et al., 2006a). Recently, differential responses of the hippocampal subregions CA1 and DG to BLA activation have been described. Vouimba and Richter-Levin (2005) could demonstrate that stimulating the BLA shortly before induction of LTP impaires a potentiation in the CA1, whereas LTP in the DG is increased. As described in the behavior paradigms, this enhancing effect of BLA activation on the DG is mediated by glucocorticoid and noradrenergic action in the BLA (Vouimba et al., 2007).

These results clearly point out that stressful experiences modulate the amygdalo-hippocampal interactions and give rise to the possibility to use the overtraining protocol from Laxmi et al. (2003) for studying the role of this interaction in salience-dependent modulation of hippocampus-dependent memory. Moreover, it was demonstrated that familiarization to the context prior to fear conditioning training diminishes the modulating effects of the BLA on contextual fear memory (Huff et al., 2005), thus raising another possibility for modulating the salience of the context in a fear conditioning paradigm.

1.3. Aim of the study

The appropriate determination of stimulus saliency during memory formation is critical for the adaptive response of an individual towards its environment. Disturbances of this function are evident in psychopathological states such as mood and anxiety disorders.

Firstly, I used laser capture microdissection and subsequent quantitative polymerase chain reaction to locate fear conditioning-induced NCAM mRNA expression changes in the subregions of mouse amygdala and hippocampus.

Secondly, to determine the functional relevance of NCAM expression in these brain regions, I conducted a behavioral analysis of mice with ablation of the NCAM gene, focusing on the amygdala-mediated determination of stimulus salience during contextual fear conditioning. To this end, I took advantage of the fact that amygdalo-hippocampal interaction varies dependent on complexity of the learning task and its emotional relevance, and employed contextual pre-exposure, different training intensities, as well as background and foreground contextual fear conditioning task.

2. Material and Methods

2.1 Animals

2.1.1 Animal housing

All mice used in this study were housed under standard laboratory conditions in the Instiute of Biology and the Institute of Physiology, Otto-von-Guericke University Magdeburg. The animals were kept in groups of two to six individuals in Macrolon cages (36,2 cm x 16 cm x 14,3 cm; Ebeco, Castrop-Rauxel, Germany) with standard bedding (Lignocel BK8/15, J. Rettenmaier & Söhne, Rosenberg, Germany). Room temperature and moisture was regulated by an air conditioning system with 21°C air temperature and 50% air moisture, respectively. Lights went on automatically at 7:00 PM with a 30 min dawn phase, resulting in an inverse light/dark cycle with a phase duration of twelve hours. Access to food, a standard pellet diet (Ssniff R/M-H V-1534, Ssniff Spezialdiäten, Soest, Germany), and water was ad libitum.
One week before the experiments began the mice were separated into single cages with the possibility of visual and acoustical contacting within each level of the rack. The experiments were conducted during 9:00 AM and 18:00 PM, hence in the active phase of the animals.

2.1.2 Animal welfare

Animal housing and experiments in this study were conducted in accordance with the European and German regulations for animal experiments and approved by the Landesverwaltungsamt Saxony-Anhalt (AZ 2-441 and 2-618).

2.1.3 Animals for the gene expression analysis

For the gene expression analysis in naïve animals and after fear conditioning, adult male C57B/6BomTac (M&B Taconic, Berlin, Germany) were obtained at an age of seven weeks. The animals were housed in our animal facility for two weeks before the experiments started to adapt them to the inverse 12 h light/dark cycle. As mentioned above, mice were separated into single cages during the second week.

Shortly before experiments started the animals were randomly assigned to one of four training groups (naïve control, cued standard conditioning, cued overtraining and contextual standard conditioning).

2.1.4 NCAM mutant mice

2.1.4.1 Breeding

NCAM mutant mice and their wildtype littermates descended from a NCAM mutant line which carries a targeted disruption of the NCAM gene by inserting a neomycin cassette that replaces most of exons three and four and the introns between them ($NCAM^{-/-}$ mice; Cremer et al., 1994). This mutant line was backcrossed to a C57Bl/6 genetic background for more than twelve generations. For experiments, male $NCAM^{+/-}$ mice were bred with female $NCAM^{+/-}$ mice obtaining $NCAM^{-/-}$ and $NCAM^{+/+}$ offspring in one litter. After weaning at the age of four weeks, the offspring was raised in groups of two to six animals.

2.1.4.2 Genotyping

2.1.4.2.1 Tail biopsy

Shortly after weaning tail biopsies of 0.5 cm length were taken from the adolescent animals accompanied by marking of individual animals via ear holes. The tail tissue was stored -20°C or processed immediately by transferring them to 500 µl tail lysis buffer.

2.1.4.2.2 Isolation of genomic DNA

For processing of DNA, 10 µl Proteinase K (10 mg/ml) was added to the lysis buffer containing the individual tail cut. After overnight incubation at 55°C in a rotor incubator, the lysate was centrifuged briefly at the next day. 350 µl of the supernatant were transferred in a new 1.5 ml Eppendorf tube containing 350 µl isopropanol and were mixed manually by inverting the tubes repeatedly. After centrifugation (15 min at 10 000 rpm, room temperature), the supernatant was removed and the pellet washed briefly with 500 µl 70% ethanol. Again, samples were centrifuged (5 min at 10 000 rpm). The supernatant was removed and the DNA-pellet air dried at room temperature. The dry DNA-pellet was dissolved in 50 µl TE buffer. The genomic DNA of each animal was stored at 4°C.

2.1.4.2.3 Polymerase chain reaction for NCAM genotyping

Genotypes were determined by multiplex polymerase chain reaction (PCR) on genomic DNA, as described previously (Stork et al., 2000). The polymerase chain reaction is a widely used standard technique to amplify DNA fragments of a known sequence *in vitro*. Initially, a heating phase from 94°C up to 98°C will denature the DNA double strand in single DNA strands and allow the primers, synthetic oligomers of a certain sequence which are highly concentrated in the PCR mix, to hybridize to their complementary sequence at the 3'-end of the DNA when temperature is lowered. The specific annealing temperature will thereby depend on the primer melting temperature (T_m). During extension a heat stable polymerase will elongate the primers according to the 5'- to 3'-sequence of the template DNA by adding the complementary dNTP. Since a primer pair is flanking the target DNA region repetition of these denaturation-annealing-extension cycles will increase only the number of copies from the target DNA sequence exponentially from cycle to cycle whereas other sequences where primer pairs cannot bind remain unamplified. For genotyping of NCAM mutants three different primers were used in a multiplex PCR. The NCAM ko primer is a 3'-primer specific for the neomycin cassette whereas the NCAM wt primer was complementary to a 3'-sequence in exon three which is substituted by the neomycin resistance gene in the NCAM mutant mice. The 5'-primer NCAM all binds a sequence in exon 2.

Tab. 2.1. Primer Sequences (5' to 3') for genotyping of NCAM mutant mice

Primer name	**Sequence**
NCAM all	CCA GCA GCG GAT CTC AGT GGT GTG G
NCAM ko	TGC AAT CCA TCT TGT TCA ATG GCC G
NCAM wt	GAT GGT TGG AGG CAG GGA GCT GAC C

For NCAM genotyping PCR Phusion high-fidelity DNA polymerase (Finnzymes OY, Espoo, Finnland) was used, which is derived from the thermostabil polymerase of *Pyrococcus furiosus* combined with a processivity-enhancing domain, and the appropriate buffer together with dimethyl sulfoxide (DMSO) to improve denaturation. In a 100 µl reaction tube 1 µl of the isolated genomic DNA of the individual animal was added to 24 µl of the PCR master mix consistent of the following components (for 1 reaction):

Tab. 2.2. PCR master mix.

5 µl	5x Phusion HF buffer
2 µl	dNTP mix (2.5 mM each)
0.1 µl	$MgCl_2$ (25 mM)
2.5 µl	Primer NCAM all
2.0 µl	Primer NCAM ko
0.5 µl	Primer NCAM wt
0.75 µl	DMSO
11 µl	H_2O
0.25 µl	Phusion Polymerase (2 U/µl)

Next to the individual genomic DNA from animals determined for genotyping, a negative control with H_2O as a template as well as a positive control with template DNA from a $NCAM^{+/-}$ mouse were included.

The PCR was performed with a hot start, i.e. the mix with the DNA was kept on ice until the thermocycler reached the denaturation temperature to prevent formation of unspecific PCR products before initial denaturation. After initial denaturation (3 min at 98°C) gaining complete dissociation of DNA double strands, 35 cycles were conducted to amplify the specific target fragments, followed by a final extension phase (7 min at 72°C) for terminal amplicon synthesis (Thermocycler: Verity, Applied Biosystems, Darmstadt, Germany). The PCR products were stored at 4°C until analysis per gel electrophoresis.

Tab. 2.3. Thermo Cycler Program.

Phase	**duration**	**temperature**	**Number of steps**
Initial denaturation	3 min	98°C	1
Denaturation	10 s	98°C	35
Annealing	30 s	62°C	35
Extension	1 min 30 s	72°C	35
Final extension	7 min	72°C	1
Storage	∞	4°C	1

2.1.4.2.4 Gel electrophoresis.

To analyse the DNA fragments derived from the genotyping PCR they were separated via electrophoresis on a 1% agarose gel. For this, 1 g agarose pulver was solved in 100 ml 1x TAE buffer under heating in a microwave (3 min at 600 Watt). After cooling in a water bath, 10 µl ethidium bromid (0.5 mg/ml) were added to the agarose. The fluid agarose was then poured into the gel preparation chamber and a gel comb was inserted to obtain pockets for applying DNA to the gel. After hardening, the comb was carefully pulled out of the gel and the gel was unhinged from the gel preparation chamber and transferred to the electrophoresis chamber containing TAE buffer covering the gel completely. Each PCR-product was then mixed with loading buffer (10 µl DNA + 2 µl 6x loading buffer) and transferred to an individual gel pocket. One of the pockets contained a DNA length standard (DNA Molecular Weight Marker VII; Roche Diagnostics, Mannheim, Germany). At the plus pole of the electrophoresis chamber 10 µl ethidium bromid (0.5 mg/ml) were added to prevent diffusion of the ethidium bromid in the gel. Electrophoresis was performed at 100 mV for ca. 30 min. Detection and documentation of the DNA fragments was conducted with the InGenius LHR gel documentation and analysis system (Syngene, Cambridge, UK). As in other documentation systems, ultraviolet light exposure of the gel elicits a fluorescence signal of the ethidium bromid incorporated in the DNA fragments. Thereby, applying three primers in a multiplex PCR for NCAM genotyping resulted in a 1.1 kb fragment from the wildtype allel and a 1.4 kb fragment from the targeted allele in $NCAM^{-/-}$ mice.

2.2 Fear conditioning apparatus

Fear conditioning towards a tone or to the context was applied. Conditioning was performed in an apparatus (TSE, Bad Homburg, Deutschland) comprising a sound isolated cubicle connected with a control module and a personal computer. The box contained the cuboid shaped arena made of acrylic glass (36 cm x 21 cm x 20 cm) with a grid floor for delivering of the foot shock. Above the arena a loud speaker for delivering of the tone was installed. Inside the box, the light intensity was 10 lux. A ventilation fan providing fresh air within the box produced a background noise of 70 dB sound pressure level (SPL). The duration of the single phases of the conditioning experiments as well as the strength of the foot shock and the frequency of the tone were controlled via the Fear Conditioning Software (FCS, TSE, Bad Homburg, Germany) and the control module. During the whole experiment, a video camera

installed above the arena recorded the animal for off-line behavioral analysis. Additionally, light beams installed around the arena in two levels above the grid floor monitored activity and behavior of the mouse on-line.

2.3 NCAM gene expression analysis

2.3.1 Fear conditioning protocols

Naïve control, cued standard conditioning and cued overtraining groups were pre-exposed to the conditioning apparatus over two days in two daily sessions of five minutes each. The contextual standard training group was exposed to a novel standard cage serving as neutral context. Contrary to the pre-exposure procedure used in the differential mutant conditioning paradigms, the presentation of a neutral test stimulus was surrendered for the sake of best possible distinction between cue and context effects, as a separate testing of cue and context effects like during the different retrieval phases in the mutant behavior analysis was not possible in the gene expression study.

In the conditioning session, animals were given two minutes of adaptation. Afterwards, mice in the naïve control group (n = 6) were exposed to three CS+ (10 kHz tone, 85 dB SPL, 10 s duration with inter stimulus intervals, ISI, of 20 s) but no US. The cued standard conditioning group (n = 6) in the same manner received three CS (10 kHz tone, 85 dB SPL, 9 s duration) that each co-terminated with a US (0.4 mA foot shock, 1 s, ISI 20 s), whereas cued overtraining (n = 6) was conducted with ten such CS/US pairings and increased US intensity (0.6 mA, 1 s). The contextual standard conditioning group (n = 6) was exposed to three US (0.4 mA, 1 s, ISI 29 s) but no CS during training (see also Fig. 2.1).

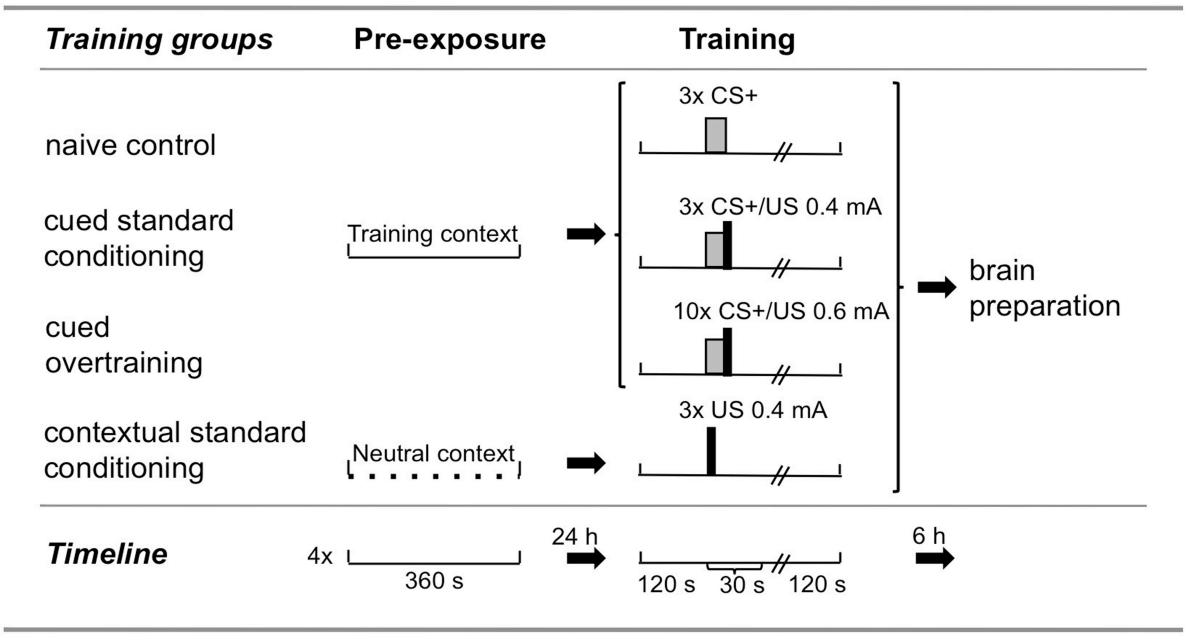

Fig. 2.1. Different fear conditioning protocols used in the NCAM gene expression analysis. After four pre-exposure sessions on the first two days of the experiment, training was done on the third day. The gray bar indicates the CS+ (a 10 kHz tone), the black bar the US (a footshock for 1 s with either 0.4 mA or 0.6 mA intensity). Brain preparation was conducted 6 hours after training.

All protocols engaged have been firmly established and were shown to produce context fear memories of different salience (Laxmi et al., 2003; Bergado-Acosta et al., 2008). Efficient training was further confirmed by monitoring electric current flow during foot shock delivery as well as the animals' immediate behavioral response to shock (flight, vocalization). Two minutes after the last US presentation animals were returned to their home cage where they remained until preparation. Before the next animal was placed in the fear conditioning box, the chamber was cleaned with 70% ethanol for disinfection and removal of olfactory traces.

2.3.2 Tissue preparation

Six hours after training animals were killed by cervical dislocation. After opening scalp and removing of the cranium, the brains were quickly removed from the skull and transferred to an aluminum cup containing the embedding medium Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe, Zoeterwoude, NL). Tissue Tek was added to the cup until it covered the brains completely. Then, the embedded brains were snap frozen in liquid nitrogen-cooled

methylbutane until the embedding compound was cured. The brains then were stored at -80°C for a maximum of 3 weeks before sectioning on a cryostat took place.

2.3.3 Tissue sectioning

2.3.3.1 Preparation of object slides

For laser capture micro dissection special glass object slides covered by a 1.35 µm thick polyethylene naphthalate (PEN) membrane (P.A.L.M., Bernried, Germany; PALM Microlaser Systems Protocols) facilitating laser cutting and catapulting of the tissue samples were used. To increase adherence of brain slices on the membrane, the PEN-slides were exposed to ultraviolet light for 30 min. Afterwards the membrane surface was covered by 1 ml 0.05% Poly-L-Lysine (PLL) solution for 30 min at room temperature. Excess PLL was washed away by incubation of the object slides for three times 5 min in 200 ml double distilled water in a staining cuvette. For removal of RNAse the object slides were soaked in „RNAse away spray“ (Molecular BioProducts, San Diego, USA) for 2 min at room temperature. Afterwards, object slides were washed five times for 5 min in 200 ml double distilled water treated with dimethyl-dicarbonate (DMDC) before (room temperature). The slides were air dried and stored in RNAse-free slide box.

2.3.3.2 Preparation of brain sections

Before cutting of sections on a cryostat the snap frozen brains were transferred from the -80°C freezer to a -20°C freezer for 30 min and than to the cryostat chamber (chamber temperature -16°C, object temperature -12°C). There, coronal sections of 20 µm thickness were cut at the level of amygdala and dorsal hippocampus and thaw mounted on the PLL-coated RNAse free membrane slides. Between every mounting, the brain sections on the object slide were allowed to dry on a warming plate at 40°C to minimize RNAse activity.

2.3.4 Staining of brain sections

For identification of amygdalar and hippocampal subregions the brain sections were first fixed with 70% ethanol cooled to -21°C for 1 min. Immediately afterwards, a brief hematoxylin-eosin staining under RNAse-minimized conditions was performed. The sections

were dipped into hematoxylin solution for 2 min, then washed in 200 ml DMDC-treated double distilled water for 1 min and again for 3 min in a fresh cuvette with 200 ml DMDC-water before sections were transferred to eosin staining reagent for 1 min. After dehydration in an increasing ethanol series (50% - 70% - 96% ethanol for 2 min each, prepared with DMDC-treated double-destilled water) sections were air dried and laser capture microdissection took place immediately after. To further minimize RNAse activity, all solutions were prepared and handled in baked glass ware (3 h at 180 °C).

2.3.5 Laser capture micro dissection and tissue lysis

Laser capture micro dissection (LCM) allows isolation of tissue and cells on pure and high resolution level. With a microscope offering possibility of light transmission or fluorescence approaches connected to a personal computer and a camera it is possible to record microscopic live images and define regions of interests on the digital images obtained. A software controlled laser beam can cut along the defined area and catapult tissue by a laser pulse into a capture device located above the slide. The short contact of the laser to the glass slide underside comprises a contact- and contamination-free sampling method with high spatial accuracy due to automated, software-controlled movement of the stage (Burgemeister, 2005; Bova et al., 2005).

On the brain sections, the dentate gyrus (DG), cornu ammonis (CA)1 and CA3 regions of the hippocampus as well as the lateral (LA), basolateral (BLA) and the central (CeA) nuclei of the amygdala were identified in a 10fold magnification under the microscope of the LCM setup and marked at the digital life image on the computer screen. All six regions were then microdissected from the left and right hemisphere of eight to twelve sections and catapulted in a capture device with the AutoLPC mode (automated laser pressure catapulting modus, laser energy at 100%). The capture device was the cap of a special 500 µl collection tube (P.A.L.M., Bernried, Germany) containing 10 µl of sterile mineral oil for better adherence of the sample fragments.

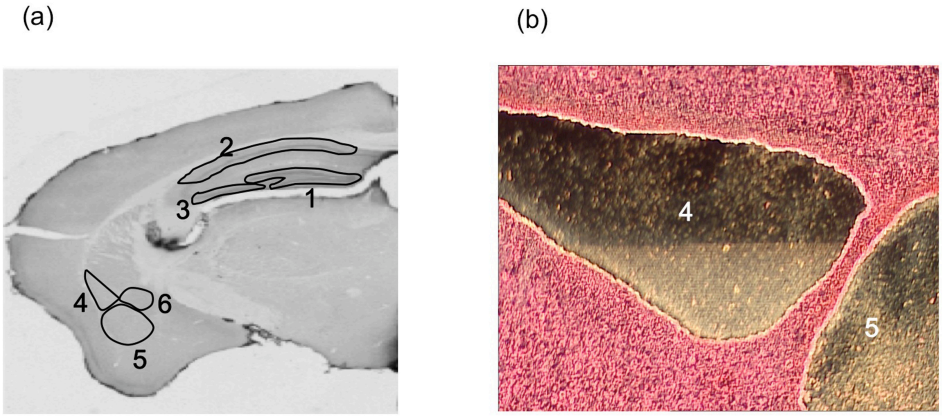

Fig. 2.2. (A) Areas for Laser Capture Microdissection in a coronal brain section. Hippocampus: (1) Dentate Gyrus (DG), (2) Cornu Ammonis (CA) 1, (3) CA3; Amygdala: (4) Lateral (LA), (5) Basolateral and (6) Central subnucleus of the amygdala. (B) Part of the LA and BLA after Laser Capture Microdissection in a hematoxylin-eosin stained brain section at 10x magnification.

Immediately after LCM, 50 µl ice-cold lysis buffer (from "Cells-to-cDNA II"-Kit, Ambion, Huntington, UK) was added to the samples in the collection tube. Tissue fragments were solved from the cap by inverting the collection tube repeatedly. Lysis was conducted by repeated pipetting. Adjacent, the sample was incubated in a water bath at 75°C for 10 min for RNAse-inactivation. After cooling down on ice, 1 µl DNAse I (2 U/µl; from "Cells-to-cDNA II"-Kit, Ambion, Huntington, UK) was added to the sample. For DNA digestion the sample was incubated at 37°C for 30 min, followed by 5 min at 75°C in a water bath for subsequent DNAse inactivation.
Until further processing the direct lysate containing the RNA was stored at -80°C for maximal four weeks.

2.3.6 Reverse transcription (RT)

For first-strand synthesis of cDNA a two-step-approach according to the "Cells-to-cDNA II" Kit (Ambion Huntingdon, UK) was performed. In a first step, 5 µl of the lysate was mixed with 11 µl of reverse transcription mix 1 containing dNTPs and oligonucleotide primer and incubated at 70°C for 3 min in a thermocycler, enabling heat denaturation of secondary structures of the template RNA. After cooling on ice and subsequent brief centrifugation, 4 µl of mix 2 were added containing the reverse transcription enzyme in the appropriate buffer as well as an RNAse inhibitor (see tab. 2.4 for details; all reagents from "Cells-to-cDNA II"-Kit,

Ambion Huntingdon, UK). The cDNA synthesis was conducted in 50 µl PCR tubes in a thermocycler at 42°C for 60 min followed by enzyme inactivation at 95°C for 10 min. The cDNA samples were stored at -20°C until real time PCR was conducted.

Tab. 2.4. Master Mix 1 & 2 for Reverse Transcription reaction

Mix 1:	5 µl	Lysate
	4 µl	dNTP Mix (2.5 mM each)
	2 µl	Oligo (dT)18 first strand primer (50 µM)
	5 µl	RNAse free water
Mix 2:	2 µl	10x RT-Buffer
	1 µl	M-MLV Reverse Transcriptase (100 U/µl)
	1 µl	RNase Inhibitor (10 U/µl)

2.3.7 Real time PCR

Quantitative PCR was performed in an AbiPrism 7000 Sequence detection system (Applied Biosystems, Darmstadt, Germany) using TaqMan® reagents. The chemistry of the real time PCR used in this study allows detection of specific PCR amplification products via a fluorescent signal depending on FRET (Förster Resonance Energy Transfer). By FRET, fluorescence occurs when a fluorescence dye is coupled to a quencher on oligonucleotide substrates and accumulates with increasing numbers of amplicons during PCR. In principle, also real time PCR consists of the standard PCR steps – denaturation, annealing, extension – described above. The real time assay contains the primer pair specific for the target sequence and an oligonucleotide probe. This probe is constructed with a fluorescence dye on the 5'-end and a quencher on the 3'-end. At the beginning of the PCR reaction the quencher and fluorescence dye are closely located to each other on the intact probe sequence. This reduces fluorescence emission on a large scale. When the primers anneal to the target cDNA sequence, the probe binds downstream to one of the primer. In the extension phase the polymerase elongates the primer according to the target sequence. Additionally, the polymerase has also a 5'-nuclease activity that cleaves now the probe sequence. Thereby, the reporter dye is dislocated from the quencher dye allowing now a strong increase in fluorescence signal emission. Moreover, the probe is removed from the target sequence and

the primer extension can continue undisturbed along the target sequence. With each PCR cycle, more reporter dye is unleashed and the fluorescence signal intensity increases proportional to the amount of amplicon. Thereby quantification can take place, because a high number of cDNA at the beginning of the PCR will produce an increase of fluorescence significantly above background levels after relatively few PCR cycles (the so called cycle thresholt, CT) while low numbers of template will need more PCR cycles until the fluorescence signal reaches threshold level (VanGuilder et al., 2008).

I used pre-designed assays for NCAM and the housekeeping gene phosphoglycerate kinase (PGK) provided by Applied Biosystems (TaqMan® Gene expression assays, Applied Biosystems, Darmstadt, Germany). As described above, based on TaqMan chemistry the assays contain next to the gene-specific primer sequences the TaqMan MGB (minor groove-binder) probe with a non-fluorescent quencher (NFQ) and 6-carboxy-fluorescine (FAM) as a fluorescence dye. The TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany) provided the PCR components needed with AmpliTaq Gold DNA Polymerase, dNTPs with UTP and optimized buffer. Another component was the AmpErase® uracil-N-glycosylase (UNG) that minimizes decontamination with carryover-PCR. All assays were run on the samples in triplicates with the reaction setup described in table 2.5.

Tab. 2.5. Reaction setup for Real-Time-PCR

Sample	5 µl	cDNA 1:5 dilution
Mix	10 µl	2x TaqMan ® Universal Master Mix
	1 µl	20x assay (NCAM or PGK)
	4 µl	H_2O

All runs were performed in the ABI Prism 7000 Real-Time-PCR system (Applied Biosystems, Darmstadt, Germany) and consisted of 50 cycles with 15 s at 95°C and 1 min at 60°C and were preceded by a 2 min step at 50°C for decontamination with UNG.

2.3.8 Data analysis and statistics

Using the ABI Prism Software (Applied Biosystems, Darmstadt, Germany) the mean cycle threshold (CT) values were determined for each triplicate assay. Relative quantification of

NCAM mRNA levels was conducted according to the ddCT method (Livak and Schmittgen, 2001). Here, NCAM expression values were normalized to the overall content of cDNA in a sample using PGK as internal control. In pre-experiments it was confirmed that PGK expression was independent of treatment groups and referred only to the starting amount of DNA in each sample. The dCT, that is the normalized NCAM expression raw value relative to the house keeping gene expression, was determined by subtraction of the mean CT of PGK from the mean CT of NCAM for each sample:

dCT (NCAM) = (mean CT (NCAM)) - (mean CT (PGK))

The dCT was calculated for each sample (with six areas in four different training groups each) and was used for further statistical analysis. Firstly, the different relative expression values of NCAM were evaluated in the single areas. Differences in expression between distinct areas were determined with an one-way-ANOVA for the factor area followed post-hoc by Fisher's protected least significant difference (PLSD) test.
Accordingly, effect of the different fear conditioning protocols on NCAM expression was determined for each area and analyzed with one-way-ANOVA for the factor training group followed by Fisher's PLSD test for post-hoc comparison. For illustration of expression differences due to fear conditioning, the ddCT value was calculated for each training group with ddCT (naïve) = 0 as a reference. Therewith, the mean dCT of each training group was normalized to the mean dCT of the naïve control group:

ddCT (training group) = (mean dCT (training group)) - (mean dCT (naïve control group))

Since Real time PCR is based on an exponential function, the ddCT value for each training group was transformed in the Relative Quantification value (RQ):

$$RQ = 2^{-ddCT(\text{training group})}$$

RQ now illustrates the relative expression of NCAM mRNA in each training group for a specific area with RQ (naïve control group) = 1.

2.4 Fear conditioning of NCAM mutant mice

$NCAM^{-/-}$ and $NCAM^{+/+}$ mice underwent background conditioning, were conditioning to a cue was included, and foreground context conditioning, i.e. without any cue presentation. Within the different training protocols that were also employed in the gene expression experiments before, the stimulus salience was systematically varied. Additionally, the training groups were amended by naïve and unpaired controls to test for the specificity of conditioned fear behavior. In order to minimize differences in the novelty-induced salience modulation of context fear memory (Huff et al., 2005) a contextual pre-exposure protocol was applied before the training. Within this group, stimulus salience was further modulated by training with increased stimulus intensity (Laxmi et al., 2003). Figure 2.3 gives a graphical overview about the different fear conditioning protocols applied in the behavioral analysis of NCAM mutant mice.

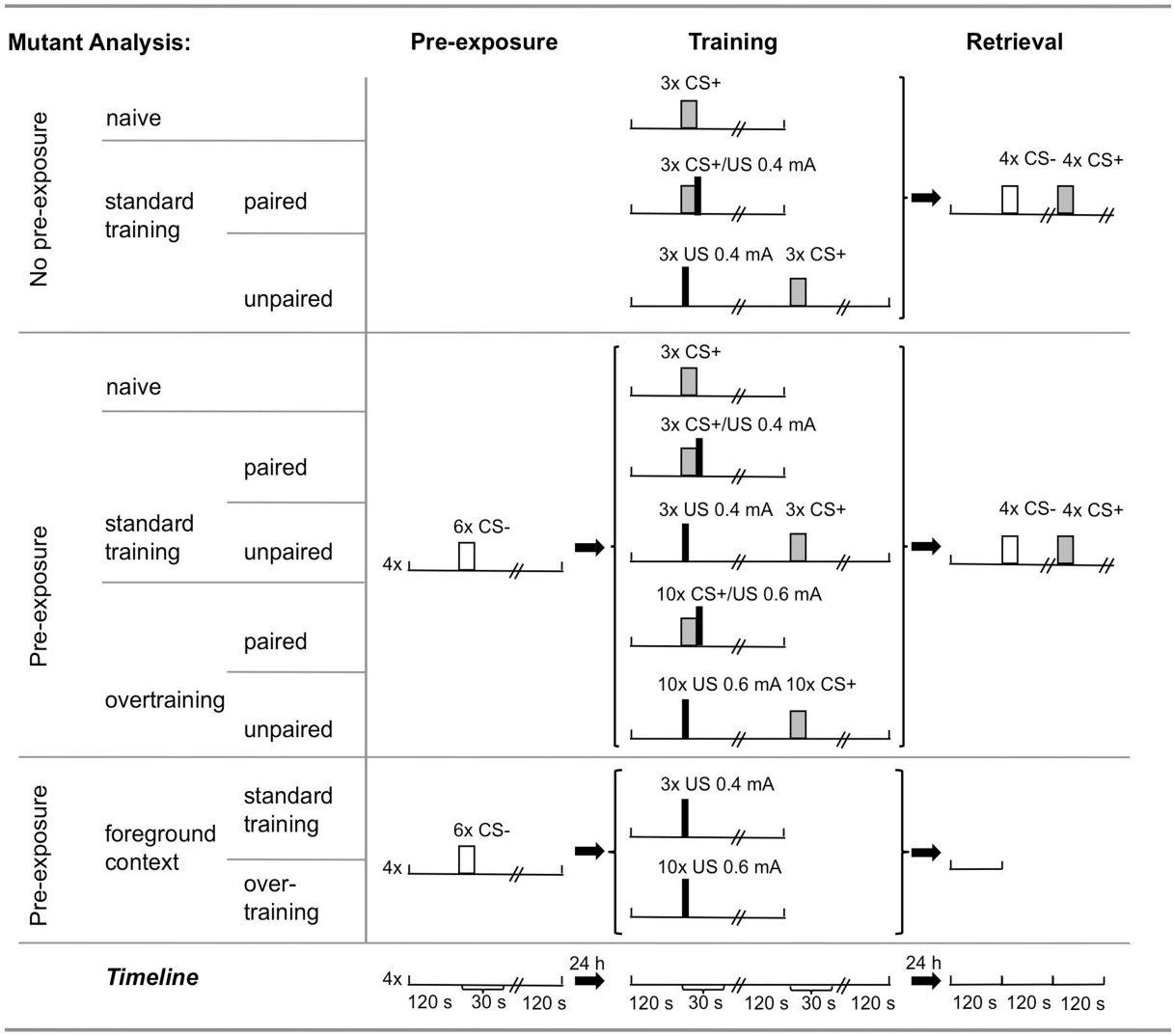

Fig. 2.3. Overview of the different protocols applied for behavioral analysis of NCAM mutant mice. The white bars indicate the CS- (a neutral tone with 2.5 kHz, 85 dB SPL, 10 s), the gray bars the CS+ (the conditioned tone with 10 kHz, 85 db SPL, 9 or10 s) and the black bars the US (the footshock, 1 s duration, with 0.4 mA in standard training and 0.6 mA intensity in overtraining). See also text for details.

2.4.1 Fear conditioning with different salience

2.4.1.1 Fear conditioning without pre-exposure

Firstly, to replicate the previously described fear memory deficits of NCAM$^{-/-}$ mice (Stork et al., 2000), animals were fear conditioned without any contextual pre-exposure. Therein, NCAM$^{-/-}$ (n = 9) and NCAM$^{+/+}$ mice (n = 9) received *standard cued conditioning*. After 2 min habituation, the animals experienced the CS+ (10 kHz, 85 dB SPL, 9 s) co-terminating with

the US (a footshock for 1 s with 0.4 mA intensity) three times (ISI 20 s). After a posttraining phase of 2 min in the fear conditioning box, the animals were returned to their home cages. In the *unpaired* control group $NCAM^{-/-}$ (n = 8) and $NCAM^{+/+}$ mice (n = 9) received three US (0.4 mA, 1 s, 29 s inter-stimulus-intervals (ISI)) followed 2 minutes later by a set of three CS+ (10 kHz, 85 dB SPL, 10 s, 20 s ISI). *Naïve control* animals (n = 5 $NCAM^{+/+}$, n = 7 $NCAM^{-/-}$) received three CS+ (10 kHz, 85 dB SPL, 10 s, 20 s ISI) without any US presentation.

2.4.1.2 Fear conditioning with pre-exposure

As demonstrated by Huff et al. (2005), contextual pre-exposure has the ability to modulate contextual fear conditioning by reducing the influence of novelty-induced salience on the memory formation. Therefore, another set of animals was familiarized with the training environment in four sessions of seven minutes each (*pre-exposure*). Here, the mice experienced the context alone for two minutes, followed by presentation of six neutral auditory stimuli (CS-; 2.5 kHz, 85 dB SPL, 10 s, 20 s ISI) and again, two minutes of context exposure only. Afterwards, the animals were subjected to one of the following training protocols proceeding on the next day: In the shock-*naïve control* groups, $NCAM^{+/+}$ (n = 7) and $NCAM^{-/-}$ mice (n = 5) received three CS+ (10 kHz, 85 dB SPL, 10 s, 20 s ISI) but no US during training. During *paired standard training* $NCAM^{+/+}$ (n = 7) and $NCAM^{-/-}$ mice (n = 10) were confronted with three CS+ (10 kHz, 85 dB SPL, 10 s, 20 s ISI) / US (0.4 mA foot shock, 1 s) pairings. During explicitly *unpaired standard training* $NCAM^{+/+}$ (n = 8) and $NCAM^{-/-}$ mice (n = 8) received first three US (0.4 mA, 1 s, 29 s ISI) followed then by a set of three CS+ (10 kHz, 85 dB SPL, 10 s, 20 s ISI) two minutes later.

2.4.1.3 Fear conditioning with increased stimulus intensity

Within the pre-exposure group, mice were also trained with increased stimulus intensity (Laxmi et al., 2003), to reveal effects of training intensity on cued and background context memory. $NCAM^{-/-}$ (n = 12) and $NCAM^{+/+}$ mice (n = 9) underwent an established *paired overtraining* protocol (Laxmi et al., 2003) with ten CS+ / US (0.6 mA foot shock, 1 s) pairings. The explicitly *unpaired overtraining* groups of $NCAM^{-/-}$ (n = 8) and $NCAM^{+/+}$ mice (n = 9) were exposed to a block of each ten US (0.6 mA, 1 s, 29 s ISI) and, two minutes later, 10 CS+ (10 kHz, 85 dB SPL, 10 s, 20 s ISI). Remarkably, compared to standard training, not only the number of CS+/ US-pairings, but also the intensity of the US was increased in the overtraining protocols.

2.4.1.4 Fear conditioning towards a foreground context

Finally, to address the effect of NCAM mutation on foreground context conditioning, $NCAM^{+/+}$ (n = 8) and $NCAM^{-/-}$ mice (n = 10) received contextual pre-exposure and were then confronted with a *foreground context standard training* procedure where they received three US of 0.4 mA (29 s ISI) without any CS+ presentation; an additional *foreground context overtraining* groups (n = 8 $NCAM^{+/+}$, n = 8 $NCAM^{-/-}$) were exposed to ten US of 0.6 mA (29 s ISI).

2.4.2 Collection of behavioral data

Fear memory was tested in a single 6 min-retrieval session, 24 h after training. In background context/cued training groups, the animals' response to the training context alone was tested for two minutes. This was immediately followed by two blocks of two minutes duration during which four CS- and four CS+, respectively, were presented with inter-stimulus-intervals of 20 s each. For the analysis of foreground context memory, conditioned animals were re-exposed for two minutes to the training context without cue presentation. Blind for genotype and training, the defensive behavior comprising risk assessment (orientation towards CS-/ CS+, alert watching with head movements, stretched attending) and freezing (immobility except respiratory movements) was monitored off-line continuously throughout each session (Blanchard et al., 2003; Laxmi et al., 2003; Seidenbecher et al., 2003; Bergado-Acosta et al., 2008; Meis et al., 2008), using the public domain software Wintrack. Thus, the duration of freezing and risk assessment behaviors was determined during context, CS- and CS+ test periods and expressed in percent of the total time (two min each, including stimuli and inter-stimulus intervals). Moreover, immobility periods of >1 s and activity bursts (>20 cm/s, sampling rate 10 Hz) were detected in the same time intervals on-line using a photo beam activity detection system, in order to confirm the reliability of visual inspection. In fact, automatically gained immobility durations and rate-evaluated freezing were significantly correlated in the different experimental groups and retrieval phases, as shown in table 2.6:

Tab. 2.6. Pearson's correlation coefficient (two-tailed) between automatically gained immobility periods and rater evaluated freezing duration. *, significant correlation, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

	Context retrieval	CS+ retrieval	CS- retrieval
No pre-exposure groups	0,811***	0,865***	0,780***
Pre-exposure groups	0,626***	0,751***	0,707***
Foreground context groups	0,580***		

Accordingly, an inverse correlation for risk assessment behavior and activity bursts was observed (table 2.7), which is in line with previous observations (Laxmi et al., 2003):

Tab. 2.7. Pearson's correlation coefficient (two-tailed) between automatically gained activity bursts (>20 cm/s) and rater evaluated duration of risk assessment behavior. *, significant correlation, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

	Context retrieval	CS+ retrieval	CS- retrieval
No pre-exposure groups	-0,725***	-0,804***	-0,797***
Pre-exposure groups	-0,785***	-0,670***	-0,793***
Foreground context groups	-0,449**		

These correlation values confirm the reliability of the rater-derived behavioral measurements.

2.4.3 Data analysis and statistics

For analyzing genotype effects and effects of the different training protocols applied a multiple analysis of variance (MANOVA) for the factors genotype and training group was used, followed by Fisher's PLSD for post hoc comparison. Planned comparisons by one-way-ANOVA were conducted to specify genotype effects as well as different training effects within either one genotype to test for training effect or in one training group to test for genotype effect. To determine cue-specific responses in the retrieval session without an overlay of the background context response, risk assessment and freezing values for the CS- and CS+ were normalized by subtracting the context values from the CS+ and CS- values.

3. Results

3.1 Gene expression analysis

Using Relative Quantification in a Real Time PCR approach, I determined NCAM mRNA levels in subregions of amygdala and hippocampus and their change after fear conditioning.

3.1.1 NCAM mRNA distribution in naïve animals

First, I analyzed the principle subregional distribution of NCAM mRNA in naïve control animals, revealing significant difference of expression levels in subareas of hippocampus and amygdala (ANOVA; $F[5,40] = 2.952$, $p = 0.025$; Fig. 3.1). Highest NCAM mRNA levels (RQ relative to the housekeeping gene PGK) were obtained in the BLA; these were more than twofold higher than in any of the hippocampal subareas ($p = 0.049$ compared to DG, $p = 0.014$ to CA1, and $p = 0.002$ to CA3, Fisher's PLSD) and more than three times higher than in the CeA ($p = 0.008$). The BLA expression levels were also elevated compared to the LA, but failed to reach significant difference ($p = 0.157$).

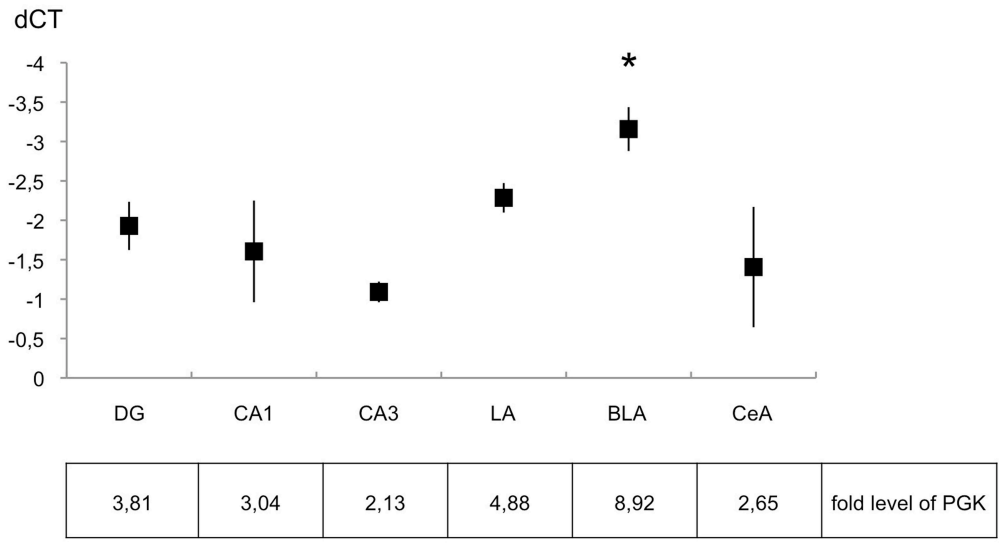

Fig. 3.1. NCAM mRNA expression in subareas of hippocampus and amygdala. NCAM mRNA distribution in naïve animals varies between subareas of the hippocampus (DG, dentate gyrus; CA1, CA3, cornu ammonis 1 and and 3) and amygdala (LA, lateral subnucleus; BLA, basolateral subnucleus; CeA, central subnucleus of the amygdala). A maximum of expression was evident in the BLA. Values in the graph are indicated as dCT (difference of cycle times, compared to the housekeeping gene phosphoglycerate kinase (PGK); mean ± SEM), with more negative values indicating higher NCAM expression. The values below indicate the expression relative to PGK (RQ, relative quantification towards PGK with PGK = 1). *, significant difference of dCT (BLA) compared to dCT of DG, CA1, CA3, CeA, $p < 0.05$.

3.1.2 NCAM mRNA expression after fear conditioning

Subsequently, I investigated effects of different fear conditioning paradigms 6 h after training. Univariate ANOVA reported a training x area interaction ($F[3,51] = 4.103$, $p = 0.012$) in the BLA and LA. Fischer's PLSD confirmed significant effects in the LA, with an increase of NCAM mRNA levels to 2.16x levels of naïve controls upon contextual standard conditioning (Tab. 3.2; $p = 0.049$ to naïve control group; Fig 3.2a). At the same time, NCAM expression in the BLA of contextually conditioned mice was reduced to 0.40x levels of the naïve group ($p = 0.013$) and similarly to 0.41x in the cued overtraining group ($p = 0.012$, compared to the naïve group). The cued standard conditioning group displayed intermediate levels in the BLA with 0.70x of controls, but the change failed to reach significance ($p = 0.335$) (Tab. 3.2; Fig. 3.2b).

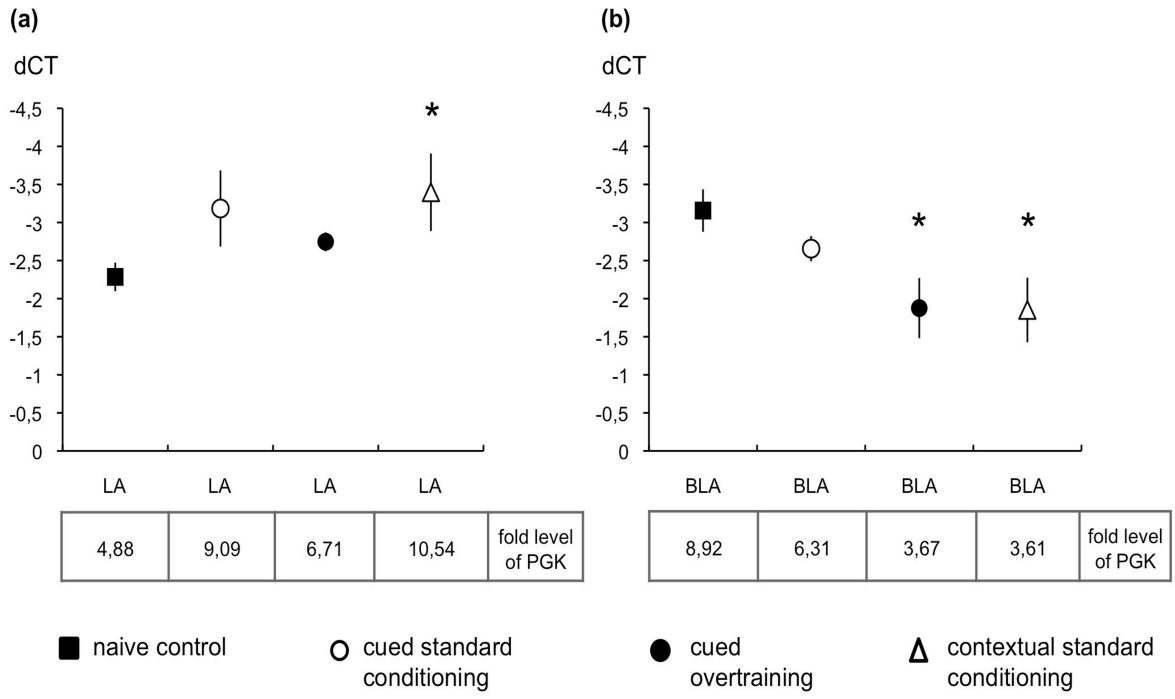

Figure 3.2. NCAM mRNA expression in hippocampus and amygdala 6 h after different fear conditioning with different paradigms. (a) NCAM mRNA expression was increased in the LA after contextual standard conditioning. However, following both, contextual standard conditioning and cued overtraining, reduced levels of NCAM mRNA were observed in the BLA. Values are indicated as dCT. The values below the graphs indicate the relative expression towards PGK (RQ), relative expression compared to the naïve controls is referred in Tab. 3.1. *, significant difference of dCT values compared to dCT of naïve control group, $p < 0.05$.

In contrast, no significant training-dependent changes of NCAM mRNA expression could be observed in hippocampal subareas ($F[6,78] = 0.513$, $p = 0.513$). Table 1 provides an overview of relative NCAM mRNA expression values (RQ) in amygdala and hippocampus subareas after different training situations.

Tab. 3.1. NCAM mRNA expression in subareas of hippocampus and amygdala 6 h after fear conditioning with different paradigms. Relative expression compared to the naïve control group (RQ) demonstrates the increase in NCAM mRNA expression in the LA after contextual standard conditioning and the decrease in the BLA after both, contextual standard conditioning and cued overtraining. No significant expression changes could be detected in any of the other areas. Values are indicated as dCT mean ± SEM and as Relative Quantification (RQ) with naïve controls = 1. *, significant differences of dCT values compared to dCT of naïve control group, $p < 0.05$ (see also Fig. 3.2 a,b).

	naive control		**cued standard conditioning**		**cued overtraining**		**contextual standard conditioning**	
	dCT	RQ	dCT	RQ	dCT	RQ	dCT	RQ
DG	-1,93 ± 0,31	1,00	-2,33 ± 0,21	1,32	-2,20 ± 0,22	1,20	-1,93 ± 0,08	1,00
CA1	-1,61 ± 0,65	1,00	-1,07 ± 0,44	0,69	-1,84 ± 0,26	1,18	-1,63 ± 0,53	1,01
CA3	-1,09 ± 0,13	1,00	-1,16 ± 0,21	1,05	-1,65 ± 0,15	1,47	-1,28 ± 0,42	1,14
LA	-2,29 ± 0,19	1,00	-3,18 ± 0,50	1,86	-2,75 ± 0,12	1,38	-3,40 ± 0,51*	2,16
BLA	-3,16 ± 0,28	1,00	-2,66 ± 0,17	0,71	-1,88 ± 0,40*	0,41	-1,90 ± 0,42*	0,40
CeA	-1,41 ± 0,76	1,00	-1,54 ± 0,82	1,10	-1,52 ± 0,69	1,08	-1,83 ± 0,63	1,34

3.2 Fear memory deficits in NCAM mutant mice

To evaluate the role of NCAM in salience modulation of context conditioning, I fear conditioned $NCAM^{-/-}$ mice and their $NCAM^{+/+}$ littermates in a background context conditioning paradigm with and without pre-exposure, with increased US intensities and in foreground context conditioning paradigms of different intensities. In agreement with previous observations (Laxmi et al., 2003), high levels of freezing behavior were generally observed during retrieval of the CS+, but also during foreground context fear memory recall whereas risk assessment behavior was predominant in retrieval of background context fear memory

(following an auditory cued fear conditioning with CS+/US pairings). Both risk assessment and freezing were evident in response to the CS-. Accordingly, changes in both freezing and risk assessment behavior were considered as adequate behavioral responses during different test situations. This approach revealed that NCAM deficient mice display deficits in both, foreground and background context memory under conditions of increased context salience.

3.2.1 Context fear memory

3.2.1.1 Background context response without pre-exposure

For the freezing response towards the of background context, I observed significant effects of training ($F [2,47] = 43.829$; $p = 0.000$) and a genotype x training interaction ($F [2,47] = 11.599$; $p = 0.000$; but no effect of genotype: $F [1,47] = 0.989$; $p = 0.326$) when conditioning was conducted without prior contextual exposure. Although general freezing levels were low even in $NCAM^{+/+}$ mice, freezing was further reduced in $NCAM^{-/-}$ animals following paired standard training (mean ± SEM in % of time: 7.081 ± 2.579% for $NCAM^{+/+}$ vs. 1.180 ± 0.917% for $NCAM^{-/-}$; ANOVA for effects of genotype in standard training paired group $F [1,17] = 4.648$; $p = 0.047$), which is in line with previous observations (Stork et al., 2000). As expected, unpaired standard training evoked a high background freezing response in animals of both genotypes with even increased freezing in $NCAM^{-/-}$ mice (ANOVA for training in $NCAM^{+/+}$: $F [2,23] = 4.591$; $p = 0.023$; 12.357 ± 2.441%; with Fisher's PLSD $p = 0.007$ to naïve control $NCAM^{+/+}$; ANOVA for training in $NCAM^{-/-}$: $F [2,24] = 89.835$; $p = 0.000$; 24.172 ± 2.256%; $p = 0.000$ to naïve control $NCAM^{-/-}$ and $p = 0.017$ to standard paired trained $NCAM^{-/-}$; ANOVA for effects of genotype in standard training unpaired group: $F [1,16] = 12.439$; $p = 0.003$). Freezing towards the background context in naïve control animals was minimal in animals of both genotypes (1.022 ± 0.694% for $NCAM^{+/+}$ and 0.048 ± 0.048% for $NCAM^{-/-}$; ANOVA for effects of genotype in naïve animals: $F [1,11] = 2.84$; $p = 0.123$).

Risk assessment towards the background context was minimal in naïve control animals as well (mean ± SEM in % of time: 8.578 ± 2.789% for $NCAM^{+/+}$ and 5.567 ± 1.488% for $NCAM^{-/-}$), suggesting successful increase of risk assessment due to standard training (effect of training: $F [2,47] = 21.106$; $p = 0.000$; effect of genotype: $F [1,47] = 0.844$; $p = 0.364$; genotype x training interaction: $F [2,47] = 1.567$; $p = 0.219$) in paired (43.839 ± 8.032% for $NCAM^{+/+}$ and

27.378 ± 8.377% for NCAM$^{-/-}$; p = 0.000 to naïve controls) as well as unpaired fear conditioned animals (48.743 ± 5.758% for NCAM$^{+/+}$ and 53.522 ± 3.676% for NCAM$^{-/-}$; p = 0.000 to naïve controls). A slight increase in risk assessment after unpaired compared to paired standard conditioning was also observed in NCAM$^{-/-}$ mice only (p = 0.004). See Fig. 3.3a,b.

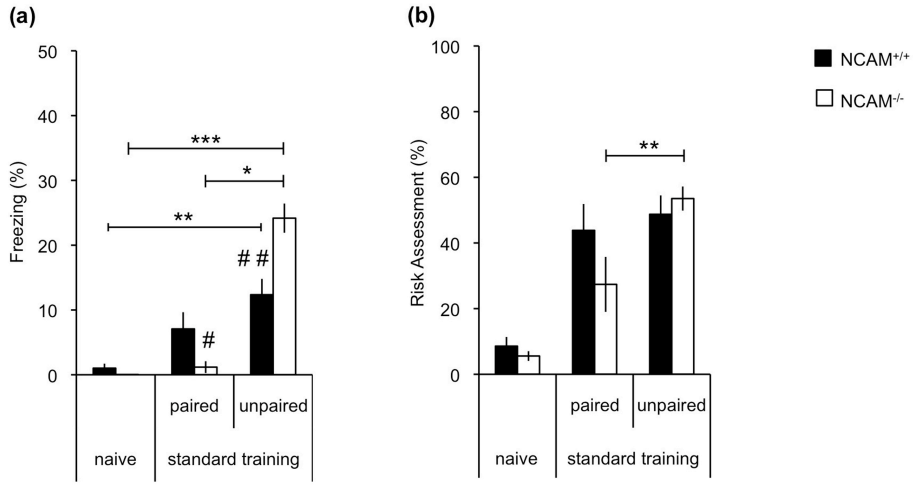

Fig. 3.3. NCAM$^{-/-}$ mice after paired auditory cued standard training without contextual pre-exposure showed severe fear memory deficits compared to their NCAM$^{+/+}$ littermates, confirming previous observations (Stork et al., 2000). These deficits were apparent in freezing towards a background context only (a), whereas no difference between genotypes was observed in risk assessment behavior (b). Values are mean ± SEM. *, significant differences between training groups, p < 0.05; **, p < 0.01; ***, p < 0.001; for improved comprehension, only the most relevant differences between training groups are indicated. #, significant differences between NCAM$^{-/-}$ and NCAM$^{+/+}$ mice, p < 0.05; ##, p < 0.01.

3.2.1.2 Background context response with different training intensities

Pre-exposure before standard training alleviated the fear memory deficits in NCAM$^{-/-}$ mice; no gene-specific effects towards freezing to background context could be observed in all pre-exposed animals, neither following a naïve, a standard training nor an overtraining (paired or unpaired) paradigm, respectively (no effect of the genotype: F [1,87] = 0.929; p = 0.338; no genotype x training interaction: F [4,87] = 0.718; p = 0.582). However, as expected, training intensities affected the freezing response significantly (effect of training: F [4,87] = 17.280; p = 0.000). As in standard training without pre-exposure, I observed high levels of background context freezing following an unpaired training protocol, with increased freezing in unpaired standard trained (17.551 ± 2.985% for NCAM$^{+/+}$ vs. 19.238 ± 4.141% for NCAM$^{-/-}$) and unpaired overtrained animals of both genotypes (16.601 ± 3.671% for NCAM$^{+/+}$ and

23.145 ± 4.237% for NCAM$^{-/-}$) compared to the naïve control group (2.545 ± 1.036% for NCAM$^{+/+}$ and 0.017 ± 0.017% for NCAM$^{-/-}$ in naïve control; p = 0.000 to standard unpaired; p=0.000 to overtraining unpaired) as well as to paired standard trained (5.151 ± 0.820% for NCAM$^{+/+}$ vs. 7.627 ± 1.857% for NCAM$^{-/-}$; p = 0.000 to standard training unpaired; p = 0.000 to overtraining unpaired) and to paired overtrained animals (7.033 ± 1.664% for NCAM$^{+/+}$ and 7.010 ± 1.948% for NCAM$^{-/-}$; p = 0.000 to standard training unpaired; p = 0.000 to overtraining unpaired; but increased compared to naïve control group (p = 0.035)). As no differences between genotypes could be observed in the standard trained group any longer, pre-exposure was able to ameliorate the deficits of NCAM$^{-/-}$ mice in background context memory formation.

This is further confirmed when analyzing risk assessment behavior. Here, significant effects of training (F [4,87] = 15.091; p = 0.000) and genotype were detected (F [1,87] = 15.044; p = 0.000), although the genotype x training interaction failed to reach significant levels (F [4,87] = 2.454, p = 0.053). Detailed analysis of genotype effects in each of the training groups revealed similar levels of risk assessment between NCAM$^{-/-}$ and NCAM$^{+/+}$ mice after standard training with pre-exposure, either in a paired (40.305 ± 7.182% for NCAM$^{-/-}$ vs. 48.852 ± 4.899% for NCAM$^{+/+}$; F [1,20] = 0.998; p = 0.330), or unpaired paradigm (51.601 ± 7.223% for NCAM$^{-/-}$ vs. 51.915 ± 5.071% for NCAM$^{+/+}$; F [1,15] = 0.001; p = 0.972). Interestingly, after intensive fear conditioning in the paired overtraining group, NCAM$^{-/-}$ mice displayed a deficit in background context memory (38.083 ± 5.380% for NCAM$^{-/-}$ vs. 70.647 ± 5.380% for NCAM$^{+/+}$; F [1,20] = 17.546; p = 0.000), that in trend was also detectable after unpaired overtraining (43.634 ± 5.103% for NCAM$^{-/-}$ vs. 56.906 ± 4.824% for NCAM$^{+/+}$; F [1,16] = 3.571; p = 0.078). Furthermore, pre-exposure led to a slight increase in risk assessment in naïve control NCAM$^{+/+}$ mice only; such a familiarization effect was not observed in NCAM$^{-/-}$ mice (3.054 ± 0.489% for NCAM$^{-/-}$ vs. 18.980 ± 4.052% for NCAM$^{+/+}$; F [1,11] = 21.862; p = 0.001). However, in animals of both genotypes risk assessment towards the background context was elevated after paired (p = 0.000) and unpaired standard training (p = 0.000) and after paired (p = 0.000) and unpaired overtraining (p = 0.000) compared to the naïve controls, proving risk assessment as appropriate behavioral read out for successful fear conditioning. See Fig. 3.4a,b.

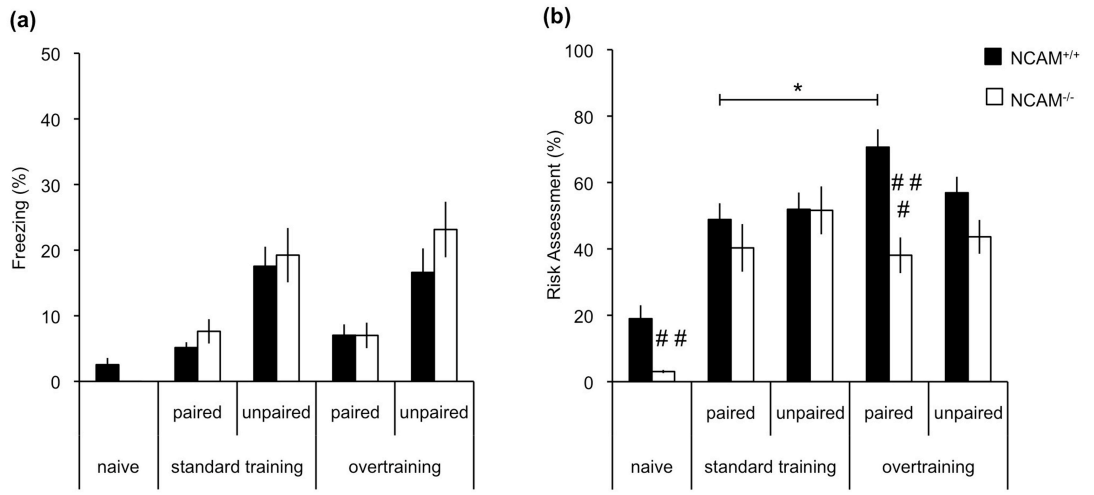

Fig. 3.4. Applying the pre-exposure protocol ameliorated the freezing deficit in NCAM$^{-/-}$ mice after standard training. Even after overtraining, levels of background context freezing were generally low. Unpaired standard training elicited high contextual freezing in mice of both genotypes (a). In contrast, NCAM$^{-/-}$ mice failed to increase risk assessment behavior upon overtraining (b). Values are mean ± SEM. *, significant differences between training groups, $p < 0.05$; for improved comprehension, only the most relevant differences between training groups are indicated. ##, significant differences between NCAM$^{-/-}$ and NCAM$^{+/+}$ mice, $p < 0.01$; ###, $p < 0.001$.

3.2.1.3 Foreground context response with different salience

The deficit in context memory following conditioning with high training intensities was also evident in a foreground contextual conditioning paradigm: I observed a significant effect of training ($F[1,34] = 7.788$, $p = 0.009$) and a strong trend towards genotype effect ($F[1,34] = 3.259$, $p = 0.081$) and genotype x training interaction ($F[1,34] = 3.063$, $p = 0.09$). While NCAM$^{-/-}$ mice trained in a contextual standard paradigm showed no significant deficit in conditioned freezing behavior (4.6 ± 1.0% for NCAM$^{-/-}$ vs. 4.7 ± 1.6% for NCAM$^{+/+}$), mutants failed to significantly increase conditioned freezing upon contextual overtraining (7.1 ± 2.6%), in contrast to their wild type littermates (15.5 ± 3.8%; $p = 0.004$ compared to NCAM$^{+/+}$ mice trained in a contextual standard paradigm). Hence after overtraining, a lower level of contextually induced freezing behavior was evident in NCAM$^{-/-}$ mice compared to their NCAM$^{+/+}$ littermates ($p = 0.02$).

At the same time risk assessment was not significantly affected by genotype ($F[1,34] = 2.581$, $p = 0.119$) or training intensity ($F[1,34] = 1.380$, $p = 0.249$), accordingly, no genotype x training interaction ($F[1,34] = 1.088$, $p = 0.305$) was detected. I observed similar levels of risk assessment in both genotypes subsequently to either contextual standard training

(52.795 ± 5.699% for NCAM$^{-/-}$ vs. 56.230 ± 6.975% for NCAM$^{+/+}$) or contextual overtraining (53.596 ± 6.992% for NCAM$^{-/-}$ vs. 69.755 ± 4.244% for NCAM$^{+/+}$). See Fig. 3.5a,b.

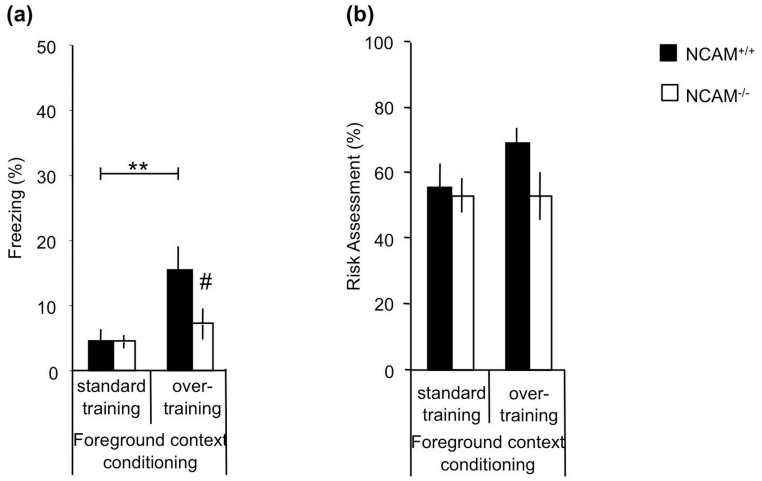

Fig. 3.5. In foreground contextual fear conditioning, overtraining led to a significantly enhanced conditioned freezing behavior in NCAM$^{+/+}$ but not in NCAM$^{-/-}$ mice (a), whereas no difference between genotypes was apparent in risk assessment behavior (b). Values are mean ± SEM. **, significant differences between training groups, $p < 0.01$; for improved comprehension, only the most relevant differences between training groups are indicated. #, significant differences between NCAM$^{-/-}$ and NCAM$^{+/+}$ mice, $p < 0.05$.

3.2.2 Cued fear memory

3.2.2.1 Cued fear memory without pre-exposure

Because of the high interference of background context memory, especially in unpaired fear conditioned animals, to the defensive behavior to the CS+, I performed a normalization of freezing and risk assessment values during the 2 min retrieval phase of the CS+ towards their corresponding background freezing values. After this normalization, the pre-described freezing deficit of NCAM$^{-/-}$ mice towards the CS+ (Stork et al., 2000) was no longer visible, suggesting a context-specific fear memory disturbance in the mutants (effect of training: $F [2,47] = 20.158$; $p = 0.000$; no significant effect of genotype: $F [1,47] = 2.397$; $p = 0.129$; no significant genotype x training interaction: $F [2,47] = 1.060$; $p = 0.356$), although freezing in NCAM$^{-/-}$ mice appeared to be somewhat reduced after paired standard training compared to their wild type littermates (11.989 ± 2.948% for NCAM$^{-/-}$ vs. 18.939 ± 3.042% for NCAM$^{+/+}$).

Furthermore, risk assessment was influenced only by the training protocol applied (effect of training: $F[2,47] = 10.767$; $p = 0.000$; no significant effect of genotype: $F[1,47] = 0.392$; $p = 0.535$; no significant genotype x training interaction: $F[2,47] = 0.620$; $p = 0.543$). The increase of freezing and risk assessment towards the CS+ after paired standard training compared to unpaired trained animals (context-normalized freezing: 4.147 ± 2.145% for $NCAM^{+/+}$ and 2.991 ± 1.628% for $NCAM^{-/-}$; $p = 0.000$; context-normalized risk assessment: 1.769 ± 1.227% for $NCAM^{+/+}$ and 0.171 ± 0.171% for $NCAM^{-/-}$; $p = 0.000$) as well as to the naïve controls (context-normalized freezing: 2.362 ± 0.745 % for $NCAM^{+/+}$ and 1.266 ± 0.780% for $NCAM^{-/-}$; $p = 0.000$; context-normalized risk assessment: 10.378 ± 2.854% for $NCAM^{+/+}$ and 11.169 ± 2.388% for $NCAM^{-/-}$; $p = 0.021$) suggests successful learning of the tone-shock-association. See Fig. 3.6a,b.

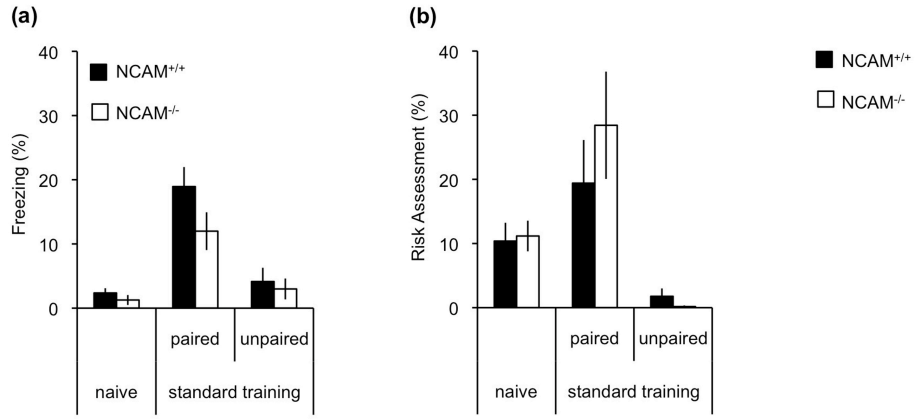

Fig. 3.6. $NCAM^{-/-}$ mice showed no deficit in auditory cued fear response, neither in context-normalized freezing (a) nor in context-normalized risk assessment (b). Low levels of freezing and risk assessment in the unpaired standard and the naïve control group suggested successful conditioning to the CS+. Values are mean ± SEM.

3.2.2.2 Cued fear memory with different training intensities

Applying fear conditioning with different training intensities subsequently to a pre-exposure protocol affected the fear memory response towards the CS+ (context-normalized) in a genotype-independent fashion (effect of training: $F[4,87] = 49.476$; $p = 0.000$; no effect of genotype: $F[1,87] = 0.401$; $p = 0.528$; or genotype x training interaction: $F[4,87] = 0.760$; $p = 0.555$). As demonstrated before (Laxmi et al., 2003), paired overtraining could increase freezing towards the CS+ (29.473 ± 2.879% for $NCAM^{+/+}$ and 32.314 ± 3.307% for $NCAM^{-/-}$) in comparison with paired standard training (17.885 ± 2.825% for $NCAM^{+/+}$ and 19.025 ± 2.400% for $NCAM^{-/-}$; $p = 0.000$). As expected and shown before in the no

pre-exposure groups, only low freezing levels could be detected in the naïve control animals (6.922 ± 2.038% for $NCAM^{+/+}$ and 1.064 ± 0.610% for $NCAM^{-/-}$; p = 0.000 to standard training paired; p = 0.000 to overtraining paired), as well as after unpaired standard training (3.797 ± 1.790% for $NCAM^{+/+}$ and 1.412 ± 0.688% for $NCAM^{-/-}$; p = 0.000 to standard training paired; p = 0.000 to paired overtraining) or unpaired overtraining (4.875 ± 2.378% for $NCAM^{+/+}$ and 3.914 ± 1.728% for $NCAM^{-/-}$; p = 0.000 to standard training paired; p = 0.000 to overtraining paired), confirming specificity of the auditory fear memory even after pre-exposure and overtraining procedures.

Risk assessment (again normalized to background context risk assessment) towards the CS+ was low in all training groups following pre-exposure, but further reduced in the unpaired standard trained (p = 0.043 to naïve controls) and the unpaired overtrained animals (p = 0.01 to naïve controls; p = 0.018 to standard training paired; p = 0.038 to overtraining paired; with effect of training: F [4,87] = 2.544; p = 0.046), supporting the specificity of defensive behavior in auditory cued fear conditioning with pre-exposure and overtraining. After paired overtraining the $NCAM^{-/-}$ mice showed even more risk assessment towards the CS+ than their wildtype littermates (15.144 ± 4.217% for $NCAM^{-/-}$ vs. 2.098 ± 1.391% for $NCAM^{+/+}$; effect of genotype: F [1,87] = 6.322; p = 0.014; no genotype x training interaction: F [4,87] = 0.836; p = 0.506; gene effect in overtraining paired group only: F [1,20] = 6.689; p = 0.018), further confirming the context-specificity of the fear memory deficit in the mutants. On the other hand, the decreased risk assessment response in $NCAM^{+/+}$ mice is reflected by high contextual freezing values, since total values for risk assessment (context + cue) were comparable between genotypes (51.872 ± 0.872% for $NCAM^{+/+}$ and 47.988 ± 3.232% for $NCAM^{-/-}$). In all other pre-exposed training groups no gene effect was observed (naïve control: 14.241 ± 2.557% for $NCAM^{-/-}$ vs. 11.102 ± 5.081% for $NCAM^{+/+}$; F [1,11] = 0.499; p = 0.496; standard training paired: 12.618 ± 5.310% for $NCAM^{-/-}$ vs. 8.816 ± 3.875% for $NCAM^{+/+}$; F [1,20] = 0.5; p = 0.488; overtraining unpaired: 2.933 ± 0.970% for $NCAM^{-/-}$ vs. 1.133 ± 1.133% for $NCAM^{+/+}$; F [1,16] = 1.418; p = 0.252), including the standard training unpaired group, where risk assessment was low in $NCAM^{+/+}$ as well, but without reaching significant difference (8.600 ± 5.429% for $NCAM^{-/-}$ vs. 0.0 ± 0.0% for $NCAM^{+/+}$; F [1,15] = 2.509; p = 0.136). See Fig. 3.7a,b.

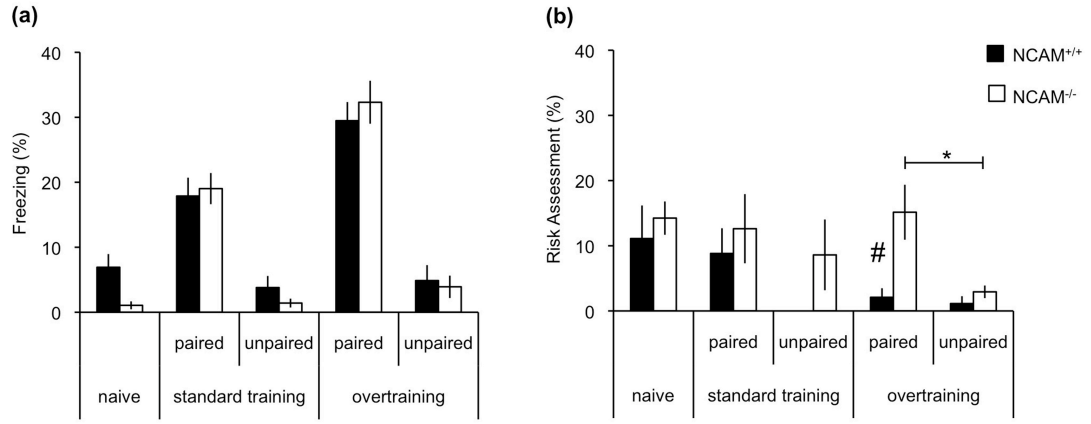

Fig. 3.7. Also with pre-exposure, there was no deficit in NCAM$^{-/-}$ mice towards the CS+ response obvious. Paired overtraining enhanced the freezing response in a genotype-independent fashion, suggesting context-specificity of the deficit in NCAM$^{-/-}$ mice (a). As freezing is the dominant response towards the CS+, risk assessment was generally low in all training groups and further decreased in the NCAM$^{+/+}$ mice after paired overtraining (b). Values are mean ± SEM. *, significant differences between training groups, $p < 0.05$; for improved comprehension, only the most relevant differences between training groups are indicated. #, significant differences between NCAM$^{-/-}$ and NCAM$^{+/+}$ mice, $p < 0.05$.

3.2.3. Generalized fear response

As demonstrated before by Bergado-Acosta et al. (2008), generalization of auditory fear memory towards a neutral CS- occurs in wild types in a context- and training intensity-dependent manner. Therefore, this neutral test stimulus was included to my analysis. As done for the CS+, cue-specific responses were dissected by subtracting the individuals' background context freezing and risk assessment values.

3.2.3.1 Generalized fear memory without pre-exposure

Following fear conditioning without pre-exposure, the generalized freezing response towards the CS- was significantly affected by training (effect of training: $F[2,47] = 8.085$; $p = 0.001$) and genotype (effect of genotype: $F[1,47] = 5.339$; $p = 0.026$), whereas a genotype x training interaction failed to reach significant levels ($F[2,47] = 2.673$; $p = 0.081$). Using ANOVA in each training group for planned comparison of the genotypes revealed a significant deficit of NCAM$^{-/-}$ mice in CS- -induced freezing after paired standard training (3.260 ± 1.994% for NCAM$^{-/-}$ vs. 11.729 ± 3.132% for NCAM$^{+/+}$; $F[1,17] = 5.203$; $p = 0.037$). No such genotype-specific effects were obvious after unpaired standard training (0.135 ± 0.135% for

$NCAM^{-/-}$ vs. 2.732 ± 1.355% for $NCAM^{+/+}$; F [1,16] = 3.213; p = 0.093) or in the naïve control group (0.806 ± 0.450% for $NCAM^{-/-}$ vs. 0.638 ± 0.470% for $NCAM^{+/+}$; F [1,11] = 0.064; p = 0.806). In animals of both genotypes freezing levels are low following unpaired standard training or the naïve control paradigm, suggesting specific intramodal generalization in classical auditory fear conditioning only. Moreover, in contrary to their $NCAM^{+/+}$ littermates (p = 0.006 compared to naïve, p = 0.008 compared to unpaired), $NCAM^{-/-}$ mice failed to enhance their freezing response subsequently to paired standard training, thereby paralleling the contextual memory deficit.

Likewise, I found elevated levels of generalized risk assessment following paired standard training without pre-exposure (13.534 ± 6.177% for $NCAM^{+/+}$ and 14.434 ± 3.849% for $NCAM^{-/-}$). However, only effects of training (F [2,47] = 7.410; p = 0.002), but neither effects of genotype (F [1,47] = 0.262; p = 0.611) nor a genotype x training interaction (F [2,47] = 0.076; p = 0.927) could be observed. Again, generalized risk assessment was only minimal after unpaired standard training (0.185 ± 0.185% for $NCAM^{+/+}$ and 3.324 ± 2.210% for $NCAM^{-/-}$; p = 0.001 compared to paired standard training) and in the naïve controls (3.050 ± 0.942% for $NCAM^{+/+}$ and 3.621 ± 1.008% for $NCAM^{-/-}$; p = 0.007 compared to paired standard training). See Fig. 3.8a,b.

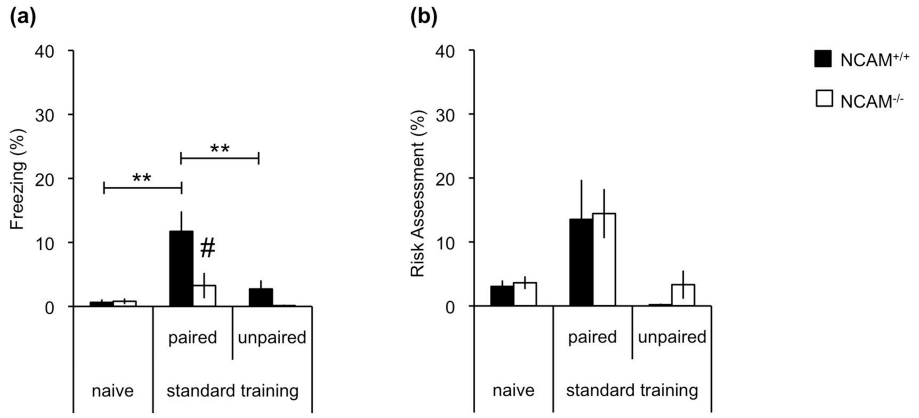

Fig. 3.8. After paired training without pre-exposure $NCAM^{-/-}$ mice displayed impaired generalized freezing to the CS- (a). In contrary, risk assessment to the CS- was equally increased in animals of both genotypes following paired standard. Values are mean ± SEM. **, significant differences between training groups, p < 0.01; for improved comprehension, only the most relevant differences between training groups are indicated. #, significant differences between $NCAM^{-/-}$ and $NCAM^{+/+}$ mice, p < 0.05.

3.2.3.2 Generalized fear memory with different training intensities

Applying pre-exposure, the generalization of fear memory subsequently to standard training was ameliorated. Here, the generalized freezing response towards the CS- was affected by training intensities only ($F[4,87] = 7.426$; $p = 0.000$), but not by genotype ($F[1,87] = 0.077$; $p = 0.783$), or by a genotype x training interaction ($F[4,87] = 0.746$; $p = 0.746$). Analysis of the training effects demonstrated that pre-exposure elicited low freezing levels after standard training, either paired ($3.169 \pm 1.07\%$ for $NCAM^{+/+}$ and $4.812 \pm 1.330\%$ for $NCAM^{-/-}$) or unpaired ($1.605 \pm 1.072\%$ for $NCAM^{+/+}$ and $1.774 \pm 0.801\%$ for $NCAM^{-/-}$), and almost not visible in the naïve control groups ($1.62 \pm 1.284\%$ for $NCAM^{+/+}$ and $0.227 \pm 0.227\%$ for $NCAM^{-/-}$). However, fear conditioning with increased training intensities in the paired overtraining group enhanced the generalized freezing response significantly ($7.546 \pm 2.012\%$ for $NCAM^{+/+}$ and $9.8 \pm 2.711\%$ for $NCAM^{-/-}$; with $p = 0.002$ to paired standard training; $p = 0.000$ to unpaired standard training; $p = 0.000$ to naïve controls). Moreover, this effect was cue-specific since no such increase could be observed after unpaired overtraining ($2.825 \pm 1.121\%$ for $NCAM^{+/+}$ and $1.628 \pm 0.798\%$ for $NCAM^{-/-}$; $p = 0.000$ to overtraining paired). Thereby, the described effects suggest a dependence of CS- generalization on context and stimulus intensity as observed previously (Bergado-Acosta et al., 2008).

Accordingly, risk assessment to CS- was only elevated in the paired overtraining group ($6.944 \pm 3.244\%$ for $NCAM^{+/+}$ and $18.213 \pm 4.038\%$ for $NCAM^{-/-}$; effect of training: $F[4,87] = 3.587$; $p = 0.01$) compared to the other training groups (naïve: $3.610 \pm 2.728\%$ for $NCAM^{+/+}$ and $5.740 \pm 1.666\%$ for $NCAM^{-/-}$; $p = 0.013$; standard training paired group: $5.310 \pm 2.871\%$ for $NCAM^{+/+}$ and $9.002 \pm 4.482\%$ for $NCAM^{-/-}$; $p = 0.029$; standard training unpaired group: $1.165 \pm 0.910\%$ for $NCAM^{+/+}$ and $4.039 \pm 1.549\%$ for $NCAM^{-/-}$; $p = 0.001$; overtraining unpaired group: $3.050 \pm 1.866\%$ for $NCAM^{+/+}$ and $3.645 \pm 2.001\%$ for $NCAM^{-/-}$; $p = 0.001$). Interestingly, I observed an effect of genotype on generalized risk assessment behavior ($F[1,87] = 4.096$; $p = 0.046$), but no genotype x training interaction ($F[4,87] = 0.972$; $p = 0.428$). A detailed analysis of the training effects for each genotype revealed that only $NCAM^{-/-}$ mice showed increased generalization after paired overtraining ($p = 0.048$ compared to standard training paired; $p = 0.004$ compared to overtraining unpaired). However, planned comparisons could not detect significant effects of genotype in the single training groups (naïve controls: $F[1,11] = 0.499$; $p = 0.496$; paired standard training: $F[1,20] = 0.5$; $p = 0.488$; unpaired standard training: $F[1,15] = 2.56$; $p = 0.132$; paired overtraining $F[1,20] = 4.265$; $p = 0.053$; unpaired overtraining: $F[1,16] = 0.047$; $p = 0.831$). See Fig. 3.9a,b.

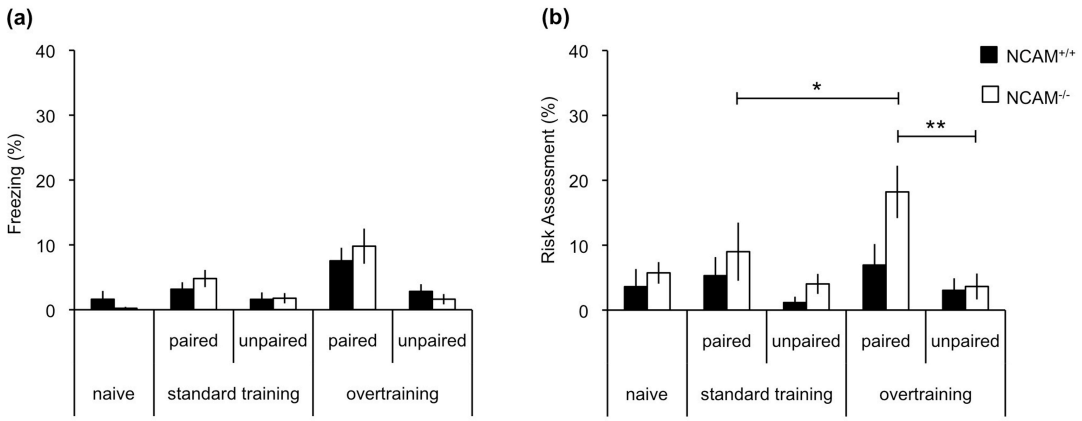

Fig. 3.9. Applying pre-exposure ameliorated the previously observed mutant deficit to enhance their generalized freezing response upon paired standard training. Moreover, cued overtraining caused intramodal generalization with similarly enhanced fear response to the CS- in both genotypes (a). After pre-exposure, a generalized risk assessment response to the CS- was observed in paired overtrained animals only. Hence, a detailed analysis revealed that mainly NCAM$^{-/-}$ mice contributed to this effect. Values are mean ± SEM. *, significant differences between training groups, $p < 0.05$; * $p < 0.01$; for improved comprehension, only the most relevant differences between training groups are indicated

4. Discussion

The neural cell adhesion molecule NCAM is thought to play a key role in adaptive and maladaptive cellular changes in the central nervous system that underlie memory formation, stress response and changes in emotional state (Sandi, 2004). This study provides evidence for an involvement of NCAM in amygdalo-hippocampal interactions during consolidation of fear memory by demonstrating expression changes of NCAM mRNA in the amygdala of fear conditioned mice and disturbance of amygdala-mediated modulation of hippocampus-dependent fear memory achieved by applying either pre-exposure or overtraining. Accompanying work by Jorge R. Bergado-Acosta (Albrecht et al., 2009) demonstrated a reduced amygdalo-hippocampal theta synchronization during fear memory retrieval of NCAM null mutant mice. Together, these data suggest an involvement of NCAM-mediated processes in information processing of the amygdalo-hippocampal system and in the amygdala-dependent modulation of contextual fear memory according to stimulus salience.

4.1. Amygdala NCAM mRNA expression is regulated by fear conditioning in a context- and training-intensity-dependent manner

Various studies have demonstrated before that event-related changes in NCAM expression depend on the time period past the events, the stress intensity and the brain regions observed. Most studies conducted expression analysis on the protein level of NCAM and its polysialylated form, PSA-NCAM, in hippocampal subareas (Bisaz et al., 2009a). It could be demonstrated that PSA-NCAM protein expression is increased in the DG 24 h after contextual fear conditioning and it is even further enhanced when conditioning occurs with high training intensities (Lopez-Fernandez et al., 2007). However, at a 12 h posttraining time point PSA-NCAM levels are decreased after intensive training but enhanced after moderate fear conditioning (Sandi et al., 2003). In synaptosomal preparations from the whole hippocampus NCAM protein levels are reduced 12 h after contextual fear conditioning; PSA-NCAM is also reduced at this time point, but only after intensive training. 24 h later again only intensive training reduces PSA-NCAM, but now NCAM is increased in this group (Merino et al., 2000). As these data demonstrate, increasing the spatial resolution unwinds a differential regulation pattern. So far, expression changes on the transcriptional level were

analyzed only after chronic stress exposure (21 days) in hippocampal subregions (Venero et al., 2002), but not in relation to learning events, and information about NCAM expression regulation especially in the amygdala is still rare (Markram et al., 2007b). Now in this study, to gain insights in learning-induced NCAM mRNA expression regulation, subareas of amygdala and hippocampus were isolated per laser capture microdissection and NCAM mRNA levels were quantified with real time RT-PCR six hours after applying different fear conditioning paradigms. This time point - six hours after training - was chose because the fear memory is in its consolidation stage. During consolidation, transcriptional regulators of memory-related intracellular transductional pathways are activated, as described for the extracellular signal-regulated kinase (ERK) 1/2 (Trifilieff et al., 2007). Six hours after fear conditioning, ERK1/2 is activated in LA, BLA and CA3 in a discriminative pattern related to foreground vs. background conditioning. Moreover, six hours after training a strong induction of mRNA expression of various molecular factors takes place (Stork et al., 2001; Stork et al., 2004; Mei et al., 2005; Bergado-Acosta et al., 2008).

As a first step, I analyzed NCAM expression profiles in amygdalar and hippocampal subregions of naïve control mice. I observed high NCAM mRNA levels in the LA and BLA, suggesting a strong transcriptional control of NCAM in these areas. Accordingly, following foreground context conditioning, NCAM mRNA expression was moderately increased in the LA and, more prominent, decreased in the BLA. Similarly, after cued overtraining, a procedure known to evoke generalization towards the background context in auditory cued fear conditioning (Laxmi et al., 2003), NCAM mRNA levels were decreased in the BLA as well. Markram and colleagues (Markram et al., 2007b) reported an increased expression of PSA-NCAM subsequent to intensive auditory cued fear conditioning. Although these observations seem contradictory at the first glance, both effects might serve similar cellular functions as reducing the expression of the core protein as well as increasing polysialylation would decrease NCAM-mediated cell adhesion. However, neither acute pre- and posttraining removal of PSA nor a genetically induced deficiency in polysialylation in ST8SialIV mutant mice disturbed auditory cued fear conditioning (Markram et al., 2007a; Markram et al., 2007b). On the contrary, NCAM null mutant mice and mice that overexpress a soluble extracellular fragment of the NCAM molecule display deficits in contextual and cued fear memory (Stork et al., 2000; Pillai-Nair et al., 2005). Together, the findings suggest that NCAM function in the amygdala is predominantly determined by expression of the core protein rather than its polysialylation status.

No expression regulation on the NCAM mRNA level could be observed in the hippocampal subregions. Until now, there is evidence that NCAM function in the hippocampus is regulated downstream of mRNA transcription through posttranslational modifications such as polysialylation and also ubiquitination. It has been demonstrated that deficits in LTP in CA1 region of hippocampus and contextual fear memory deficits in NCAM null mutant mice can be rescued by injection of PSA-NCAM but not by an extracellular NCAM fragment alone (Senkov et al., 2006). Accordingly, an upregulation of PSA-NCAM is observed subsequently to contextual fear conditioning and enzymatic removal of hippocampal PSA abolishes the contextual fear response (Lopez-Fernandez et al., 2007). Another hippocampus-dependent learning task, Morris water maze, relies on PSA-NCAM as well, since mice lacking the polysialylating enzyme ST8SialIV show deficits in this task, but not in auditory cued fear conditioning as mentioned above (Markram et al., 2007a). Moreover, six hours after passive avoidance training, internalization and ubiquitination of the NCAM protein in the DG is observed, revealing an additional learning-induced mechanism to regulate cell adhesion on NCAM protein levels (Foley et al., 2000).

Together, these results suggest a regulation of NCAM expression on different levels - transcriptional, translational and posttranslational. Therefore, it will be interesting to uncover the cellular specificity of these changes and their distinct contribution on memory formation in hippocampus and amygdala. Thus, further microdissection studies addressing smaller subregions or additional time points may allow to dissect the pathways of fear memory related NCAM transcriptional changes more precisely. So the contribution of NCAM mRNA to hippocampal cellular processes could be resolved in detail.

However, a robust expression change identified in my experimental groups was the reduction of NCAM mRNA levels in the BLA after cued overtraining as well as contextual standard conditioning. Indeed, fear conditioning with increased salience increases background context fear memory (Laxmi et al., 2003), whereas the contextual standard paradigm establishes fear memory formation towards a foreground context. Both processes require amygdalo-hippocampal interactions. According to the classical view of fear conditioning, contextual aspects of fear memory are mediated by the hippocampus, whereas the amygdala is required for both, contextual and cued fear conditioning (Phillips and LeDoux, 1992). However, such amygdalo-hippocampal interaction is not static. In a subsequent study, Philips and LeDoux (1994) demonstrated that background but not foreground context conditioning depends on the integrity of the dorsal hippocampus. Moreover, the interplay between lateral and basolateral subnuclei of the amygdala contributes to the balance of foreground vs.

background context memory, whereas foreground context memory can be enhanced by lateral amygdala lesion (Calandreau et al., 2005). Likewise, plasticity-relevant molecular factors as ERK1/2 are expressed in a regional specific pattern following different fear conditioning protocols (Trifilieff et al., 2007), thus reflecting differential activation of amygdalar and hippocampal subregions. However, my data do not show any significant change of NCAM expression in the dorsal hippocampus after either foreground context conditioning or background context conditioning of different intensities. The BLA, in contrast, showed a comparable reduction in NCAM expression in both paradigms. The BLA is a critical structure for contextual learning towards both, the foreground and the background context (Calandreau et al., 2005; Trifilieff et al., 2007; Maren, 2008). It features extensive reciprocal and partly direct connectivity to the hippocampal formation (Pitkänen et al., 2000) and appears to be suitable to mediate such contextual aspects of fear memory (Maren and Fanselow, 1995; Akirav and Richter-Levin, 2002 ; Malin and McGaugh, 2006). On the other hand, various studies have shown that the BLA mediates effects of behavioral stress and of the stress hormones corticosterone and norepinephrine on hippocampal plasticity and hippocampus-dependent learning (Akirav and Richter-Levin, 2002; Roozendaal et al., 2006b; Vouimba et al., 2007). Together with the expression data, these findings suggest a role of NCAM-mediated cell recognition in the mediation and salience-dependent modulation of hippocampus-mediated background and foreground context fear memory through the BLA.

4.2 NCAM$^{-/-}$ mice display a contextual fear memory deficit that can be overcome by pre-exposure

The results from the expression analysis of NCAM mRNA indicate an involvement of NCAM background and foreground context conditioning mediated by the BLA; further, it highlights the role of the BLA in amygdalo-hippocampal interaction and the contextual modulation of fear memory. Huff et al. (2005) showed that pre-exposure can render a hippocampal context representation independent of modulation through the amygdala. Applying pre-exposure in an auditory cued fear conditioning paradigm in mice deficient for the NCAM molecule allows to determine the contribution of the NCAM core protein to cued and background fear memory. For analyzing the behavioral outcome of altered NCAM function, the fear memory was tested at a consolidated stage 24 h after training. In contrast, the gene expression data was obtained six hours after training in order to evaluate the contribution of NCAM during consolidation. A

first set of experiments applying standard training without a pre-exposure protocol reproduced the previously observed context fear memory deficit of NCAM$^{-/-}$ mice (Stork et al., 2000). In all further experiments, I employed a pre-exposure protocol in which mice were confronted with the conditioning context and a neutral auditory stimulus before conditioning took place. In wild types only the generalized fear response towards the CS- was altered, but there was no interference with cued or contextual fear memory, hence excluding an induction of latent inhibition by the pre-exposure paradigm (Laxmi et al., 2003; Wheeler et al., 2003).
However, pre-exposure allowed the NCAM$^{-/-}$ mice to overcome the previously reported fear memory deficits (Stork et al., 2000). As pre-exposure is not related to painful events, a context-specific memory deficit rather than potential differences in pain threshold detection in the mutants is to be held accountable for the observed behavioral differences. Furthermore, applying pre-exposure would also decrease the stress level the mice experience entering the training session, because, amongst others, the animals are handled and familiarized with the environment. In NCAM$^{-/-}$ mice increased stress levels imposed during training in a novel environment may have specifically interfered with the (strongly amygdala-dependent) acquisition and / or consolidation of fear memory. Contextual pre-exposure would abrogate this disturbance.

4.3 NCAM$^{-/-}$ mice failed to increase their contextual fear response after salient overtraining

Accordingly, chronic or acute stress pre-treatment (Sandi et al., 2001; Cordero et al., 2003; Rodríguez Manzanares et al., 2005) and post-training administration of the stress hormones corticosterone and norepinephrine facilitate cue and context memory formation (Roozendaal, 2002). Thereby, the BLA is thought to mediate such stress effects on hippocampus-dependent memory (Conrad, 2005; Roozendaal et al., 2006b).
Following this line of evidence NCAM mutant mice and their wild type littermates underwent salient overtraining. Cued overtraining with a high number of tone-foot shock-pairings at increased shock intensities enhanced the fear response towards the cue and, additionally, to the background context (Laxmi et al., 2003), suggesting enhanced involvement of the amygdala (Maren, 2000; Pelletier et al., 2005). This is also reflected by increased anxiety levels following overtraining as described by Laxmi et al. (2003). Indeed, upon overtraining NCAM$^{+/+}$ mice enhanced their conditioned fear response, but again NCAM$^{-/-}$ animals failed to

increase the behavioral response to the context. In detail, NCAM$^{-/-}$ mice displayed decreased levels of freezing during retrieval of foreground context conditioning and decreased levels of risk assessment during retrieval of the background context after auditory cued conditioning.

Both, risk assessment and freezing, are forms of defensive behavior displayed by mice according to the intensity and proximity of a given threat (Blanchard et al., 2003; Laxmi et al., 2003). In this line, freezing is expressed in response to more salient events, i.e. the CS+ after auditory cued fear conditioning or the conditioning chamber after foreground context conditioning, whereas risk assessment occurs predominately during retrieval of the less salient background context. According to the corresponding retrieval test NCAM$^{-/-}$ mice were able to display the predominant type of defensive behavior appropriately (freezing > risk assessment during CS+ and foreground context, freezing < risk assessment during background context), suggesting that NCAM$^{-/-}$ mice are principally able to adapt to an aversive condition and to form specific fear memories. In fact, they adequately increased freezing to the CS+ and generalized freezing and risk-assessment to the CS- following cued overtraining comparable to their wild type littermates. Together, these observations strongly support the idea that NCAM null mutation evokes deficits in the salience-dependent modulation of context fear memory through the amygdala.

The mossy fiber system in the CA3 region of the hippocampus contributes to the acquisition and consolidation of contextual fear memory (Daumas et al., 2004). In NCAM deficient mice a disturbed fasciculation of the mossy fiber tract and laminar growth of CA3 occurs (Cremer et al., 1998), which may adversely affect CA3 function in the consolidation of contextual fear memories. However, this structural abnormality cannot explain the salience-dependence of the observed NCAM mutant deficits in context fear memory, nor their deficits in cued fear memory when lacking contextual pre-exposure. In summary, these results suggest salience-dependent deficits in amygdalo-hippocampal information processing.

Very recently, Bisaz and Sandi (2010) analyzed auditory cued fear conditioning in conditional NCAM mutant mice, in which NCAM is abolished in glutamatergic forebrain neurons within the second postnatal week. In this mice freezing to CS+ is reduced following auditory cued fear conditioning with a protocol resembling my standard conditioning without pre-exposure. Deficits to the cue were described in constitutive NCAM$^{-/-}$ mice as well (Stork et al., 2000), but after correction for background freezing they were not longer obvious in this present study. Interestingly, the conditional NCAM mutant mice do not display the morphological alterations shown in constitutive NCAM knock out mice (Bukalo et al., 2004), adding further evidence for fear conditioning deficits independent from altered CA3 morphology.

Furthermore, chronic uncontrollable stress does not affect freezing towards an auditory cue in conditional NCAM mutants. However, Bisaz and Sandi (2010) mention increased freezing towards the neutral context 24 h after training in chronically stressed conditional NCAM knock out mice. This could point towards a generalization of background contextual memory, but testing explicitly foreground and background contextual fear memory formation with adequate protocols is required to resolve this issue. Also, these results appear to be rather contra-intuitive to the observation that fear conditioning with increased salience would decrease the contextual fear response in constitutive mutant mice. However, one should bear in mind that chronic unpredictable stress applied in the study from Bisaz and Sandi and acute innate stress that may result from increased salience of the conditioning stimuli trigger different molecular events and morphological changes in distinct brain areas (Sandi, 2004; McLaughlin et al., 2007), hence complicating a direct comparison of the results from Bisaz and Sandi and the results obtained in the present study. Nevertheless, several unique features of the conditional mutants including lack of morphological changes, a relative polysialylation level comparable to their wild type littermates, and reduced but not completely abolished NCAM levels make them a valuable tool to further investigate the role of NCAM in salience-dependent modulation of fear memory formation.

4.4 Disturbance of amygdalo-hippocampal Theta synchronization in NCAM$^{-/-}$ mice

To gain insight into potential changes in neural activity patterns in the amygdalo-hippocampal pathway, Jorge Ricardo Bergado-Acosta analyzed theta synchronization in overtrained NCAM mutant mice amendatory to the behavioral testing described above (Albrecht et al., 2009). During retrieval of fear memory, amygdala and hippocampus synchronize their neuronal activity at the theta frequency range, as it can be measured by recording of field potentials in freely behaving animals (Seidenbecher et al., 2003). This phase-locking between the two key regions of fear conditioning occurs in either cued or the contextual fear memory at a long term stage and may reflect thereby transient changes in ensemble activity in relation to memory consolidation and circuit refinement within the amygdalo-hippocampal system (Narayanan et al., 2007). Most likely, theta synchronization between amygdala and hippocampus is strongly influenced by stress factors during fear memory consolidation and retrieval, since stress is able to enhance the power of hippocampal theta activity via

mineralocorticoid receptor activation (Murphy et al., 1998) and induces theta activity in the amygdala (Pare and Collins, 2000).

Indeed, after intensive overtraining, $NCAM^{+/+}$ mice showed pronounced theta synchronization throughout the retrieval session, accompanied by high freezing responses towards the background context and the CS+. On the contrary, $NCAM^{-/-}$ mice after overtraining displayed only low levels of amygdalo-hippocampal theta synchronization towards the background context along with diminished contextual freezing compared to their wild type littermates. These results can further support the hypothesis of disturbed information processing within the amygdalo-hippocampal pathway. Furthermore, the described reduction in amygdalo-hippocampal theta synchronization was observed not only during contextual but also during cued retrieval despite any accompanying deficits in freezing towards the CS+ or the CS-. This is in agreement with the observations by Seidenbecher et al. (2003) where similar levels of theta synchronization during cued and contextual fear memory retrieval have been described. Additionally, cue-related synchronization was also reduced in a contextual blocking experiment (Narayanan, Stork and Pape, unpublished observations) providing further evidence for the contextual nature of the information relayed by this pathway. On the other hand were the electrophysiological changes in $NCAM^{-/-}$ mice not restricted to conditions of behavioral dysfunction. Thus, the genetic alterations in $NCAM^{-/-}$ mice may induce a disturbance in network function that is not consequential, but may rather be causal to the observed fear memory deficits. Interestingly, untreated, naive $NCAM^{-/-}$ mice display a reduction of the phosphorylated form of the cAMP response element binding protein (CREB) in the BLA and the CA3 (Aonurm-Helm et al., 2008). These basic changes in the molecular configuration of NCAM mutant mice suggest an additional causality factor for the fear memory deficits on a cellular level.

4.5 Conclusion

The findings presented here provide insight into the involvement of NCAM in amygdalo-hippocampal interactions during the consolidation of fear memory applying distinct levels of analysis. Using laser capture microdissection and real time PCR, NCAM mRNA expression changes during the consolidation phase of fear memory were unraveled. Hence, different fear conditioning paradigms induced expression changes in subregions of the amygdala; conditioning to the foreground context increased NCAM mRNA levels in the LA,

whereas expression was reduced in the BLA after both, foreground context conditioning and cued overtraining. This suggests the involvement of NCAM in amygdala-dependent memory formation. Moreover, a similar regulation pattern in the BLA, a key structure for interaction with the hippocampus, in contextual conditioning and stress-dependent modulation of fear memory points towards a function of NCAM in amygdalo-hippocampal interaction. The behavioral analysis of NCAM mutant mice provides further evidence for this relationship. Increasing the salience of cue and context and thereby the activation of the amygdala resulted in a relative deficit of hippocampus-dependent fear memory in $NCAM^{-/-}$ mice compared to their wild type littermates. So, contextual pre-exposure, a procedure that diminishes the activation of the amygdala, abolished the fear memory deficit of $NCAM^{-/-}$ mice towards the background context. In contrast, overtraining, which enhances amygdalar activation during training, resulted in a fear memory deficit in $NCAM^{-/-}$ mice displayed towards either the foreground or the background context, but not towards the auditory cue. The disturbance of the amygdalo-hippocampal system suggested by these findings was further confirmed by *in vivo* electrophysiological experiments conducted in collaboration with Jorge R. Bergado-Acosta. There, reduced amygdalo-hippocampal theta synchronization was observed in $NCAM^{-/-}$ mice during retrieval of fear memory.

Together, these findings strongly suggest an involvement of NCAM-mediated processes in the amygdala in determination or coding of salience information during contextual fear memory formation through an interplay with the hippocampus.

4.6 Clinical Implications

Classical Fear conditioning is a widely used paradigm for modeling anxiety orders. Within this group of neuropsychiatric disorders including generalized anxiety disorder, phobia, panic disorder and posttraumatic stress disorder (PTSD) a disturbed salience determination and altered balance between fear memory components is evident (Rau et al., 2005; Garakani et al., 2006).

Until now a direct link for genetic association of NCAM and anxiety disorders in human is still missing. However, previous studies reported increased anxiety-like behavior, serotonergic hypersensitivity and impulsivity of NCAM mutants (Stork et al., 2000). Also, a dysregulation of NCAM was demonstrated in a juvenile stress model of PTSD (Tsoory et al., 2008). PTSD is elicited by a traumatic, stressful event of great salience for the individual. The

role of NCAM in salience coding suggested by the results of the presented study therefore add further evidence for an involvement of NCAM in the pathology of PTSD. On the other hand, the well established responsiveness of NCAM to stress refers to a contribution of NCAM in depression, a mood disorder which can appear in co-morbidity with PTSD, and which is also linked to (chronic) stressful experiences (Sandi and Bisaz, 2007). In this line, a modulation of NCAM expression by antidepressant drugs (Varea et al., 2007; Conboy et al., 2009) and an increased content of certain NCAM isoforms in the cerebrospinal fluid of depressive patients has been described (Jorgensen, 1988; Poltorak et al., 1996).

The results of my study give insights into the role of amygdalo-hippocampal interactions in the formation of memory towards highly salient, emotional arousing events. NCAM was identified as one possible key factor in these processes. On a clinical perspective, interfering with NCAM during early stages of memory formation could provide therefore a valuable tool for minimizing PTSD-associated symptoms like generalized fear memory states, although this application needs further careful evaluation.

5. Summary

5.1 Summary (English)

Evidence suggests that the neural cell adhesion molecule (NCAM) is an important molecular constituent of adaptive and maladaptive circuit (re-)organization in the central nervous system. In the current study I further investigate its putative involvement in amygdala and hippocampus function during context fear memory formation. Using laser capture micro dissection and quantitative RT-PCR, I could show high NCAM mRNA expression levels in the lateral and basolateral subnuclei of the amygdala, as well as their training intensity- and context-dependent regulation during fear memory consolidation. Moreover, I demonstrate that deficits of $NCAM^{-/-}$ mice in context fear memory can be overcome through contextual pre-exposure, i.e. by reducing the modulatory influence of the amygdala on this hippocampus-dependent memory. On the reverse, $NCAM^{-/-}$ mice failed to increase contextual fear memory after salient overtraining, although they adequately increased their response to auditory cued fear stimuli. Together with the observation of a disturbance in amygdalo-hippocampal theta synchronization, these results suggest an involvement of NCAM-mediated cell recognition processes in information processing of the amygdalo-hippocampal system and in the amygdala-mediated modulation of context fear memory according to stimulus salience.

5.2 Summary (German) – *Zusammenfassung*

Das Neural Cell Adhesion Molecule (NCAM) spielt eine wichtige Rolle in der adaptiven und maladaptiven (Re-) Organisation neuronaler Schaltkreise im zentralen Nervensystem. In der vorliegenden Studie untersuchte ich den Beitrag der Expression von NCAM in Amygdala und Hippokampus zur Bildung eines kontextuellen Furchtgedächtnisses. Mit Laser-gestützter Mikrodissektion und quantitativer Polymerasekettenreaktion bestimmte ich hierzu zunächst Veränderungen der NCAM- mRNA-Expression in Subarealen von Amygdala und Hippokampus nach Furchtkonditionierung. Dabei zeigte sich ein generell hohes NCAM mRNA-Expressionsniveau in den lateralen und basolateralen Nuclei der Amygdala, sowie deren trainingsintensitäts- und kontextabhängige Regulierung nach Furchtkonditionierung. Darüber hinaus konnte ich zeigen, dass Mäuse mit einer gezielten Ausschaltung des NCAM-

Gens (NCAM$^{-/-}$ Mäuse) ihr bereits bekanntes Defizit in kontextueller Furchtkonditionierung durch eine Prä- Exposition an den Trainingskontext überwinden können - ein Vorgang, bei dem der modulatorische Einfluss der Amygdala auf diese hippokampusabhängige Gedächtnisleistung vermindert wird. Darüber hinaus zeigten NCAM$^{-/-}$- Mäuse im Unterschied zu Wildtyp-Kontrolltieren keine Verstärkung des kontextuellen Furchtgedächtnisses durch ein Training mit gesteigerter Stimulussalienz und erhöhter Amygdalaaktivierung. Eine gesteigerte Furchtantwort auf den konditionierten Ton fand sich jedoch auch in NCAM$^{-/-}$- Mäusen unter diesen Bedingungen. Meine Ergebnisse werden ergänzt durch die Beobachtung einer verminderten Synchronisierung oszillatorischer Aktivität im Theta-Frequenz-Bereich zwischen Amygdala und Hippokampus furchtkonditionierter NCAM$^{-/-}$- Mäuse. Zusammen weisen diese auf eine Beteiligung NCAM-vermittelter Zellerkennung an der Informationsverarbeitung im amygdalo-hippokampalen System und an der Amygdala-vermittelten Salienzkodierung des kontextuellen Furchtgedächtnisses hin.

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Statement of interest - *Erklärung*

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

Rolle des *Neural Cell Adhesion Molecule* (NCAM) in amygdalo-hippocampaler Interaktion und der Salienzkodierung des kontextuellen Furchtgedächtnisses

im Institut für Biologie der Fakultät für Naturwissenschaften

mit Unterstützung des Instituts für Physiologie der Fakultät für Medizin der Otto-von-Guericke-Universität Magdeburg

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe die Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 29.03.10

Anne Albrecht

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Überhaupt möchte ich der gesamten Arbeitsgruppe der Abteilung Genetik und Molekulare Neurobiologie des Instituts für Biologie für die gute Zusammenarbeit in einer freundschaftlichen und anregenden Atmosphäre herzlich danken – in den letzten Jahren sind aus Kollegen Freunde und aus der Arbeit im Labor neben dem Studium ein zweites zu Hause und fast eine zweite Familie geworden.

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Curriculum vitae

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2005-2007	Seminarvorträge im Rahmen der Wahlpflichtveranstaltung Physiologie im Studiengang Medizin der Otto-von-Guericke-Universität Magdeburg
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WS 2009/10	Vorbereitung und Betreuung des der Wahlpflichtveranstaltung Genetik sowie des *Basic Practical Course Molecular Neurobiology* im Studiengang *Integrative Neuroscience* der Otto-von-Guericke-Universität Magdeburg

List of Publications - *Publikationsbeiträge*

Original publications and Review articles – *Original- und Übersichtsartikel*:

Albrecht A, Bergado-Acosta JR, Pape HC, Stork O: Role of the neural cell adhesion molecule (NCAM) in amygdalo-hippocampal interactions and salience determination of contextual fear memory. Int J Neuropsychopharmacol. 1-14. (2009)

Stoppel C, Albrecht A, Pape HC, Stork O: Genes and neurons: molecular insights to fear and anxiety. Gens Brain Behav. 5 Suppl 2. 34-47 (2006)

Contribution to Symposia and Meetings – *Kongressbeiträge*

Talks - *Vorträge*

Expression of Cholecystokinin, Neuropeptide Y and Somatostatin in Fear conditioned mice. O*utstanding young investigator awardee session*. Abstract book (p. 27) of the 9th Annual Meeting of the International Behavioral and Neuronal Genetics Society (IBANGS) 2007 in Doorwerth, Netherlands.

Poster presentations - *Posterpräsentationen*

Albrecht A, Bergado-Acosta JR, Pape HC, Stork O: Generalisation of conditioned fear in mice deficient for the neural cell adhesion molecule NCAM. Proceedings of the 6th Meeting of the German Neuroscience Society/ 30th Göttingen Neurobiology Conference 2005 eds: H. Zimmermann and K. Krieglstein, Neuroforum 2005, 1 Suppl.: (#234B)

Albrecht A, Bergado-Acosta JR, Pape HC, Stork O: Generalisation of conditioned fear in mice deficient for the neural cell adhesion molecule NCAM. Abstract book (p. 68) of the XIth Magdeburg International Neurobiological Symposium 2005, Magdeburg, Germany.

Albrecht A, Bergado-Acosta JR, Pape HC, Stork O: Generalisation of conditioned fear in mice deficient for the neural cell adhesion molecule NCAM. Abstract book (p. 20) of the 7th

Annual Meeting of the International Behavioral and Neuronal Genetics Society (IBANGS) 2005 in Sitges, Spain.

Albrecht A, Bergado-Acosta JR, Pape HC, Stork O: Generalisation of conditioned fear in mice deficient for the neural cell adhesion molecule NCAM. FENS Abstr. vol 3, A055.2, 2006

Albrecht A, Schulz M, Stoppel C, Pape HC, Stork O: Expression of Cholecystokinin, Neuropeptide Y and Somatostatin in Fear conditioned mice. Abstract book (p. 29) of the FENS 2008 satellite Symposium "Cell adhesion mechanisms: From development of synaptic networks to cognitive dysfunction" 2008, Villars-sur-Olon, Switzerland

Albrecht A, Schulz M, Stoppel C, Pape HC, Stork O: Expression of Cholecystokinin, Neuropeptide Y and Somatostatin in Fear conditioned mice. FENS Abstr., vol.4, 057.2, 2008.

Appendix

A.1 Solutions and buffers

DNA loading buffer:	0.25%	bromophenol blue
	0.25%	xylene cyanol FF
	15%	Ficoll in H_2O
DMDC-treatment of water:	0.1%	Dimethyldicarbonate in double destilled water
		Stir for 3 hours
		autoclave
Lysis Buffer for tail cuts:	50 mM	Tris, pH 8.0
	100 mM	NaCl
	100 mM	EDTA
	1%	SDS
Poly-L-Lysine:	1:2 dilution of Poly-L-Lysine 0.1% in double destilled water	
50x TAE-Buffer:	242 g	Tris base
	57,1 ml	acetic acid
	100 ml	0.5 M EDTA, pH 8.0
1x TE-Buffer:	1 mM	EDTA, pH 8.0
	10 mM	Tris/HCl, pH 7.4
Tris/HCl:	1 M	stock solution, calibrated with HCL to pH 7.4 or 8.0

A.2 DNA length standard

DNA molecular weight marker VII, Roche diagnostics, Mannheim, Germany

A.3 Kits and assays

„Cells-to-cDNA-Kit II“:

Ambion, Huntington, UK

Cell Lysis II Buffer
DNase 1 (2 U/µl)
dNTP Mix (2.5 mM each dNTP)
Oligo(dT)18 Primers (50 µM)
Nuclease-free Water
10x RT Buffer
M-MLV Reverse Transcriptase (100 U/µl)
RNase Inhibitor (10 U/µl)

Phusion Polymerase High-Fidelity DNA Polymerase:

Finnzymes, Espoo, Finnland

Phusion DNA Polymerase 2 U/µl
5x Phusion HF buffer
DMSO
50 mM $MgCl_2$ solution

TaqMan reagents:

Applied Biosystems, Darmstadt, Germany

2x TaqMan Universal Master Mix
TaqMan gene expression assays (predesigned):
NCAM (Assay ID: Mm00456815_m1)
PGK (Assay ID: Mm00435617_m1)

A.4 Chemicals (not provided in a kit)

Acedic Acid	Carl Roth, Karlsruhe, Germany
Agarose	Serva, Heidelberg, Germany
Bromophenol blue	Sigma, Taufheim, Germany
Dimethyl dicarbonat (DMDC)	Sigma, Taufheim, Germany
di-Nucleotide-Tri-Phosphate (dNTP)	Perkin-Elmer, Waltham, MA, USA

Ethylendiamintetraessigsäure (EDTA)	Sigma, Taufheim, Germany
Eosin Y	Sigma, Taufheim, Germany
Ethanol 96%	Carl Roth, Karlsruhe, Germany
Ethidium bromid	Merck, Darmstadt, Germany
Hematoxylin	Sigma, Taufheim, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Methylbutane	Fluka, Neu-Ulm, Germany
Mineral oil	Sigma, Taufheim, Germany
NaCl	Carl Roth, Karlsruhe, Germany
Poly-L-Lysine 0.1%	Sigma, Taufheim, Germany
Custom primer NCAM all	Life technologies, Karlsruhe, Germany
Custom primer NCAM ko	Life technologies, Karlsruhe, Germany
Custom primer NCAM wt	Life technologies, Karlsruhe, Germany
Proteinase K	Roche Diagnostics, Mannheim, Germany
RNAse away	Molecular BioProducts, San Diego, CA, USA
Sodium-Dodecyl-Sulfate (SDS)	Serva, Heidelberg, Germany
Tissue Tek O.C.T. Compound	Sakura Finetek Europe, Zoetwerwoude, Netherlands
TRIS-hydrochloride	Carl Roth, Karlsruhe, Germany
Xylene cyanol	Sigma, Taufheim, Germany

A.5 Instruments and consumables

Animal care

Macrolon standard cages	Ebeco, Castrop-Rauxel, Germany
Ssniff R/M-H V-1534	Ssniff Spezialdiäten, Soest, Germany
Lignocel BK 8/15	J. Rettenmaier & Söhne, Rosenberg, Germany

Plastic ware

Safe lock tubes (1.5 ml)	Eppendorf, Hamburg, Germany
MicroAmp Fast Reaction Tubes	Applied Biosystems, Darmstadt, Germany
MicroAmp 8-cap strip	Applied Biosystems, Darmstadt, Germany
MicroAmp Fast Optical 96-Well plate	Applied Biosystems, Darmstadt, Germany
MicroAmp Optical Adhesiv Film	Applied Biosystems, Darmstadt, Germany
Micro tube 500 for LCM	PALM, Bernried, Germany
Falcon tube 50 ml	Greiner Bio-one, Frickenhausen, Germany
Object slide box	Carl Roth, Karlsruhe, Germany

Glass ware

glass bottles	Carl Roth, Karlsruhe, Germany
Erlenmeyer flasks	Carl Roth, Karlsruhe, Germany
Beaker	Carl Roth, Karlsruhe, Germany
graduated cylinders	Carl Roth, Karlsruhe, Germany
staining cuvettes	Carl Roth, Karlsruhe, Germany
slide holder	Carl Roth, Karlsruhe, Germany
MembraneSlides 1.0 PEN	PALM, Bernried, Germany

Pipettes

Pipettes	Brand, Wertheim, Germany
Pipette tipps	Brand, Wertheim, Germany
Pipette tips with filter	Brand, Wertheim, Germany

Freezers & Fridge

Liebherr KU 2407	Liebherr Hausgeräte, Ochsenhausen, Germany
Liebherr GU 4506	Liebherr Hausgeräte, Ochsenhausen, Germany
Sanyo Ultra Low	Ewald Innovationstechnik, Bad Nenndorf, Germany

Scales

Sartorius TE 1535	Sartorius AG, Göttingen, Germany
Sartorius TE 212	Sartorius AG, Göttingen, Germany
Sartorius TE 2101	Sartorius AG, Göttingen, Germany

Centrifuges

centrifuge 5424	Eppendorf, Hamburg, Germany
centrifuge 5430	Eppendorf, Hamburg, Germany
VWR Galaxy Mini	VWR International, Darmstadt, Germany

Autoclave

Systec DB-23	Systec Labortechnik, Wettenberg, Germany

Oven

Binder FP53	Binder, Tuttlingen, Germany

pH meter

inoLab pH720	WTW, Weilheim, Germany

Magnetic Stirrer	
IKA RET basic	IKA-Werke, Staufen, Germany
magnetic stir bar	Brand, Wertheim, Germany
Rotor incubator	
Hybrid 2000	H. Saur Laborbedarf, Reutlingen, Germany
PCR Hood	
Captair bio	Erlab, Köln, Germany
Thermocycler	
Veriti Thermal Cycler	Applied Biosystems, Darmstadt, Germany
Microwave	
Clatronic MWG 746 H	Clatronic International, Kempen, Germany
Gel electrophoresis system	
AGT3 & Maxi-VG	VWR International, Darmstadt, Germany
Gel documentation system	
InGenius LHR	Syngene, Cambridge, UK
Cryostat	
CM 1950	Leica, Nussloch, Germany
Hot plate	
Medite OTS 40.2530	Medite, Burgdorf, Germany
Laser capture microdissection system	
PALM MicroBeam	PALM, Bernried, Germany
Water bath	
LAUDA A103	Lauda Dr. R. Wobser, Lauda-Königshof, Germany
Real-time-PCR	
AbiPrism 7000 sequence detection system	Applied Biosystems, Darmstadt, Germany

Fear Conditioning Apparatus

TSE Fear Conditioning System	TSE, Bad Homburg, Germany

Others

Lab clock	Carl Roth, Karlsruhe, Germany
Aluminum foil	Carl Roth, Karlsruhe, Germany

A.6 Software

SPSS	SPSS Inc., Chicago, IL, USA
Adobe Photoshop CS4 Extended	Adobe Systems, San Jose, CA, USA
EndNote	Thomson Reuters, Carlsbad, CA, USA
Microsoft Office 2007	Microsoft Cooperation, Redmond, WA, USA