Functional role of the polysialylated neural cell adhesion molecule in fear conditioning of mice

(*Mus musculus* L., 1758)

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ABSTRACT

Considerable progress has been made in uncovering the numerous functions of the neural cell adhesion molecule (NCAM) and its associated carbohydrate, polysialic acid (PSA), in ontogenetic development and in synaptic plasticity, learning and memory in the adult. Perturbation of NCAM functions by ablation of NCAM or by disruption of NCAM-mediated interactions *in vitro* or *in vivo* impaired synaptic plasticity in the hippocampus, fear conditioning, induced amnesia in a passive avoidance task, and caused spatial memory deficits. The mechanisms underlying action of NCAM and PSA at different phases of learning and memory consolidation are, however, unknown.

To investigate the contributions of PSA versus the extracellular domain of the NCAM glycoprotein backbone on learning we acutely injected NCAM, PSA-NCAM and PSA into the dorsal hippocampus of wild-type mice at different phases of auditory fear conditioning. As an internal control that injections into the hippocampus would not have unspecific effects on hippocampus-independent forms of learning, we used a fear conditioning paradigm in which an animal was simultaneously subjected to hippocampus-independent learning of tone and hippocampus-dependent learning of context. Only PSA, either attached to NCAM or alone, but not NCAM, injected before training, impaired formation of hippocampus-dependent contextual memory, as measured by the freezing response. Consolidation of contextual memory was affected only by PSA-NCAM when injected during its late, but not its early phases. None of the tested compounds disturbed extrahippocampal cued memory. Mice lacking the polysialyltransferase (ST8SiaIV/PST) responsible for attachment of PSA to NCAM in adulthood showed a mild deficit only in hippocampal contextual learning, when compared to NCAM deficient mice that were disturbed in both contextual and cued memories. Formation of contextual memory in NCAM deficient mice could be restored by pre-training injection of PSA-NCAM into the hippocampus, suggesting that a mechanism for PSA-NCAM function in learning is not mediated by modulation of NCAM-NCAM homophilic interactions but rather through its heterophilic partners. These *in vivo* results are supported by *in vitro* data showing the inhibitory effects of PSA on long-term potentiation (LTP) in the CA1 region of the hippocampus in wild-type mice and restorative effects of PSA on

impaired CA1 LTP in NCAM deficient hippocampal slices. In summary, our data support the view that polysialylated NCAM is involved in both formation and late consolidation of contextual memory, and supports synaptic plasticity.

By using tamoxifen-inducible Cre-recombinase LoxP system - which allowed us to induce temporally controlled ablation of the NCAM gene in the brain of adult mice – we show that NCAM is required for formation and/or retention of hippocampus-dependent contextual memory, whereas amygdala-contingent cued memory is less dependent on inducible ablation of NCAM. These results are intriguing since constitutive prenatal NCAM ablation causes both hippocampal- and amygdala-dependent deficits in fear conditioning, and thus raise the question whether NCAM is critical for development of the amygdala, or the levels of NCAM expression in the amygdala after inducible ablation remain sufficiently high to mediate its functions.

Strikingly, severe cognitive deficits in learning and memory of contextual and cued information in NCAM deficient mice could be rescued by facilitation of activity of the NMDA subtype glutamate receptors via systemic administration of D-cycloserine, as an agonist of the glycine site of these receptors. These results correspond to data from ongoing *in vitro* studies demonstrating a rescue of CA1 LTP in NCAM or PSA-deficient hippocampal slices by D-cycloserine. Thus, we suggest that a loss of NCAM and/or PSA leads to a deficit in NMDA receptor function during induction of synaptic plasticity and fear learning. Since lower concentrations of D-cycloserine were sufficient to restore levels of hippocampus-dependent contextual memory as compared to amygdala-based cued fear memory, there may be interesting differences in regulation of NMDA receptors by PSA-NCAM in the hippocampus and amygdala. The significance of these findings is underscored by studies uncovering a genetic link between schizophrenia and mutations in the polysialyltransferase ST8SiaII/STX, and a deficit in PSA and overproduction of soluble NCAM in schizophrenic brains.

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ZUSAMMENFASSUNG

In der Erforschung der verschiedenen Funktionen des neuralen Zellerkennungsmoleküls (NCAM) und des assoziierten Carbohydrats Polysialylsäure (PSA) wurden große Fortschritte in den Bereichen ontogenetische Entwicklung, synaptische Plastizität sowie Lernen und Gedächtnis im erwachsenen Tier gemacht. Die Störung der Funktionen von NCAM durch die Entfernung des Moleküls oder die Unterbrechung von NCAM-vermittelten Interaktionen *in vitro* oder *in vivo* beeinträchtigt die synaptische Plastizität im Hippocampus und die Furcht-Konditionierung, verursacht Amnesie in einer passiven Vermeidungsaufgabe und bewirkt Defizite im räumlichen Gedächtnis. Die Mechanismen, die den Aufgaben von NCAM und PSA in unterschiedlichen Phasen von Lernen und Gedächtniskonsolidierung unterliegen, sind allerdings noch weitgehend unbekannt.

Um die Beteiligung an Lernprozessen von PSA im Vergleich zur extrazellularen Domäne des NCAM-Glycoprotein-Rückgrats zu untersuchen, wurden NCAM, PSA-NCAM oder PSA in den dorsalen Bereich des Hippocampus' von Wildtyp-Mäusen während verschiedener Phasen der auditorischen Furcht-Konditionierung injiziert. Als interne Kontrolle dafür, dass die Injektionen in den Hippocampus keine unspezifische Wirkung auf Hippocampus-unabhängige Formen von Lernen haben, wurde ein Paradigma für die Furcht-Konditionierung gewählt, in dem die Tiere gleichzeitig Hippocampus-unabhängigem Lernen eines Tons und Hippocampus-abhängigem Lernen eines Kontextes unterworfen waren. Injektion von PSA, allein oder an NCAM gebunden, vor dem Training verminderte die Bildung von Hippocampus-abhängigem Kontext-Gedächtnis, was anhand der Erstarrungsreaktion als Furchtantwort bestimmt wurde. Injektion von NCAM allein hatte keine Wirkung. Die Verfestigung des Kontext-Gedächtnisses wurde nur durch PSA-NCAM beeinträchtigt, wenn das Molekül während der späten Phasen der Konsolidierung injiziert wurde. Eine Injektion in frühen Phasen zeigte keinen Effekt. Keine der getesteten Substanzen hatte eine Auswirkung auf extra-hippocampales Hinweisreiz-gelenktes Gedächtnis. Mäuse, denen die Polysialyltransferase (ST8SiaIV/PST), die für die Anlagerung von PSA an NCAM im erwachsenen Tier verantwortlich ist, fehlt, zeigten nur ein leichtes Defizit im Hippocampus-abhängigen Kontext-Lernen. Im Vergleich waren NCAM-defiziente Mäuse sowohl im Kontext-Gedächtnis also auch in

der Hinweisreiz-gelenkten Erinnerung beeinträchtigt. Die Fähigkeit zur Bildung von Kontext-Gedächtnis in NCAM-defizienten Mäusen konnte durch die Injektion von PSA-NCAM in den Hippocampus vor dem Training wiederhergestellt werden. Dies verdeutlicht, dass die Beteiligung von PSA-NCAM an Lernprozessen nicht auf der Modulation von NCAM-NCAM homophilen Interaktionen beruht, sondern eher durch seine heterophilen Inderaktionspartner erfolgt. Diese *in vivo* Ergebnisse wurden von *in vitro* Daten unterstützt, die inhibitorische Effekte von PSA auf die Langzeitpotenzierung (LTP) in der CA1-Region des Hippocampus' in Wildtyp-Mäusen zeigten. Außerdem hatte PSA positive Effekte auf die gestörte Langzeitpotenzierung in der CA1-Region von Hippocampus-Schnitten NCAM-defizienter Mäuse. Zusammenfassend unterstützen die Ergebnisse dieser Arbeit die Ansicht, dass PSA-NCAM sowohl an der Bildung als auch an der späten Phase der Konsolidierung des Kontext-Gedächtnisses beteiligt ist und synaptische Plastizität verstärkt.

Unter Verwendung des Tamoxifen-induzierbaren LoxP-Systems, das eine zeitlich kontrollierte Zerstörung des NCAM-Gens im Gehirn erwachsener Mäuse erlaubt, konnte gezeigt werden, dass NCAM notwendig ist für die Bildung und/oder die Aufrechterhaltung von Hippocampus-abhängigem Kontext-Gedächtnis. Währenddessen ist Amygdala-bedingte Hinweisreiz-gelenkte Erinnerung weniger abhängig von der induzierten Zerstörung von NCAM. Diese Ergebnisse sind interessant, da das konstitutive pränatale Fehlen von NCAM sowohl Hippocampus- als auch Amygdala-abhängige Defizite in der Furcht-Konditionierung mit sich bringt, und damit die Frage entsteht, ob NCAM für die Entwicklung der Amygdala notwendig ist. Eine andere mögliche Erklärung wäre, dass nach induzierter Zerstörung von NCAM die Konzentration des intakten Moleküls in der Amygdala ausreichend bleibt, um ihre Funktionen zu gewährleisten.

Faszinierend ist, dass die Verstärkung der Aktivität von NMDA-Glutamatrezeptoren mit Hilfe von D-Cycloserin, einem Agonisten der Glycin-Bindedomäne dieser Rezeptoren, schwere kognitive Defizite im Bereich von Lernen und Gedächtnis bezüglich Kontext und Hinweisreiz-gelenkter Informationen in NCAM-defizienten Mäusen ausgleichen konnte. Diese Ergebnisse decken sich mit ersten Daten aus im Moment durchgeführten *in vitro* Studien, die zeigen, dass in Hippocampus-Schnitten von NCAM- oder PSA-defizienten Mäusen LTP in der CA1-Region mit Hilfe von D-Cycloserin wiederhergestellt werden kann. Daher nehmen wir

an, dass der Verlust von NCAM und/oder PSA zu einer beeinträchtigten Funktion der NMDA-Rezeptoren während der Induktion synaptischer Plastizität und Furcht-Konditionierung führt. Da zur Wiederherstellung des Hippocampus-abhängigen Kontext-Gedächtnisses geringere Konzentrationen von D-Cycloserin ausreichten als für die Wiederherstellung der Amygdala-abhängigen Hinweisreiz-bedingten Erinnerung notwendig waren, lässt sich auf Unterschiede in der Regulation von NMDA-Rezeptoren durch PSA-NCAM in Hippocampus und Amygdala schließen. Die Bedeutung dieser Ergebnisse wird betont durch Studien, die eine genetische Verbindung zwischen Schizophrenie und Mutationen der Polysialyltransferase ST8SiaII/STX offenbarten sowie ein Defizit in der Produktion von PSA und eine Überproduktion von löslichem NCAM in Gehirnen, die Schizophrenie aufweisen, zeigten.

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PROJECT I: "The role of PSA-NCAM at different stages of fear conditioning"

REVIEW OF THE LITERATURE

Cell Adhesion Molecules (CAMs)

The immunoglobulin superfamily of CAMs

In metazoans, tissue formation, specialization, maintenance and functioning during development and adult life requires continuous direct dynamic/stable contacting of cells with each other and/or the extracellular matrix (ECM). Proteins through which these contacts are made are the cell adhesion molecules (CAMs). This is by means of the CAMs, cells are capable to recognize each other, migrate, grow and form differently specialized tissues. Among hundreds of different CAMs, there are CAMs which were united in one big immunoglobulin superfamily of CAMs (Ig-CAMs) since they share homotypic structure of their core protein, immunoglobulin (Ig)-like domains, and may have fibronectin type III repeats (see Fig. 1-IA, for review, see Crossin and Krushel, 2000).

The Ig-CAM family includes: MAG, P_0, L1/Ng-CAM, CHL1 TAG/axonin-1, F3/F11/contactin, Nr-CAM, neurofascin (for reviews, see Doherty et al., 1995; Walsh and Doherty, 1997; Juliano, 2002; Rougon and Hobert, 2003; Crossin and Krushel, 2000). The extracellular portion of these proteins contains different numbers of Ig-like domains (see Fig.1-IA) and fibronectin type III repeats, where the intracellular part has more diverse structure; it may contain a tyrosine kinase domain like in fibroblast grows factor receptor (FGF-R) or tyrosine phosphatase domain like in CRYPα1.

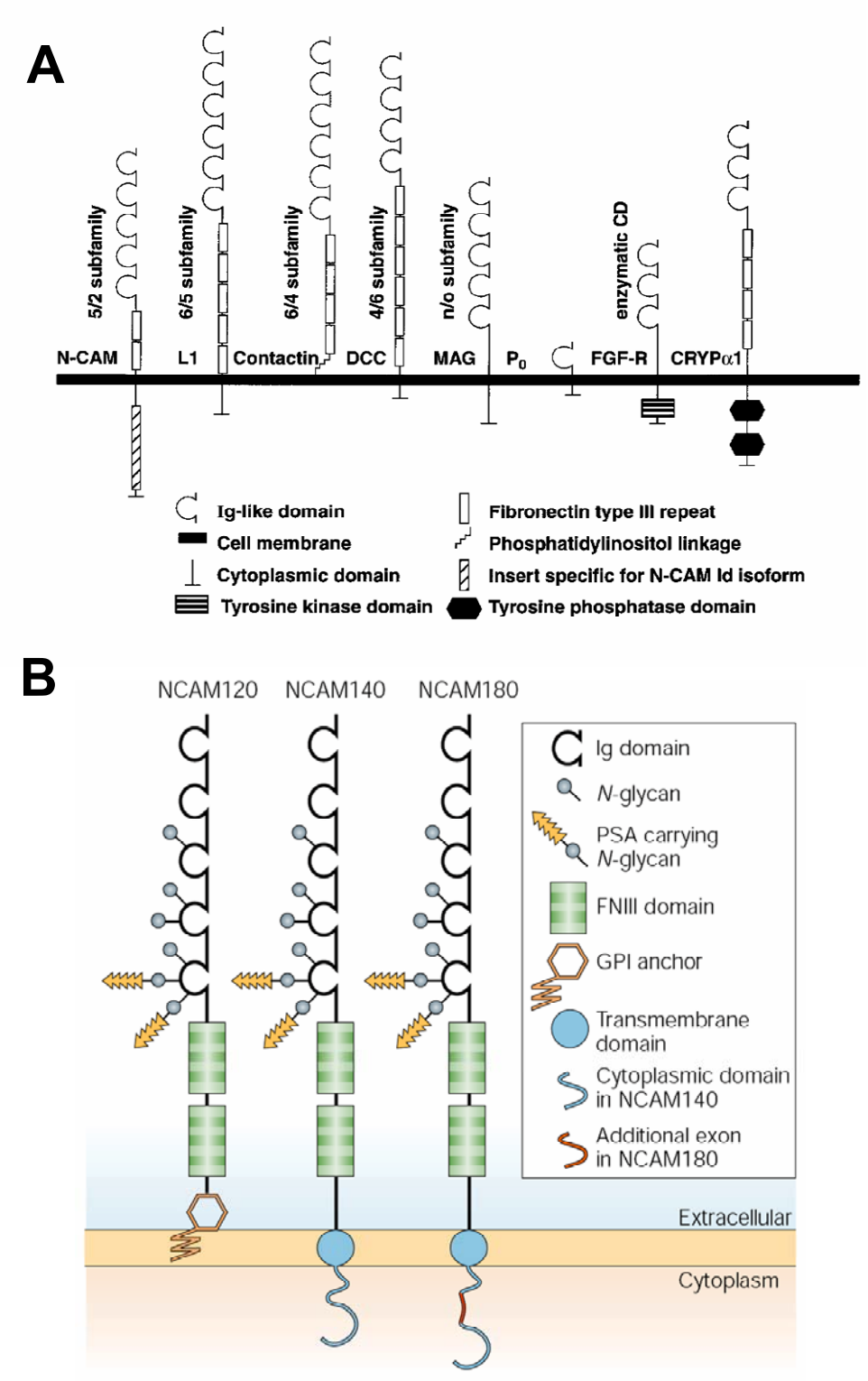


Fig. 1-I A – Immunoglobulin superfamily of CAMs which were found in nervous tissue falls into several subfamilies depending on the number of Ig-like domains and fibronectin repeats (from review Crossin and Krushel, 2000). B – Structure of three main isoforms of NCAM named according to their molecular weights (from review Kleene and Schachner, 2004).

Discovery of the Neural Cell Adhesion Molecule (NCAM)

NCAM was the first cell adhesion molecule (termed D2, N-CAM or CD56) from the immunoglobulin superfamily which had been identified almost simultaneously by two independent research groups: Elisabeth Bock's group from Copenhagen University, Denmark (Jorgensen and Bock, 1974), and Gerald M. Edelman's group, from the Rockefeller University, New York, USA (Rutishauser et al., 1976; Brackenbury et al., 1977). In following years NCAM was then isolated, cloned and characterized (Thiery et al., 1977; Cunningham et al., 1987; Hoffman et al., 1982). This protein has been found in almost all tissues with the highest expression in the central and peripheral nervous systems.

Structure of NCAM

NCAM is encoded by a single gene consisting of 26 exons, however via alternative splicing mechanism, has three major isoforms which slightly differ in their structure (see Fig.1-IB and Fig 2-I). NCAM-180 and NCAM-140 are transmembrane isoforms, able to interact with cytoskeleton and thus be more rigidly stationed in the membrane; whereas NCAM-120 is a nearly free-floating protein since it is attached to the lipid bilayer via a glycosylphosphatitylinositol (GPI) anchor. The numbers following in their names represent its approximate molecular weights. In addition, several forms of soluble NCAM exist generated by truncation or proteolysis (Olsen et al., 1993).

The NCAM gene (NCAM1) encoding all three NCAM isoforms in man is located on chromosome 11, in mouse on chromosome 9 and in rat on chromosome 8 (Nguyen et al., 1986; Walsh et al., 1986; Yasue et al., 1992). In mouse, NCAM gene consists of 0-19 exons plus 6 additional smaller ones (see Fig. 2-I); exons 0-14 encode the extracellular part of NCAM. Expression of exon 15 results in the production of the ~725 aa-long NCAM-120 molecule (M_W~80 kDa), which becomes attached to the membrane with the GPI anchor. Alternatively, the transcript can, instead of exon 15, include exon 16, which encodes a transmembrane domain, and exon 17-19, which encode the intracellular part of NCAM. If a transcript includes exon 18, the result is the ~950 aa-long NCAM-180 molecule (M_W~118 kDa). If exon 18 is excluded from the transcript, NCAM-140 molecule with the ~850 aa-long (M_W~91 kDa) will be generated (for review, see Cunningham et al., 1987).

In addition to the three main membrane isoforms of NCAM, there are several secreted forms; one (M_W~115 kDa) is produced by the expression of small so-called SEC-exon located between exons 12 and 13 (see Fig. 2-I, Bock et al., 1987; Gower

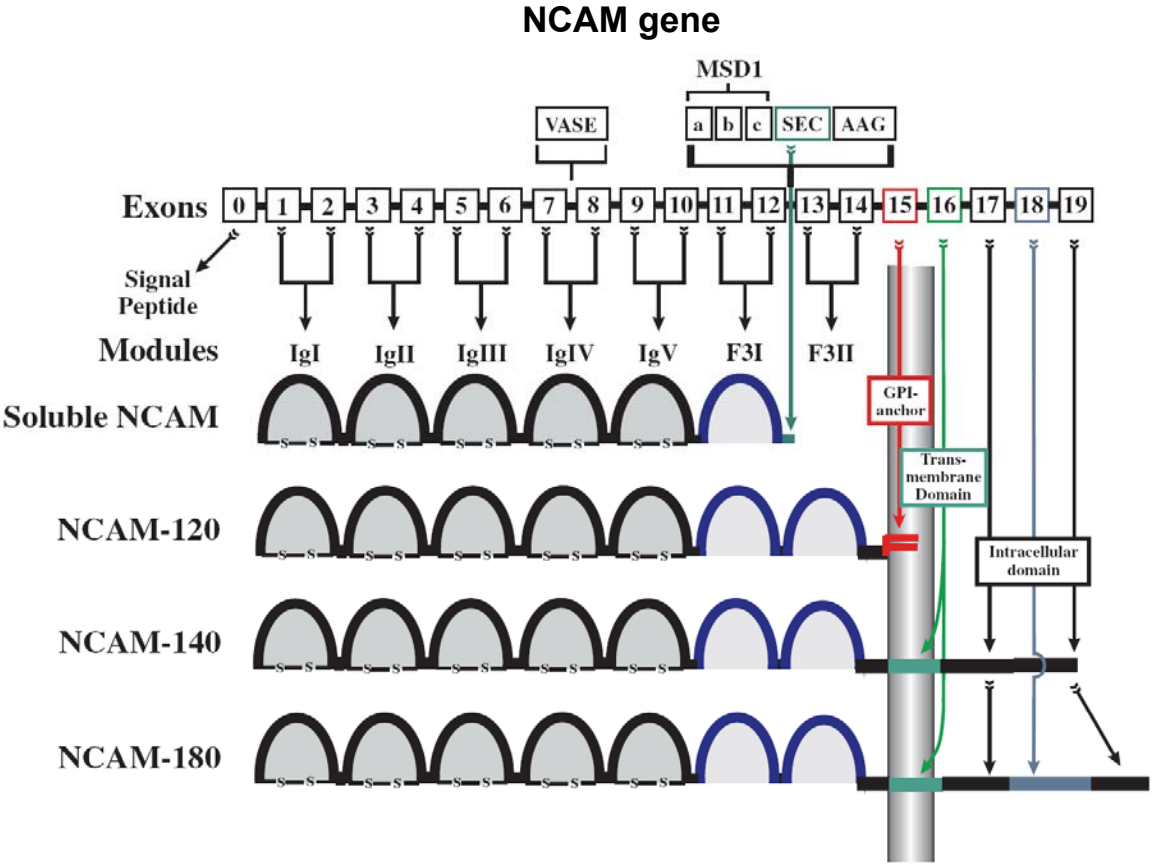


Fig. 2-I. Main NCAM isoforms. The scheme shows the exons constituting the NCAM gene, and how the translation of the respective exons is related to different isoforms of NCAM. IgI-V – immunoglobulin homology modules (black), F3-I,-II indicate fibronectin type III homology modules (blue). (From Walmod et al., 2004).

et al., 1988), the others are produced by shedding of NCAM, an enzymatic removal of NCAM-120 from the membrane by phosphatidylinositol specific phospholipase C (PI-PLC), or by proteolytic cleavage of the extracellular part of any of the three major isoforms (He et al., 1986). Transmembrane isoforms of NCAM have also been found in soluble form, e.g. in cerebrospinal fluid (Olsen et al., 1993). The extracellular part of NCAM can be slightly modified by the exclusion or inclusion of 6 small exons in the original transcript. The so-called VASE- or π-exon located between exon 7 and 8 encodes a 10 aa-long sequence, which has inhibitory effect on neurite outgrowth (Doherty et al., 1992) and abnormal expression of this exon might play a role in some psychiatric disorders (Vawter, 2000; Strekalova et al., 2006). Besides the SEC-exon,

four additional small exons are located between exon 12 and 13; three of these exons encode the so-called muscle specific domain 1 (MSD1: MSD1a, MSD1b and MSD1c), the fourth exon, AAG, consists of only a single nucleotide triplet (see e.g., Dickson et al., 1987).

NCAM-140 and NCAM-180 additionally can be posttranslationally modified by palmitoylation of four cytoplasmic cysteine residues, which help to anchor NCAM in lipid rafts. Mutation of palmitoylation sites affects distribution of NCAM within membrane bilayer as well as disrupts NCAM-mediated signaling and neurite outgrowth (Niethammer et al., 2002).

NCAM contains 6 potential N-glycosylation sites (in mouse, Asp203, Asp297, Asp329, Asp415, Asp441 and Asp470, see Albach et al., 2004), which all can be glycosylated with very dynamic spatial and temporal pattern.

All three main NCAM isoforms and their soluble derivatives can carry on their 5^{th} Ig-like domain a highly negatively charged polysialic acid (PSA), an unique sugar with an unusual α2,8 linkage of sialic acids in its chains which can be up to 200 residues long. Attachment of PSA to NCAM (glycosylation) results in a large hydration cloud appearing around the core protein, which sterically modulates homophilic binding of NCAM to NCAM on other cells, thus providing an opposite function of NCAM – deadhesion. The attachment of PSA is mediated by two polysialyltransferases (enzymes ST8SialV/PST and ST8SialI/STX).

NCAM also carries a carbohydrate epitope named HNK-1 (human natural killer cells) which is attached to NCAM by N-linked glycosylation through at least 5 different sites (Wuhrer et al., 2003). NCAM can also contain a number of non PSA/HNK-1 glycosylations; some of which are expressed in the olfactory system (for review, see Kiss and Rougon, 1997).

Interestingly, only mammalian NCAM curries such an unique sugar like PSA, expression of which on NCAM can be extremely fast up- or down-regulated by synaptic activity and/or learning (Muller et al., 1996; Sandi et al., 2003; O'Connell et al., 1997); in invertebrates, adhesion/deadhesion is regulated via tuned expression of the whole NCAM (see for review Schachner et al., 1994). Within a synapse environment, the all three NCAM isoforms have also distinct expression profiles; NCAM-120 is predominantly expressed on glia rather than on neurons (Goodman, 1996; Kiss and Muller, 2001), NCAM-180 seems to be expressed exclusively on

neurons, particularly at the postsynaptic side of synapses, whereas NCAM-140 can be found on both glia cells and neurons (Schachner, 1997).

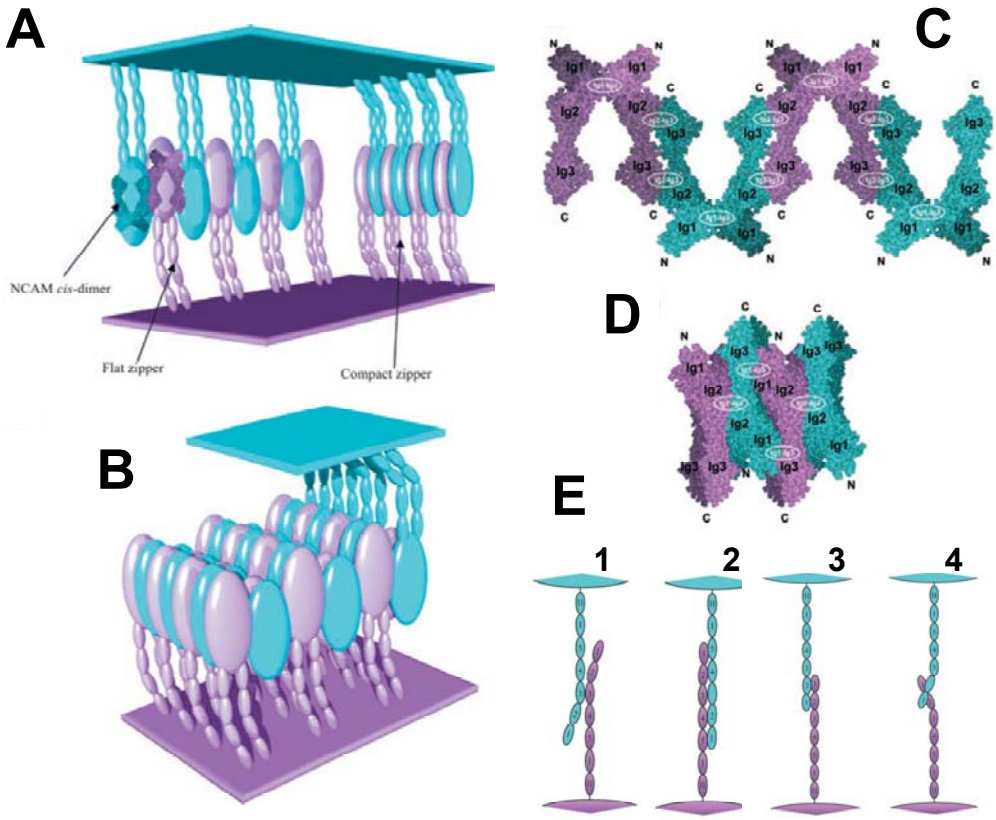


Fig. 3-I Different current models of homophilic NCAM interactions. A - a "flat" one-dimentional zipper is shown in the left part, and a "compact" one-dimentional zipper is depicted in the left; B – a two-dimentional zipper, which is a combination of the "flat" and "compact" zippers; C and D – detailed crystal structures of "flat" (C) and "compact" (D) zippers. The "compact" zipper in (D) is shown at an angle perpendicular to the "flat" zipper in (C) (modified from Kiselyov et al., 2005); E – different models of trans-homophilic NCAM interaction, 1 – trans-interaction of two anti-parallel Ig-III modules (Rao et al., 1992), 2 – all Ig modules of opposite NCAM molecules are involved in trans-interaction (Ranheim et al., 1996), 3 and 4 – models of trans-NCAM interactions by IgI and IgII modules based on differen methods (Kiselyov et al., 1997; Jensen et al., 1999; Kasper et al., 2000).

NCAM is very conservatively encoded in different genomes, ranging from 70% to 98% residue identities from human to frog. More distantly spaced NCAM homologues are fasciclin II of the fruit fly, *Drosophila melongaster* and apCAM of the sea mollusk, *Aplysia californica*, which both share about 25% of homology with vertebrate NCAM.

Homophilic NCAM interactions

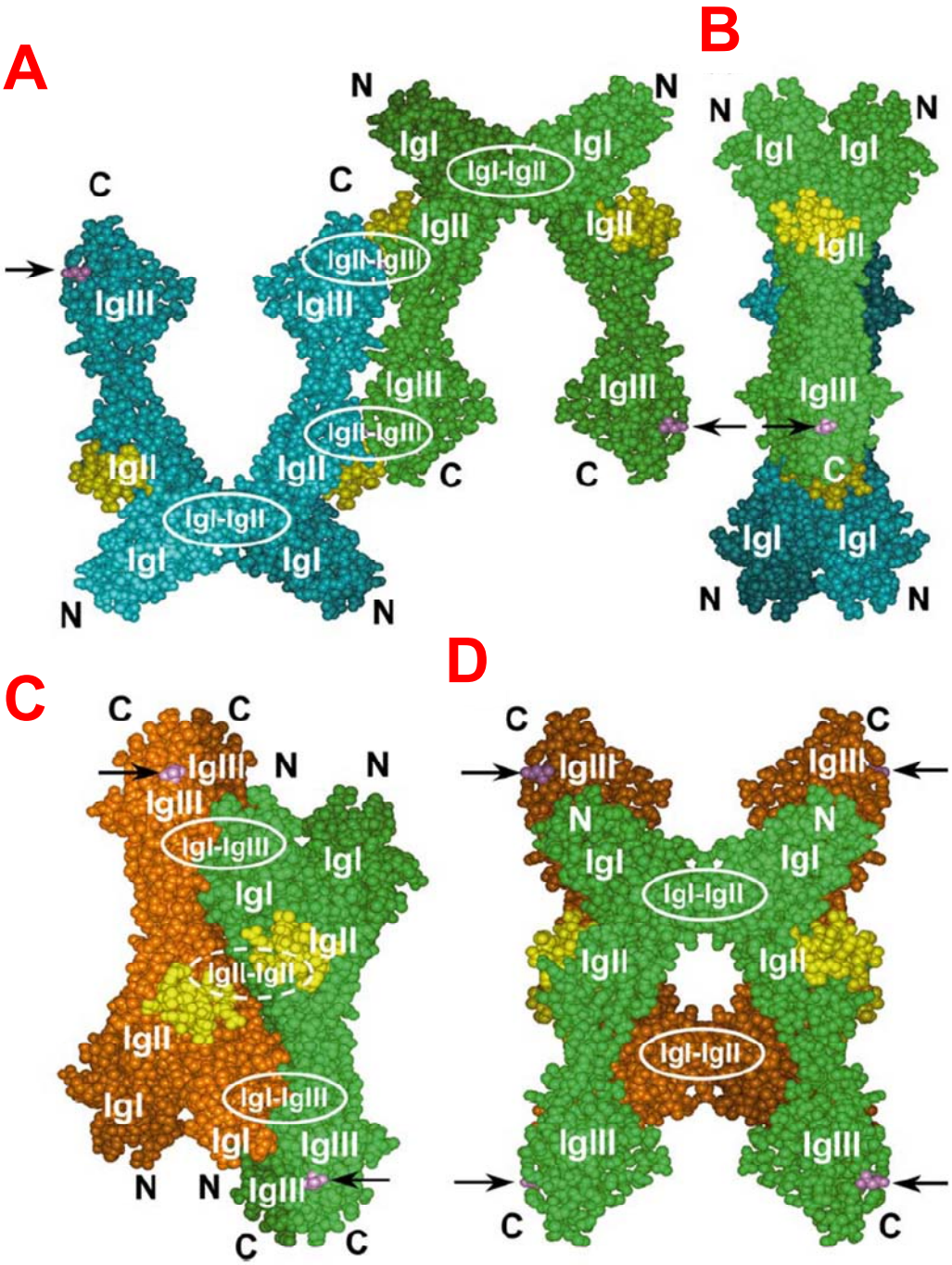
NCAM has long been proved to interact in homophilic trans-fashion mode (NCAM-NCAM located on opposite cells, see Fig. 3-IA, B, and Rutishauser et al., 1982). One of the oldest models of how two NCAMs interact with each other is that all IgI-V domains participate in homophilic trans-interactions in a reciprocal manner where IgI of one NCAM binds to IgV of the other NCAM, IgII binds to IgIV and so on, see Fig. 3-IE2 (Ranheim et al., 1996). Then it was proposed that NCAM homophilic interaction is realized via interaction of only one IgIII domain with corresponding IgIII of the other molecule (see Fig. 3-IE1), but this was later disproved (Atkins et al., 2001). However, the IgIII domain has been shown by X-ray crystallography in homophilic binding with IgI (Soroka et al., 2003). Moreover, IgI may also interact with IgII module (see Fig. 3-IE3, 4), and the combined IgI-IgII modules can interact reciprocally with the same IgI-IgII modules of another NCAM molecule (Jensen et al., 1999). The IgI-IgII interaction is about 30 times higher for interactions between IgI-IgII double modules than between single modules, and this is not affected by IgIII domain (Atkins et al., 1999; 2001). The model of the IgI-IgII double structure has been further supported by crystallography (Kasper et al., 2000).

Interestingly, in a triple IgI-IgII-IgIII module structure that there are at least four different inter-module interactions (See Fig. 4-IA, B) (Soroka et al., 2003). First, IgI-IgII binding between parallel triple IgI-IgII-IgIII complexes, which most likely represents a homophilic NCAM cis-interaction, responsible for dimerization of triplets. The other three are formed in anti-parallel fashion of triplets, most likely corresponding to NCAM trans-homophilic interactions: first, a reciprocal interaction between IgII and IgIII, second, IgI between IgIII, and third, self-interaction of IgII modules of two anti-parallel triplets (see Fig. 4-IA, C). Their biological relevance to NCAM-driven neurite outgrowth *in vivo* was further proved by specific small peptides, which interfere with corresponding module interactions (Soroka et al., 2003). Based on data mentioned above, a “double zipper model” of NCAM interactions has been proposed; the first – a “flat zipper” is formed between NCAM cis-dimers from one cell

surface interacting in trans through IgII-IgIII contacts with NCAM cis-dimers from another cell surface (see Fig. 3-IA, B, C), the second – "compact zipper" is built between NCAM cis-dimers from one cell surface interacting in trans through IgI-IgIII and IgII-IgII sites with NCAM cis-dimers from another cell surface (see Fig. 3-IA, B, C).

Importantly to note, that both "zipper" models utilize cis-dimerization of NCAM, which is a facilitating state for NCAM-mediated neurite outgrowth, and therefore maybe a necessary prerequisite for subsequent trans-interactions of NCAM. Another observation was drawn by authors of the model, that the "compact" and "flat" zippers are formed in different directions to each other, perpendicularly, and are therefore not exclusive. They proposed a simple three-step process of homophilic NCAM-mediated adhesion: the first step – cis-dimerization, the second – formation of "compact zipper" (since this zipper is formed at larger distance between opposite cell membranes than the "flat" zipper), and the third step is the formation of a "compact-flat double zipper" involving numerous NCAM molecules (Walmod et al., 2004).

Fig 4-I. 3D-model of NCAM IgI, IgII and IgIII modules. The picture shows possible interactions between the recombinant first-three-Ig-domains construct as demonstrated by X-ray crystallography. The structure reveals four different intermodular interactions indicated by ellipses around the respective interaction site. Arrows indicate the position of N-linked glycosylation at Asn203 (pink), the yellow region corresponds to the heparin binding domain. A, B – Two cis-interacting dimers (shown in cyan and green, respectively) connected by trans-interactions in a "flat zipper". It is likely that the cis-interaction is mediated by reciprocal IgI-IgII interactions, whereas the trans-interactions are mediated by reciprocal IgII-IgIII interactions. C, D – Two cis-interacting dimers (shown in green and brown) connected by trans-interactions in a "compact zipper". As in a flat zipper the cis-interaction is mediated by IgI-IgII interactions on one side of the structure, and an IgII-IgII interaction on the other side of the structure. (From Walmod et al., 2004).



← Fig. 4-I (the figure legend is on the previous page)

Heterophilic NCAM interactions

First of all, NCAM can bind to other CAMs, for example, it has been shown that NCAM may interact with L1 molecule in cis-fashion (see Fig. 5-I), which triggers phosphorylation of tyrosine and serine residues in L1 (Heiland et al., 1998). In this binding carbohydrates expressed on L1 and the IgIV module of NCAM are involved. Such interaction is shown to be facilitating for L1-L1 homophilic trans-binding and thus for L1-mediated cellular aggregation and adhesion (Kadmon et al., 1990a,b). L1-L1 interaction is dependent on simultaneous NCAM-NCAM interaction, since neurite outgrowth triggered by L1-L1 trans-binding can be inhibited by application of recombinant NCAM-IgI, -IgII and –IgIII modules, which thereby interfere with NCAM-NCAM cis- and trans-interactions, but not with L1-L1 trans-complexes (Kristiansen et al., 1999). However, L1-triggered neurite outgrowth is not impaired in NCAM-negative environment (Gabriele Loers et al., personal communication), suggesting that NCAM is not solely required for L1-L1 interactions.

NCAM also can bind to another Ig-CAM, TAG-1 (see Fig. 5-I), which was reported to bind to L1 in cis-fashion; thus all three types of interactions L1-NCAM-TAG-1, L1-TAG-1, and NCAM-TAG-1 are quite possible (Milev et al., 1996; Malhotra et al., 1998).

Interestingly, that NCAM was shown (Dzhandzhugazyan and Bock, 1993) to bind directly to ATP (adenosine three phosphate, see Fig. 5-I), which in brain among other functions can act as a neurotransmitter, since it was found in synaptic vesicles in mM concentrations (Dubyak et al., 1993). NCAM can bind to about 1000 molecules of ATP per minute, causing Ca^{2+}, Mg^{2+}-dependent ATP-hydrolysis, thus indicating that NCAM can work as an ATPase. The role of such NCAM-ATP interaction is not yet well understood, however, it was shown that it leads to inhibition of trans-homophilic NCAM-NCAM interactions (Dzhandzhugazyan and Bock, 1997). Recently, ATP-binding site of NCAM has been dissected to be in F3II module of NCAM, suggesting that disruption of trans-homophilic NCAM-NCAM interactions by ATP is due to some structural alterations in the NCAM protein core, rather than a sterical interference between Ig-modules of opposite NCAM molecules.

NCAM may bind directly to Fibroblast Growth Factor Receptor (FGFR), which bears tyrosine kinase activity (see Fig. 5-I). It was shown that FGFR contains so-called CAM-homology domain with homology to VASE-sequence of NCAM; disruption of functioning of this domain via antibodies against it or synthetic peptide

corresponding to this domain, abolishes NCAM-mediated neurite outgrowth (Williams et al., 1994a). Furthermore, NCAM-NCAM binding leads to phosphorylation of FGFR (Saffell et al., 1997), suggesting that NCAM binds to FGFR and can stimulate it, directly or indirectly. It was proposed on basis of surface plasmon resonance analysis that F3-I and F3-II modules of NCAM can bind directly the FGFR Ig-modules, D2 and D3. Indeed, NMR analysis found that NCAM F3-II module interacts with the D3 module of FGFR via the so-called FG loop. Application of either NCAM-F3II recombinant protein or a 15 aa-long mimicking peptide corresponding to the FGFR interaction site of this domain to FGFRs leads to phosphorylation of this receptor followed by neurite outgrowth. Interestingly, in the same study, it was shown that the ATP-binding site on NCAM is overlapping with the FGFR binding site, and not surprisingly, ATP could inhibit NCAM-triggered signaling mediated through FGFRs (Kiselyov et al., 2003).

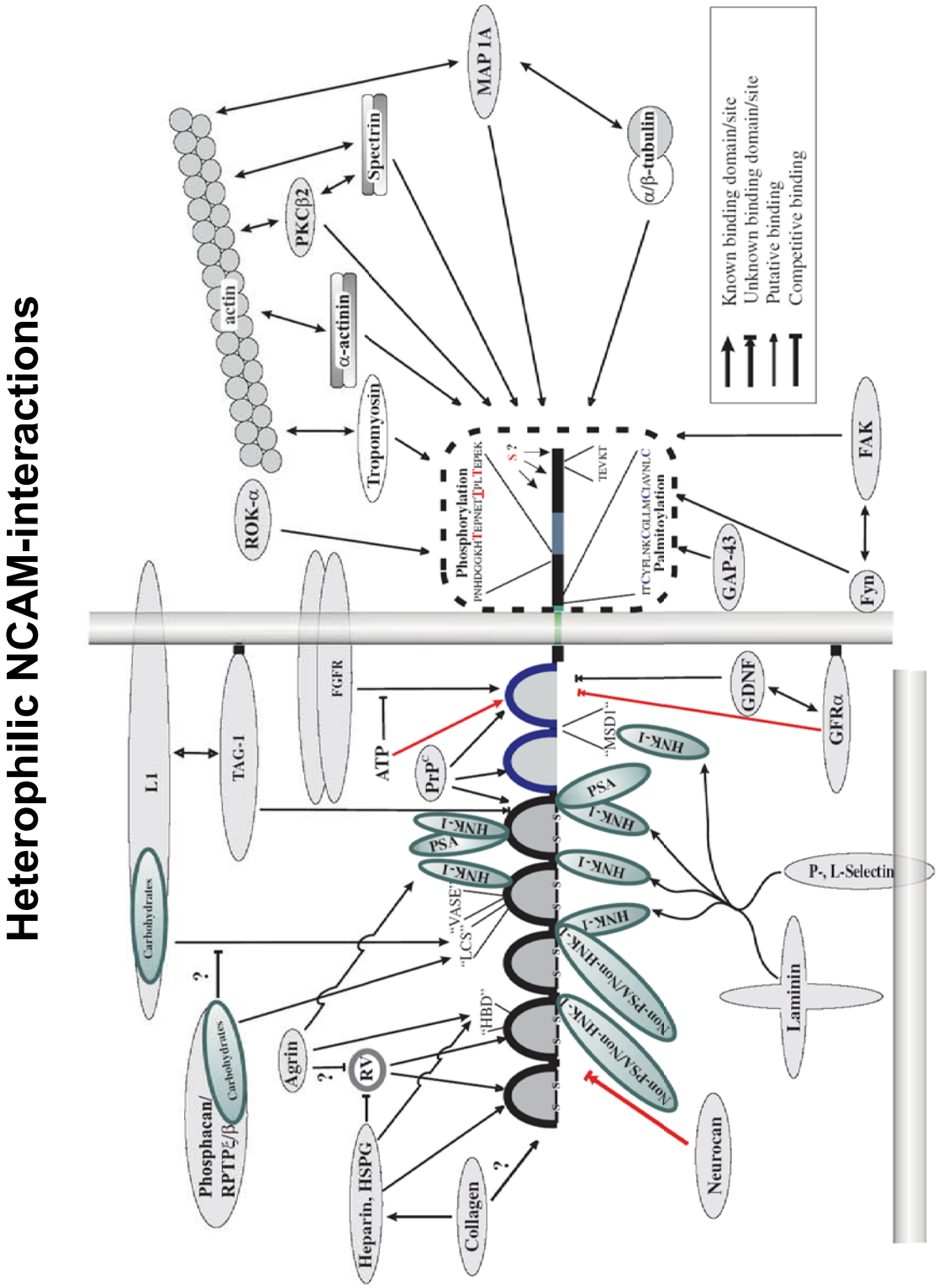
Another growth factor (see Fig. 5-I), the glial cell line-derived neurotrophic factor (GDNF) and its receptor, the GPI-linked GDNF family receptor-α (GFRα), both have been demonstrated to bind to NCAM directly (Paratcha et al., 2003). The binding of GDNF is independent of the presence PSA on NCAM and does not interfere with homophilic NCAM-NCAM interactions, whereas binding of GFRα to NCAM inhibits homophilic NCAM-NCAM trans-interactions, but at the same time potentiates binding of GDNF to NCAM. NCAM-GFRα-GDNF interactions have been shown to induce neurite outgrowth and Schwann cell migration, in an FGFR-independent manner involving intracellular signaling mediated via the kinase Fyn, indicating that NCAM-FGFR and NCAM-GFRα interactions and signaling may take place independently of each other.

NCAM may also interact with some components of the extracellular matrix (ECM). For example, NCAM carries a short, 17 aa-long sequence in the IgII module which can bind to heparin, a glycosaminoglycan of ECM, and it occurs independently from homophilic NCAM-NCAM interactions (Cole et al., 1986, 1989).

NCAM also can bind (see Fig. 5-I) to some chondroitin sulfate proteoglycans (CSPG) and heparan sulfate proteoglycans (HSPGs), including agrin, neurocan and phosphacan (Cole et al., 1986; Burg et al., 1995; Grumet et al., 1993). As it has been shown, for example, that agrin is important for synaptogenesis and axonal growth, interacting with NCAM through its heparin-binding domain in the IgII module and via PSA on the IgV module (Storms and Rutishauser, 1998).

Another example, (see Fig. 5-I) neurocan, which can interact with NCAM through many its sites, inhibiting homophilic NCAM-NCAM adhesion and neurite outgrowth (Retzler et al., 1996). NCAM may also bind to collagens I-V and IX, however, it occurs probably indirectly through heparan sulfate binding site of NCAM (Probstmeier et al., 1989, 1992).

Fig. 5-I. A summarizing scheme of main heterophilic NCAM-interactions. Carbohydrate moieties are shown in green. Putative Thr-phosphorylation sites in the cytoplasmic part of NCAM are shown in red (the most likely site is underlined). "S?" indicates unknown Ser-phosphorylation sites. Palmitoylation sites in the cytoplasmic part of NCAM are shown in blue. "TEVKT" – indicates the sequence of a putative binding site between NCAM and cytosolic signaling molecules. Interactions known to affect homophilic NCAM-NCAM interactions are shown with red arrows. For details, see the text. (From Walmod et al , 2004).



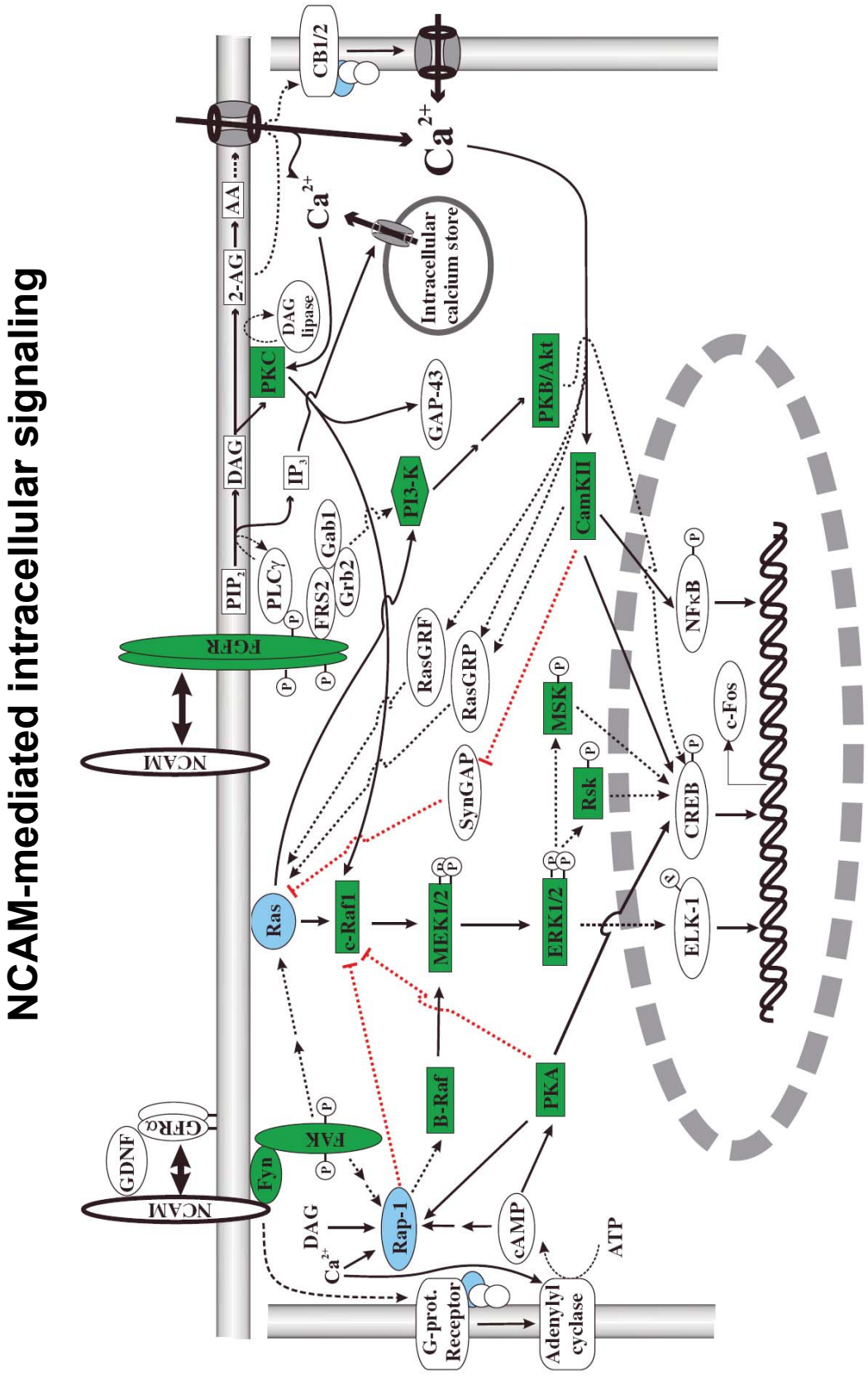
← Fig. 5-I (the figure legend is on the previous page)

Intracellular heterophilic NCAM interactions

NCAM has been demonstrated to participate in a number of direct or indirect interactions with many intracellular molecules (see Fig. 5-I). For example, in some studies NCAM-180, but not NCAM-140, can be anchored in the plasmatic membrane through intracellular interaction with a cytoskeletal linker-protein spectrin (Pollerberg et al., 1987). However, in recent studies have been shown that all three isoforms of NCAM can immunoprecipitate spectrin, NCAM-140 and NCAM-180 could be immunoprecipitated with a recombinant construct containing the spectrin repeats 2-3 of βI spectrin, NCAM-120 appeared to interact indirectly with spectrin via lipid rafts (Leshchynska et al., 2003). Another study showed that NCAM-180 and NCAM-140, both can be associated with α- and β-tubulin and α-actinin, (see Fig. 5-I) major components of the cytoskeleton (Büttner et al., 2003). In contrast, β-actin, tropomyosin, microtubuli-associated protein MAP 1A, and rhoA-binding kinase-α preferentially associate with NCAM-180.

It has been shown that NCAM-140 can co-immunoprecipitate with several kinases (see Fig. 5-I), e.g., a member of the Src-family non-receptor tyrosine kinases, Fyn, and the focal adhesion kinase FAK, and GAP-43 (He and Meiri, 2002). FAK is believed to interact indirectly with NCAM through SH2 domain of Fyn, but whether GAP-43 and Fyn interact directly or indirectly, it is not yet well known.

Fig. 6-I. A scheme of NCAM-mediated intracellular signaling pathways. In dashed lines, pathways, whose role in NCAM-mediated signaling remains to be determined. Kinases are shown in green, Ser/Thr-kinases or dual-kinases as squares, Tyr-kinases as ellipses. GTPases are shown in blue. Inhibitory pathways are indicated in red. (From Walmod et al., 2004).



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NCAM-mediated intracellular signaling.

Activation of NCAM-mediated intracellular signaling via tyrosine kinase receptors may lead to a wide diversity of cellular events (see Fig. 6-I). For instance, neuronal differentiation and axonal growth can be induced by NCAM via interaction with FGFRs, which triggers dimerization of FGFRs and its subsequent autophosphorilation (Doherty and Walsh, 1996). These phosphorylated sites of FGFRs on its cytoplasmic part become able to dock and activate several proteins, including an enzyme – phospholipase Cγ (PLCγ). After activation of the membrane form of PLCγ, it can cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) generating two second messengers – inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The first second messenger, IP_3 diffuses through the cytosole and binds to intracellular Ca^{2+}-channels, triggering of the release of Ca^{2+} from its intracellular stores and subsequently a rapid increase in intracellular calcium concentration; whereas, the second, DAG remains to be attached to the plasmatic membrane, and can either activate protein kinase C (PKC) or be converted by DAG-lipase to 2-arachidonylglycerol (2-AG), and arachidonic acid (AA), which can trigger various downstream signaling events (see Fig. 6-I).

There have been done several studies supporting described above model of NCAM-FGFRs signaling. First, it was shown that NCAM can directly interact with FGFRs inducing their autophosphorilation (Kiselyov et al., 2003). Furthermore, NCAM-mediated neurite outgrowth triggered via homophilic NCAM trans-interactions or by application of synthetic peptide ligands of NCAM, does require FGFRs activation (Rönn et al., 1999, 2000). Either of inhibitors to PLCγ or 2-AG or AA can block NCAM-driven neuritogenesis (Williams et al., 1994c; Kolkova et al., 2000). Interestingly, that AA may modulate specific Ca^{2+} channels located in the plasma membrane, triggering Ca^{2+} influx into cytosole and subsequent induction of neurite outgrowth (Williams et al., 1994b). Another lipid, 2-AG, was shown also be capable to induce FGFRs-mediated axonal outgrowth (see Fig. 6-I) via activation of G-protein-coupled cannabinoid receptors CB1 and CB2 followed by calcium influx through N- and L-types Ca^{2+} channels (Sugiura and Waku, 2002; Williams et al., 2003) which has been proved pharmacologically, with specific inhibitors of these subtypes of Ca^{2+} channels. Another study showing a link between NCAM and Ca^{2+} signaling was performed recently by Ronn and colleagues (2002) using single cell calcium imaging; it was nicely demonstrated that incubation of primary hippocampal neurons with a

synthetic peptide ligand of NCAM triggers Ca^{2+} increase in the cytosole, and such increase can be abolished via TMB-8, an inhibitor of intracellular calcium mobilization.

In addition to signaling through the receptor tyrosine kinase FGFRs, NCAM can induce downstream signaling via non-receptor tyrosine kinases leading to triggering of the mitogen-activated protein (MAP) kinases cascade (see Fig. 6-I). Such kinases could be Fyn and FAK, which can both form a functional complex with NCAM, activation of which leads to neuritogenesis. This idea is supported by experiments showing that NCAM-mediated neurite outgrowth is selectively blocked in neurons from Fyn knockout mice (Beggs et al., 1994), and by treatment with inhibitors of the MAP kinase kinases MEK1/2, which are targets for GTPase Ras, which is in turn a target for the FAK kinase (Schmid et al., 1999). Furthermore, as it has been shown, NCAM can trigger a phosphorylation of the MAP kinases ERK1/2 downstream of MEK1 and MEK2, and of the transcription factor CREB (cyclic-AMP response-element binding protein), which is activated either by Rsk kinase or via MSK1 kinase (mitogen and sress-activated kinases), both downstream of ERK1/2 kinases (Schmid et al., 1999). It has been also demonstrated that ERK1/2-mediated neuritogenesis can be induced via NCAM-NCAM homophilic trans-interaction or by NCAM peptide ligands (Kolkova et al., 2000). NCAM-mediated increase in intracellular Ca^{2+} can also lead to an activation of another CREB-phosphorylating kinase, calcium/calmodulin-dependent kinase II (CaMKII), since NCAM-FGFRs-mediated neuritogenesis can be blocked by the CaMK-inhibitors (Williams et al., 1995).

NCAM-induced neuritogenesis is also mediated via another signaling pathway (see Fig. 6-I) – cAMP/PKA, leading to activation of two transcription factors, CREB and c-Foc, which are downstream of PKA, since cAMP and PKA inhibitors can selectively abolish NCAM-mediated axonal outgrowth (Jessen et al., 2001).

Polysialic acid (PSA)

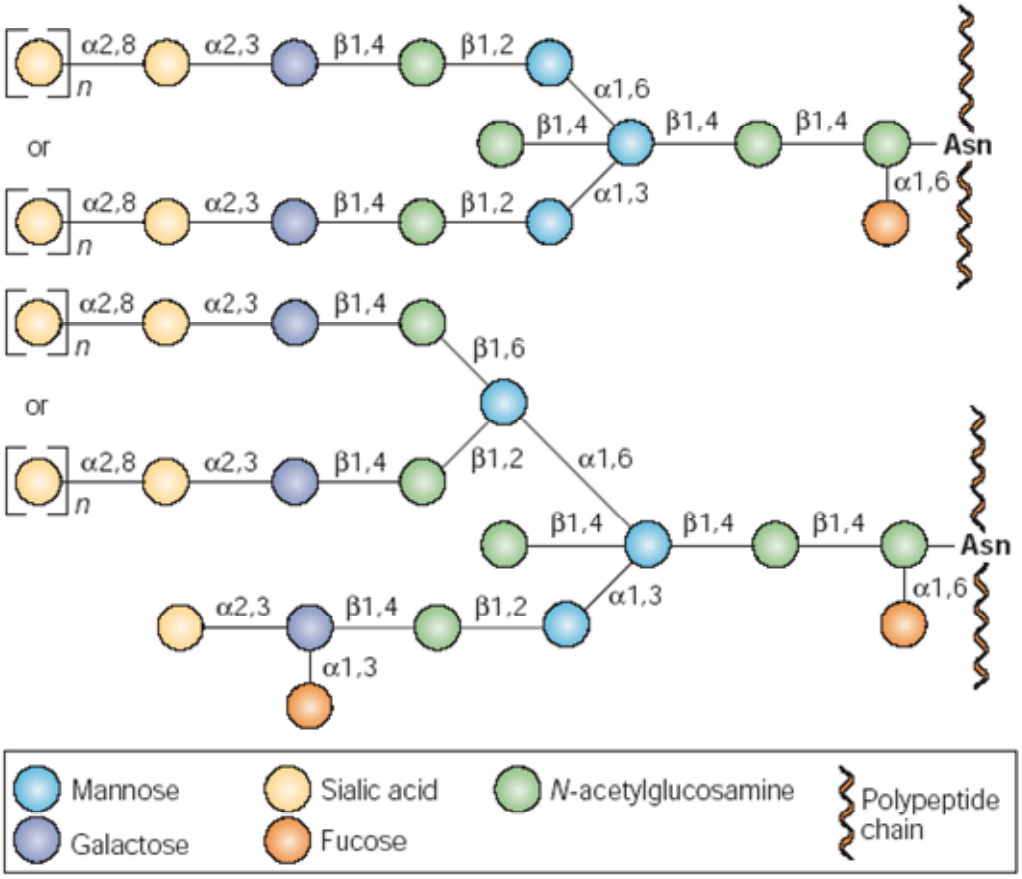

Fig. 7-I. Structure and attachment of polysialic acid (PSA) to the NCAM protein core through two *N*-glycosylation sites within the 5th Ig-like domain of all three isoforms of NCAM (from review, Kleene and Schachner, 2004).

Polysialic acid (PSA) and PSA-NCAM

NCAM can be post-translationally modified via polysialylation that is a highly spatially and temporally regulated process in neuronal and glial cells. The polysialylation of NCAM occurs in all vertebrates but appears to be absent in invertebrates (for review see Rutishauser, 1996; Rutishauser and Landmesser, 1996; Schachner, 1994). Polysialic acid (PSA) (see Fig. 7-I) can be attached to all three NCAM isoforms (including soluble ones), to their 5^{th} Ig-domain, associating with three amino acids Asn-430, Asn-459 and Asn-404 (Nelson, et al., 1995; Finne et al., 1983). PSA is a highly negatively charged sugar with an unusual α2,8 linkage in its chains which can be up to 200 residues long (see Fig. 7-I). Attachment of PSA to NCAM results in a large hydration cloud appearing around the core protein, which sterically inhibits homophilic binding of NCAM-NCAM on other cells.

The expression of PSA in the brain is very high during development; however it decreases drastically after birth. Nevertheless, PSA-NCAM expression persists in certain regions of the adult brain known to exhibit physiological plasticity or self-renewal (Theodosis et al., 1991; Seki et al., 1993): mouse olfactory bulb (Miragall and Schachner, 1988), rat periform and entorhinal cortices (Seki et al., 1991a; O'Connell et al., 1997), suprachiasmatic nucleus and hippocampus (Seki et al., 1991b, 1995; Murphy et al., 1996), hypothalamus-neurohypophysial system (Theodosis et al., 1991, 1994), mouse subventricular zone (SVZ) (Rousselot et al., 1995), nuclei of spinal cord and the rostral migratory stream, a pathway by which precursor cells migrate throughout life from SVZ of the forebrain to olfactory bulbs (Chazal et al., 2000).

In the adult hippocampus, PSA-NCAM expression persists in the mossy fibers (axons of the granule neurons), in the hilus of dentate gyrus, in the CA3a and CA3b stratum lucidum (Seki et al., 1991b, 1999), in the alveus and fimbria, above the hippocampal fissure on the molecular layer of CA1 (Seki et al., 1991b), in the subiculum and in Schaffer collaterals and neurons in CA3 (O'Connell et al., 1997). Non granule neurons express PSA-NCAM in the adult rat hippocampus, mainly in its ventral region, and most of these cells are also immunoreactive for GABA (indicating that they are inhibitory interneurons) (Nacher et al., 2002). Number of PSA-NCAM immunoreactive neurons and proliferating cells is increased after NMDA receptor antagonist administration in both the DG and periform cortex (Nacher et al., 2001).

As neurogenesis and cell death occur simultaneously in the adult dentate granular layer, a self-renewing capacity has been proposed for this region (Biebl et al., 2000).

There are many studies showing numerous roles of PSA during CNS development. For example, PSA-NCAM has been implicated in several morphogenetic processes; these include axonal growth (Doherty et al., 1990), sprouting (Zhang et al., 1992; Muller et al., 1994) and cell migration (Ono et al., 1994; Wang et al., 1994). PSA decreases neurite fasciculation of neuronal fibers promoting an opposite process – defasciculation by affecting the avidity of NCAM and other recognition molecules (Hoffman et al., 1983; Rutishauser and Landmesser, 1991). However, in the optic tract, the presence of PSA results in increased fasciculation of retinal axons (Yin and Rutishauser, 1995). Similarly, the hippocampus of adult NCAM-deficient mice shows highly disorganized and defasciculated axons of the CA3 region of the hippocampus compared to controls (Cremer et al., 1997).

There have been discovered two enzymes which catalyze attachment of PSA to NCAM protein backbone – ST8SiaII/STX is involved in polysialylation of NCAM during development, and in adults, in stem cell-derived immature granule cell neurons; and ST8SiaIV/PST is involved in polysialylation of NCAM in mature neurons. Both PST and STX can synthesize PSA on α-2,3- or α-2,6-linked sialic acid on NCAM without an initiator (Muhlenhoff, 1996; Kojima, 1996) and both are potentially involved in the biosynthesis of PSA associated with NCAM in mammalian tissues.

Enzymatic removal of PSA

Endoneuraminidase N (Endo-N), an enzyme isolated from bacteriophages, which specifically recognizes the 3D-structure of sialic acid polymers in α-2,8-linkage and cleaves units of eight sugar residues (Finne and Mäkelä, 1985). There were several studies *in vivo* and *in vitro*, in which it has been used Endo-N for acute reversible removal of PSA from NCAM. For example, elimination of PSA by Endo-N injected via chronically implanted cannulas directly into the rat hippocampi led to a significant impairment in the formation of spatial memory of rats in the Moris water maze (Becker et al., 1996) (see Table 1-I). Also, Endo-N treatment completely prevented the induction of LTP and LTD in organotypic cultures made from the CA1 region of hippocampus, not affecting other cellular and synaptic parameters such as resting or action potentials (Muller et al., 1996). Also in hippocampal slices Endo-N treatment

reduced LTP (Becker et al., 1996). Removal of PSA from NCAM by Endo-N also disrupted neuronal migration, axonal sprouting, branching and fasciculation (Durbec and Cremer, 2001; Yamamoto et al., 2000), and synaptogenesis (Dityatev et al., 2000, 2004). Interestingly, PSA reexpression after Endo-N treatment is Ca^{2+} dependent and can be modulated by neuronal impulse activity in cultures of cortical neurons (Kiss and Rougon, 1994) and at synapses in hippocampal organotypic cultures (Muller et al., 1996).

PSA-NCAM, synaptic plasticity and learning

Considerable progress has been made in understanding the numerous functions of NCAM and its associated carbohydrate, polysialic acid, in ontogenetic development and in synaptic plasticity, learning and memory in the adult (for reviews, see Panicker et al., 2003; Kleene and Schachner, 2004). Perturbation of NCAM functions by ablation of NCAM or by disruption of NCAM-mediated interactions *in vitro* and *in vivo* impaired synaptic plasticity in the hippocampus, induced amnesia in a passive avoidance task and caused spatial memory deficits (Muller et al., 1996; 2000; Luthi et al., 1994; Cremer et al., 1994, 1998; Doyle et al., 1992b; Arami et al., 1996; Bukalo et al., 2004; Stoenica et al., 2006).

PSA attached to NCAM is thought to modulate binding of the NCAM glycoprotein backbone by steric interference, thus promoting plastic changes (see refs in Weinhold et al., 2005). Removal of PSA by endosialidase-N, an enzyme which specifically cleaves PSA, disturbs neuronal migration, axonal sprouting, branching and fasciculation (Durbec and Cremer, 2001; Yamamoto et al., 2000), synaptogenesis (Dityatev et al., 2000, 2004), synaptic plasticity (Becker et al., 1996; Muller et al., 1996) and spatial memory (Becker et al., 1996). The expression of PSA is high during development and up-regulated in the adult brain by neuronal activity (Muller et al., 1996), and during learning-induced neuroplasticity in the hippocampus (Murphy et al., 1996) and entorhinal cortex (O'Connell et al., 1997).

Polysialyltransferases ST8SiaII/STX (immature neurons) and ST8SiaIV/PST (mature neurons) which are important for polysialylation of NCAM during early phases of development, and in adults, respectively, can be ablated in knockout mice showing that PST is required for LTP and LTD recorded in the CA1 area of hippocampal slices (Eckhart et al., 2000), but not for dentate gyrus LTP recorded in anaesthetized animals (Stoenica et al., 2006), whereas the embryonic form of this

enzyme (STX) is required neither for CA1 (Angata et al., 2004) nor for dentate gyrus LTP (Stoenica et al., 2006), however it is important for processing of contextual and tone memories (Angata et al., 2004).

Mice deficient in NCAM or PSA

During last 12 years, several NCAM-related transgenic mouse lines have been generated (see Table 1-I for description of phenotypes of main transgenic strains), which greatly advanced our understanding of multiple roles of NCAM in synaptic plasticity, learning and memory. In the mid 90th there were genetically constructed knockout mice lacking all isoforms of NCAM (Cremer et al., 1994), phenotype of which (see Table 1-I) appeared to be surprisingly mild; mice were found to have size-reduced olfactory bulbs, due to a defect in migration of olfactory neuron precursors (Ono et al., 1994) and increased lateral ventricles and deficits in spatial learning and exploratory behaviour (Cremer, 1994). Other studies showed that these mice have cognitive deficits in contextual and cued fear conditioning (Stork et al., 2000), impaired LTP in the CA1 and CA3 regions *in vitro* (Muller et al., 1996; Cremer et al., 1998) and abolished dentate gyrus LTP *in vivo* (Stoenica et al., 2006).

Another mutant – conditional NCAM knockout mouse (see Table 1-I), in which ablation of NCAM gene occurs postnatally approximately at day P22, shows decreased CA1 LTP *in vitro* and deficits in special memory (like constitutively NCAM deficient mice), but normal CA3 LTP and normal mossy fibers morphology (in contrast to NCAM-/- mice) (Bukalo et al., 2004).

Interestingly, mice deficient only in the 180 kDa isoforms of NCAM (see Table 1-I) have impaired granular cell migration in the rostral migratory stream from SVZ of the forebrain to olfactory bulbs (Tomasiewicz et al., 1993) and decreased prepulse inhibition and increased lateral ventricles (Wood et al., 1998).

Overexpression of NCAM-180 isoform in the brain of NCAM+/+$^{180+}$ transgenic mice (see Table 1-I) improved neither spatial memory in water maze nor contextual and tone memories in fear conditioning. Surprisinly, specific expression of NCAM-180 isoform in NCAM-/- mice rescued neither their severe deficits in spatial memory nor fear conditioning, only aggressive behaviour was improved, and also failed to normalize olfactory bulbs and mossy fibers morphology (Stork et al., 2000).

Recently, several new interesting mutants were generated (see Table 1-I). One of them is NCAM-EC mouse that has overexpressed soluble extracellular part of NCAM in the brain (mimicking situation in schizophrenic patients). These mutants

have profound deficits in both contextual and cued fear conditioning, and abnormal formation of GABAergic synapses in the cingulated, frontal association cortex, and amygdala, but surprisingly not in the hippocampus (Pillai-Nair et al., 2005). A double knockout NCAM+/+$^{PSA-}$ which lacks both polysialyltransferases (PST and STX) and therefore do not express PSA in the brain show severe deficits, including multiple brain wiring defects, progressive hydrocephalus, postnatal growth retardation, plus morphological abnormalities exhibited by NCAM-/- mice. Strikingly, these drastic defects could be selectively rescued by additional deletion of NCAM gene in triple NCAM-/-$^{PSA-}$ knockout, demonstrating that they originated from a gain of NCAM functions because of PSA deficiency (Weinhold et al., 2005).

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Table 1-I. Summary of studies showing different effects of NCAM and PSA manipulations on synaptic plasticity, learning, memory and morphology.
NCAM Abs – injection of anti-NCAM antibodies; C3d – injection of synthetic peptide interfering with NCAM-NCAM homophilic interactions; FGL – a peptide corresponding to the binding site of NCAM for the fibroblast growth factor receptor 1 (FGFR1); Endo-N – an enzyme which cleaves PSA from NCAM; NCAM-/- - mutants in which all NCAM isoforms are deleted; NCAM+/+$^{180+}$ - mutants in which NCAM-180 is overexpressed; NCAM-/-$^{180+}$ - mutants in which only NCAM-180 is expressed; NCAM-180$^{-/-}$ - mutants in which only NCAM-180 isoform has been deleted; NCAMff, cre^{T} – tamoxifen-inducible mutants; PST-/- - mutants in which one (adult form) from two polysialyltransferases has been ablated; STX-/- - mutants in which one (embryonic form) from two polysialyltransferases has been ablated; NCAM-EC – mutants with overexpression of soluble extracellular fomain of NCAM; NCAM+/+$^{PSA-}$ - double mutants in which both polysialyltransferases have been ablated resulting in the total absence of PSA in the brain; NCAM-/-$^{PSA-}$ - triple mutants in which both polysialyltransferases have been ablated together with NCAM gene.

Effects of different NCAM & PSA manipulations on synaptic plasticity, learning and memory

Manipulation	Electrophysiology				Learning & Memory			Morphology	Others
	CA1		CA3	DG	MWM	fear conditioning			
	LTP in vitro	LTD in vitro	LTP in vitro	LTP in vivo	spatial memory	context memory	tone memory		
NCAM Abs	↓ 5,13				↓6				
C3d peptide					↓21	c↓22, f= 22,			
FGL peptide					↑ 24	↑ 24	↑ 24		
Endo-N	↓ 7,8	↓ 7,8			↓7,21				
NCAMff+ CaMKII promoter(P22)	↓9	↓9	=9		↓9			*mf* = 9	
NCAM-/-	↓1		↓3	↓12	↓2	↓ 4	↓ 4	*mf* ≠ 3, *ven* ≠ 3, *olfb* ≠ 16,18,2	↑anxiety 17, ≠ sensitization to startle 19, = PPI 20, ↑aggression 4
NCAM+/+$^{180+}$					=4	= 4	= 4	*olfb* = 4, *mf* = 4	= aggression 4
NCAM-/-$^{180+}$					=4	↓ 4	↓ 4	*olfb* ≠ 4, *mf* ≠ 4	= aggression 4
NCAM-180$^{-/-}$								cell migration in *ven* and *olfb* ≠ 15,, *ven* ≠ 23	↓ acoustic PPI 23
NCAMff, cre^{T} PrP promoter (adult), inducible						↓S	=S ?		
PST-/- no PSA in mature neurons	↓ 10	↓ 10		=12		↓S	=S		
STX-/- no PSA in immature neurons	=11		=11	=12		↓ 11	↓ 11	*mf* ≠ 11	
NCAM-EC						↓ 14	↓ 14	↓ GABA intern. in *FC*, *amyg* but = *hip* 14	↓ acoustic PPI 14
NCAM+/+$^{PSA-}$								hydrocephalus 25, *mf* ≠ 25, multiple brain wiring defects, 25,	
NCAM-/-$^{PSA-}$								Rescue of wiring defects 25	

References

1 – Muller et al., 1996;
2 – Cremer et al., 1994;
3 – Cremer et al., 1998;
4 – Stork et al., 2000;
5 – Lüthi et al., 1994;
6 – Arami et al., 1996;
7 – Becker et al., 1996;
8 – Muller et al., 2000;
9 – Bukalo et al., 2004;
10 – Eckhart et al., 2000;
11 – Angata et al., 2004;
12 – Stoenica et al., 2006;
13 – Rönn et al., 1995;
14 – Pillai-Nair et al., 2005;
15 – Tomasievicz et al., 1993;
16 – Ono et al., 1994;
17 – Stork et al., 1999;
18 – Chazal et al., 2000;
19 – Plappert et al., 2006;
20 – Plappert et al., 2005;
21 – Venero et al., 2006;
22 – Cambon et al., 2003;
23 – Wood et al., 1998;
24 – Cambon et al., 2004;
25 – Weinhold et al., 2005

S – Senkov et al., unpub.

= normal
≠ abnormal
↓ decreased or impaired
↑ Increased or facilitated
mf Mossy Fibers
FC Frontal Cortex
amyg. Amygdala
hip. Hippocamus
ven Ventricles,
Olfb Olfactory Bulbs
PPI – prepuls inhibition

← Table 1-I (the table legend is on the previous page)

Pavlovian Classical Fear Conditioning

Learning and memory principles

Learning is the mechanism by which new information about the world is acquired, and memory as the mechanism by which that knowledge is retained. There are two

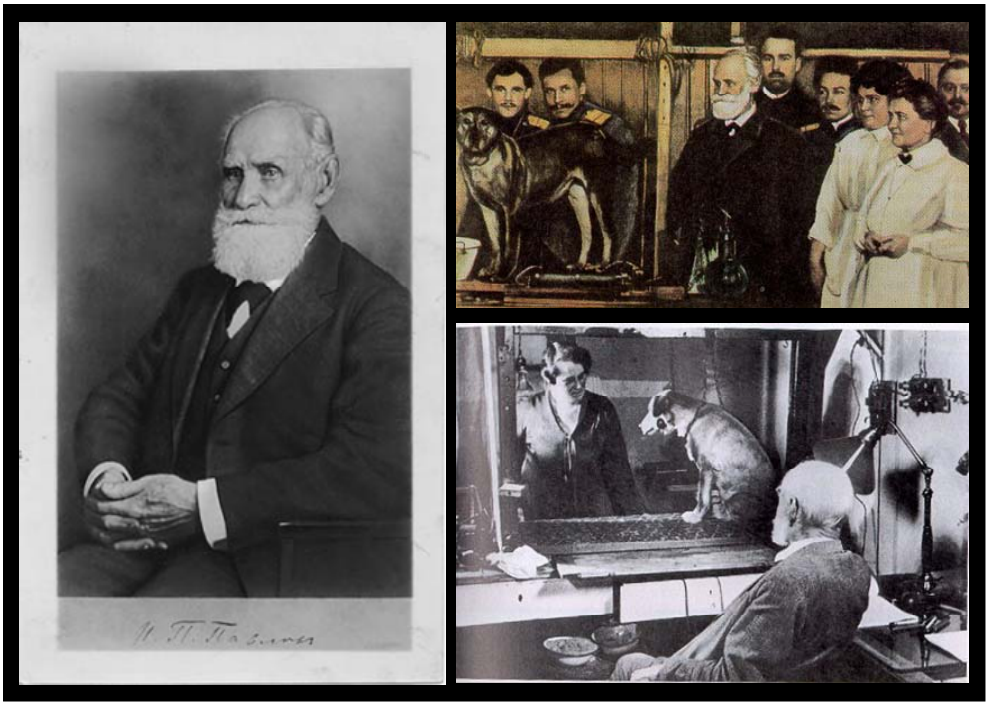


Fig. 8-I. Ivan Pavlov, a Nobel Prize winner 1904 (physiology and medicine), during his experiments with dogs and classical conditioning.

systems of memory: explicit (declarative), which is defined as that involved in the conscious recall of information about people, places, events and things, or implicit (procedural), which is characterized by perceptual learning and the nonconscious recall of tasks such as motor skills etc.

Explicit memory depends on the integrity of many brain areas: temporal lobe and diencephalic structures such as the hippocampus, subiculum, and entorhinal cortex. Implicit memory includes simple associative forms of memory, such as classical conditioning, and nonassociative forms, such as habituation, and relies on the integrity of the cerebellum and basal ganglia. However, for rodents, contextual and cued fear conditioning is an example of explicit learning, since during training they have to make association of particular context or stimulus with aversive

unconditional stimulus such as footshock and then afterwards be able to consciously recall and discriminate it.

Fear conditioning

Fear conditioning is a form of learning in which fear is associated with a particular neutral context (e.g., an experimental cage) or neutral stimulus (e.g., a tone, a light). This can be done by pairing the neutral stimulus with an aversive stimulus (e.g., a

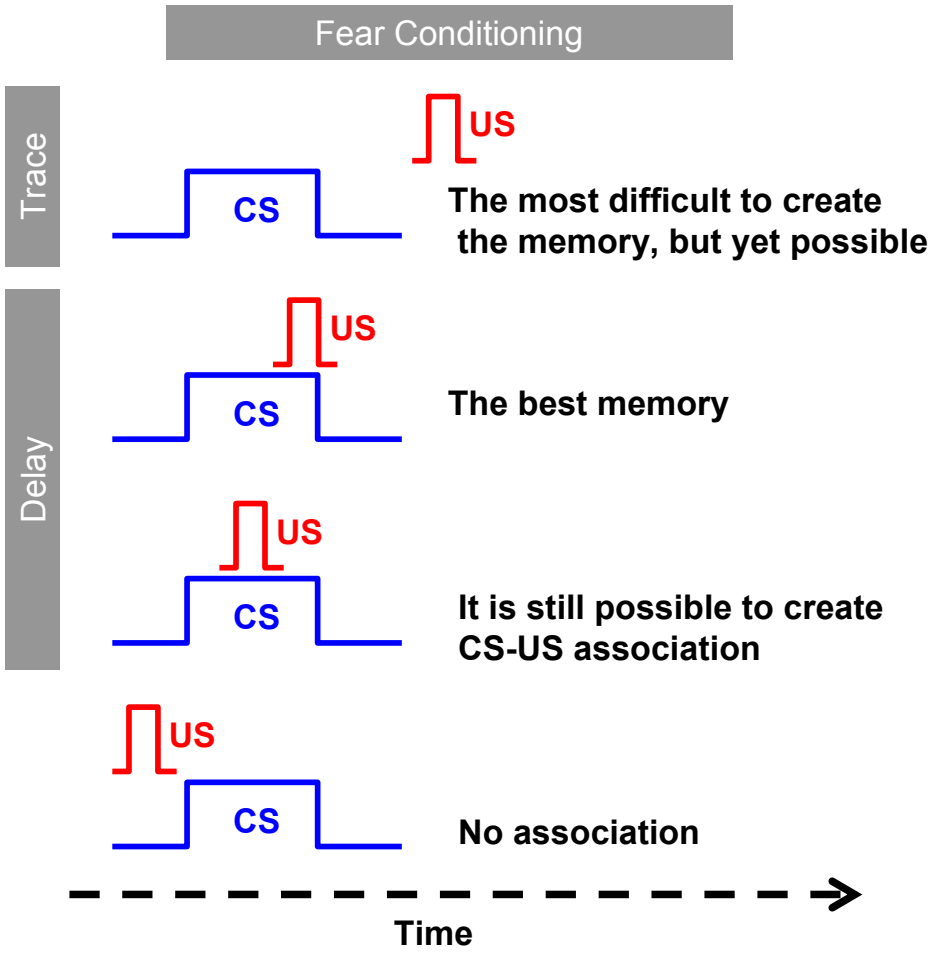


Fig. 9-I. The explanation of different temporal cases of CS-US associations which can lead to different memory strength. CS – conditioned stimulus, a tone; US – unconditioned stimulus, a footshock; Delay and Trace conditioning – are two most studied forms of fear conditioning; the trace fear conditioning is dependent on the hippocampus, whereas the delay fear conditioning relies mostly on the amygdala.

shock, loud noise, or unpleasant odour). Eventually, the neutral stimulus alone can elicit the state of fear. Fear conditioning has been studied in different species, from snails to humans. In humans, conditioned fear is often measured with verbal report

and galvanic skin response. In other animals, conditioned fear is often measured with freezing (a period of immobility) or fear potentiated startle (the augmentation of the startle reflex by a fearful stimulus). Changes in heart rate, breathing, and muscle responses via electromyography can also be used to measure conditioned fear.

Classical conditioning had been invented like a theoretical and then experimental concept by Russian physiologist Ivan Pavlov (see Fig. 8-I) in the late 20th of the last century (Pavlov, 1927), long after when he received his Nobel Prize in physiology or medicine (1904) for outstanding work on physiology of digestion. Afterwards, described by him classical conditioning theory became to bear his name “pavlovian classical conditioning”. But, as many believe, the discovery had been made nearly by an anecdotic case; when Pavlov observed that hungry dogs (see Fig. 8-I) salivated not only at the taste or sight of food, but rather at the sight or sound of the caretaker who regularly fed them, he described this salivation as a “conditional reflex”. Salivation was inborn, genetically wired reflexive response (unconditioned response, UR), unconditionally, requiring no learning, elicited by food (unconditioned stimulus, US) in the mouth, but which could be elicited by other stimuli conditionally, after certain numbers of pairings e.g. a bell, metronome, or flashing light (conditioned stimulus, CS) with USs.

Pavlovian fear conditioning is nowadays a leading behavioural paradigm for investigating different forms of associative learning and memory. The main idea behind it is that two different stimuli can be associated with each other in a way that one acquires a meaning of the other by temporal coincidence. After as little as one single pairing of a conditioned stimulus (CS, an unimodal cued stimulus: tone, light, smell) with unconditioned stimulus (US: mild footshock, air puff), the former starts to elicit defensive fear responses, such as freezing, acoustic startle, elevated blood pressure, thus becoming a predictor for the danger (Pavlov, 1927; LeDoux, 2000; Maren, 2001; Dityatev and Bolshakov, 2005). In addition to the cued, temporally restricted CSs (clicks of tone, flash of light), the US can be also associated with a more complex and long-lasting polymodal stimuli perceived by subject in conjunction as a context. Pavlovian fear conditioning involves learning that certain environmental stimuli predict aversive events. There are two major ways (see Fig. 9-I) to create CS-US association: the first, so-called “delay” hippocampus-independent fear conditioning of cued stimuli (see Fig. 9-I), when CS and US temporally coincide, and which is the most easier case to create a strong association; the second – a “trace”

hippocampus-dependent fear conditioning (see Fig. 9-I), when between the CS offset and the US onset, there is a time interval, such association is much more difficult to learn than the delay procedure.

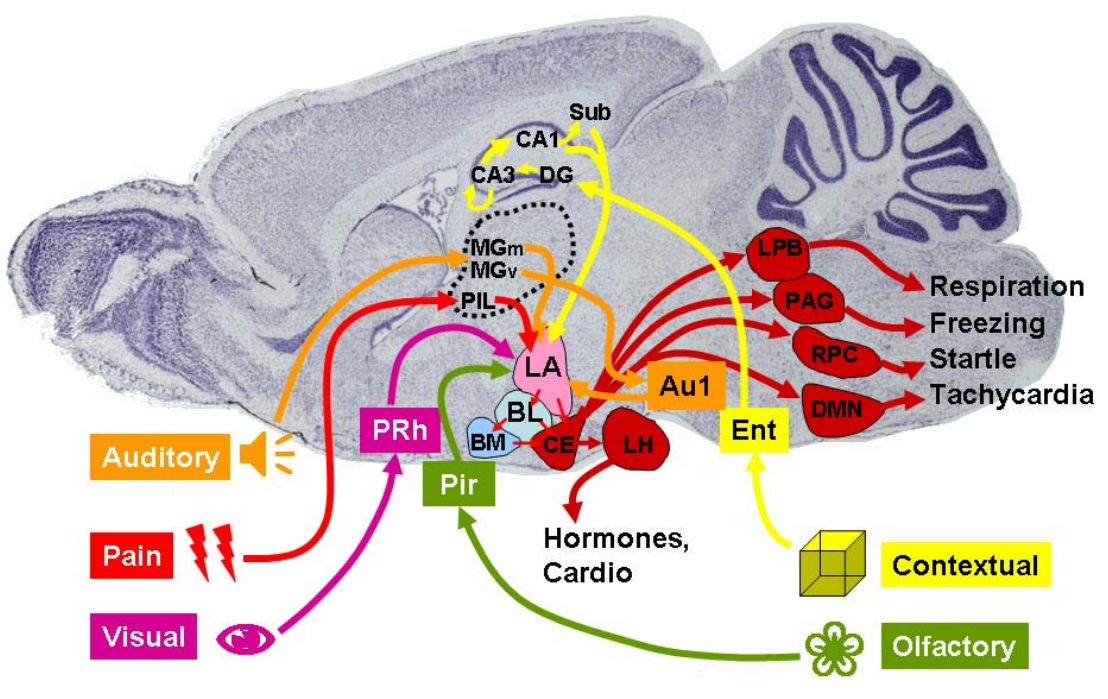


Fig 10-I. Schematic drawing of fear conditioning circuit involved in convergence of different CS modalities with US within the amygdala. Amygdala: LA – Lateral, BL – basolateral, BM – basomedial CE – central nuclei; Hippocampus: CA1, CA3 regions, DG – Dentate Gyrus, Sub – Subiculum; Thalamus: MGm – Medial Geniculate nucleus, medial part; MGv – Medial Geniculate nucleus, ventral part; PIL – Posterior Intralaminar nucleus; Cortices: PRh – Perirhinal, Pir – Piriform, Au1 – Auditory Primary, Ent – Entorhinal; Midbrain: LPB – Lateral Parabrachial nucleus, PAG – Periaqueductal Gray, RPC – Reticularis Points Caudalis nucleus, DMN – Dorsal Motor Nucleus of the Vagus; LH – Lateral Hypothalamus.

Hippocampus

Sensory contextual information is thought to be transmitted from the cerebral associational neocortex via a chain of rhinal cortices into the hippocampus through three different pathways (see Fig. 10-I and 11-I); the main excitatory input into the hippocampal trisynaptic circuit is formed by axons of cells originated from layer II of the entorhinal cortex and ending their terminals on granule cells of the dentate gyrus

(DG) through the perforant path. Two additional inputs into the hippocampus start from the same entorhinal cortex (layers II and III) but they convey information either to the CA3 pyramidal cells or directly to the CA1 area through the same perforant pathway. Further (see Fig. 11-I), from granule cells of DG contextual information flows through the mossy fibres into CA3 pyramidal cells, which are massively interconnected by recurrent collaterals that run both ipsilaterally and contralaterally. Among the three types of excitatory input that CA3 pyramidal cells receive, these recurrent collateral inputs are the most numerous (about 12,000 synapses per one pyramidal cell in the rat), whereas the perforant path and mossy fiber inputs provide only about 4,000 and 50 synapses per cell, respectively (MacVicar and Dudek, 1980a,b). From CA3 pyramidal cells information is then transmitted into the CA1 area of the hippocampus through the Schaffer collaterals, whereas CA1 pyramidal cells finally send their axons back to the entorhinal cortex either directly or through the subiculum.

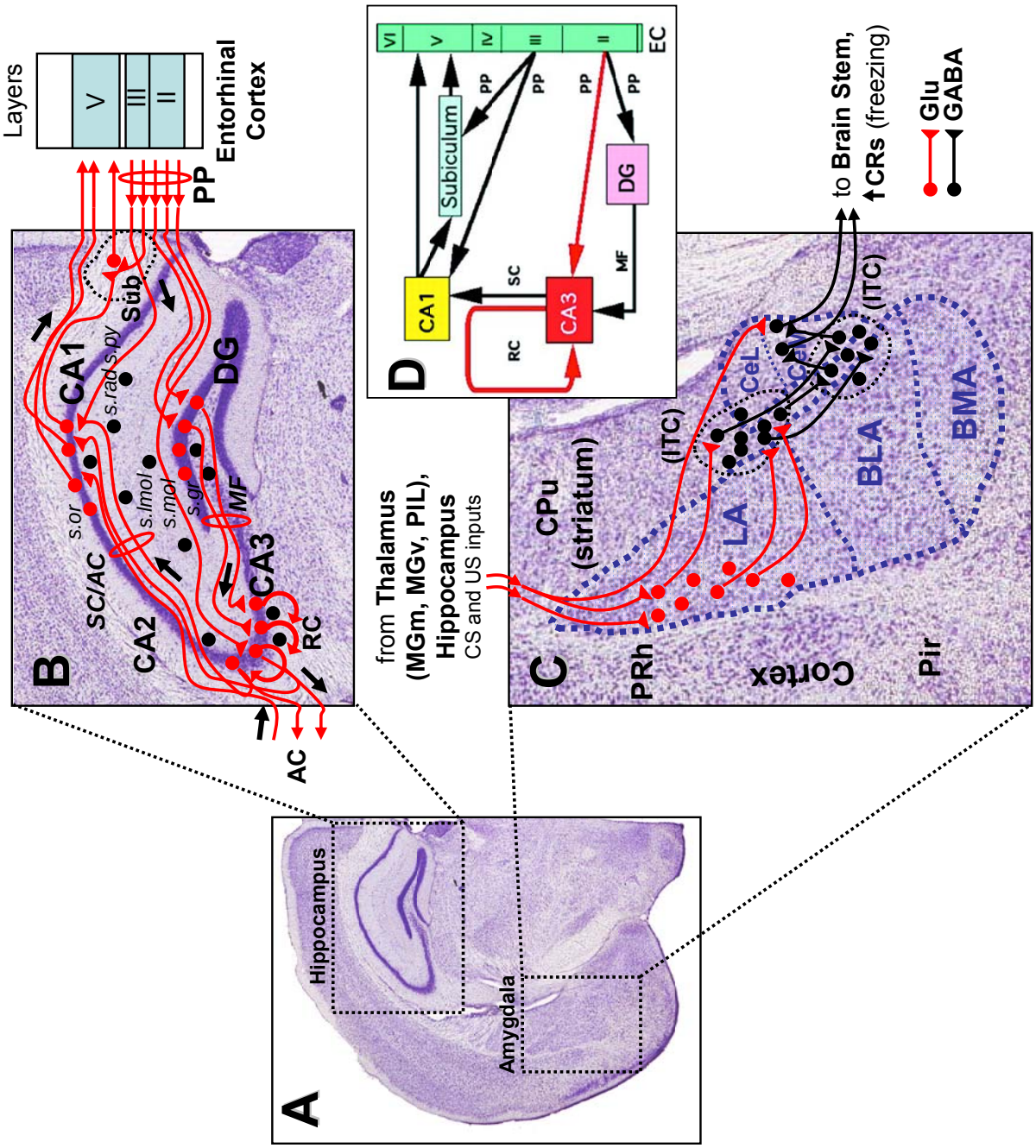
A number of theoretical studies have proposed a distinct mnemonic role for each of the hippocampal subfields and inputs. For instance, it has been postulated that recurrent network in CA3 area (see Fig. 11-I) is crucial for the storage of autoassociative memory and it works via a pattern completion mechanism, whereas the DG is involved in separation of similar memories (pattern separation) and CA1 network is utilized in recognizing the novelty and/or familiarity of an object or context (pattern recognition) (McClelland and Goddard, 1996; McNaughton and Morris, 1987; O'Reilly and McClelland, 1994; Marr, 1971; O'Reilly and Rudy, 2001; Rudy and O'Reilly, 2001).

However, the hippocampus can be involved not only in processing of very complex polymodal information like a context, but also in more simple unimodal analysis; e.g. in the hippocampus locally can be detected by electrodes almost all modalities of evoked potentials (EPs), including auditory, olfactory and visual evoked potentials. Interestingly, in the CA1 area of the hippocampus of freely moving mice auditory EPs elicited by CS after fear conditioning undergo long-lasting potentiation that correlates with freezing responses and potentiation of auditory APs in the amygdala (Tang et al., 2003).

The current most plausible model of how the hippocampus is involved in fear conditioning is that the hippocampus is required for assembling the elemental unimodal cues within a particular training context (smell, geometry of training cage,

illumination, tactile information, visual cues, etc.) into a configural representation (for review see O'Reilly and Rudy, 2001; Fanselow and Poulos, 2005), what becomes associated with US in the amygdala during fear conditioning. Support for this model comes from the finding that hippocampal lesion-induced deficits in contextual fear conditioning can be rescued by a preexposure to this context a month prior to conditioning (Young et al., 1994). However, when the hippocampus is damaged and unable anymore assemble contextual information into unified configural representation, context-US association can occur by utilizing another scenario, - individual contextual cues can be yet bound separately with aversive information (Maren et al., 1997, 1998; Rudy and O'Reilly, 1999).

Fig 11-I. Hippocampal and amygdaloid circuits involved in fear conditioning.
A – A frontal section of the mouse brain, left hemisphere;
B – Hippocampus: CA1, CA2, CA3 hippocampal areas, Sub – Subiculum, DG – Dentate Gyrus, SC/AC – Schaffer collateral pathway and Associational Commissural pathway, MF – Mossy fibers pathway, RC – Recurrent Connections, PP – Perforant Path; layers: s.or – Stratum Orients, s.py – Stratum Pyramidale, s.rad – Stratum Radiatum, s.lmol – Stratum Lacunosum Moleculare, s.mol – Stratum Moleculare, s.gr – Stratum Granulare.
C - Amygdala: LA – Lateral Amygdala, BLA – Basolateral Amygdala, BMA – Basomedial Amygdala, CeL – Central nucleus, lateral, CeM – Central necleus, medial, ITC – intercalated neurons, CPu – Caudate Putamen, PRh – Perirhinal cortex, Pir – Piriform cortex, Thalamus: MGm – Medial Geniculate nucleus, medial part, MGv – Medial Geniculate nucleus, ventral part, PIL - Posterior Intralaminar nucleus. (a current model of the amygdala circuitry, adopted from Pare et al., 2004).
D – A scheme of trisynaptic hippocampal circuitry inputs and outputs originated and ended in the Entorhinal cortex.



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Amygdala

In classical fear conditioning paradigm, the amygdala is a key anatomical structure in the brain (see Fig. 10-I), providing a bridge between many sensory inputs and nociceptive system. In rodents, the amygdala consists of approximately 12 anatomically and functionally distinct nuclei (see Fig. 10-I), including the lateral (LA), basolateral (BL), basomedial, and central (CE) amygdaloid nuclei (for review see Dityatev and Bolshakov, 2005). There are two different subsystems within the amygdala which are involved in fear conditioning: the basolateral complex (BLA) that is comprised of LA, BL and basomedial nuclei and serve as the primary sensory interface, a gateway into of the amygdale. Lesions of BLA produce deficit in the acquisition and expression of fear conditioning independent of stimulus modality (Campeau and Davis, 1995; Maren et al., 1996). The second subsystem is the CE nuclei which represent the amygdala's interface to fear response systems (Maren et al., 1996). Electrical stimulation of the CE produces behavioral responses similar to those evoked by stimuli paired with footshock (Iwata et al., 1987), and its lesion – deficits in conditional fear responses, rather than an associative deficit (Fanselow and Kim, 1994; Goosens et al., 2000).

The pathways which convey CS information into the amygdala have been studied extensively in recent years, especially auditory CS inputs. It is known that auditory CSs reach LA equally through both the auditory thalamus and auditory cortex (see Fig. 10-I and 11-I) (LeDoux et al., 1990), however cortical input is thought to be involved in processing more complex auditory stimulus patterns and thus it transmits synaptic plasticity and learning in fear conditioning more slowly than does thalamic input (Quirk et al., 1995; 1997).

Hippocampus-dependent contextual information could become associated with US during fear conditioning within the BLA complex by means of direct projections between the hippocampus and amygdale, which bidirectionally exhibit synaptic plasticity (Kim and Fanselow, 1992; Maren, 1999; Maren and Fanselow, 1995; Phillips and LeDoux, 1992). Visual CS (e.g. flash of light) can be transmitted to the BLA from the perirhinal cortex (Campeau et al., 1995).

Many lesions studies showed that amygdala is essential for processing of contextual (Antoniadis and McDonald, 2000; Maren, 1998), auditory (Campeau and Davis, 1995; Maren et al., 1996), olfactory (Cousens and Otto, 1998), and visual CSs (Sananes and Davis, 1992).

Nociceptive system

In classical fear conditioning paradigm, for conditioning to occur, pathways transmitting the CS and US have to converge in the brain. It is widely proved that the amygdala is one of the major sites of plasticity during fear conditioning. LA receives afferents from thalamic areas which convey nociceptive information from spino-thalamic tract (for review, see LeDoux, 2000). Furthermore, some of the LA cells are responsive to both pain pathway and auditory/visul stimulations (Romanski et al., 1993), which indicates that the amygdala is a locus of convergence for information about CSs and US, an integrator of information from a variety of sensory domains.

Nociceptive information comes to the amygdala not only via thalamus, but as well through cortical areas that process somatosensory stimuli, and recent behavioural studies showed that conditioning can be mediated by US inputs to LA from either of two nociceptive inputs coming from thalamic or cortical areas. BLA receives US inputs from posterior thalamus (see Fig. 10-I) (LeDoux, 2003), which is a terminal region of the nociceptive spinothalamic tract. Central nuclei of amygdala receive nociceptive inputs from parabrachial area and directly from the spinal cord (for review, see Maren, 2001).

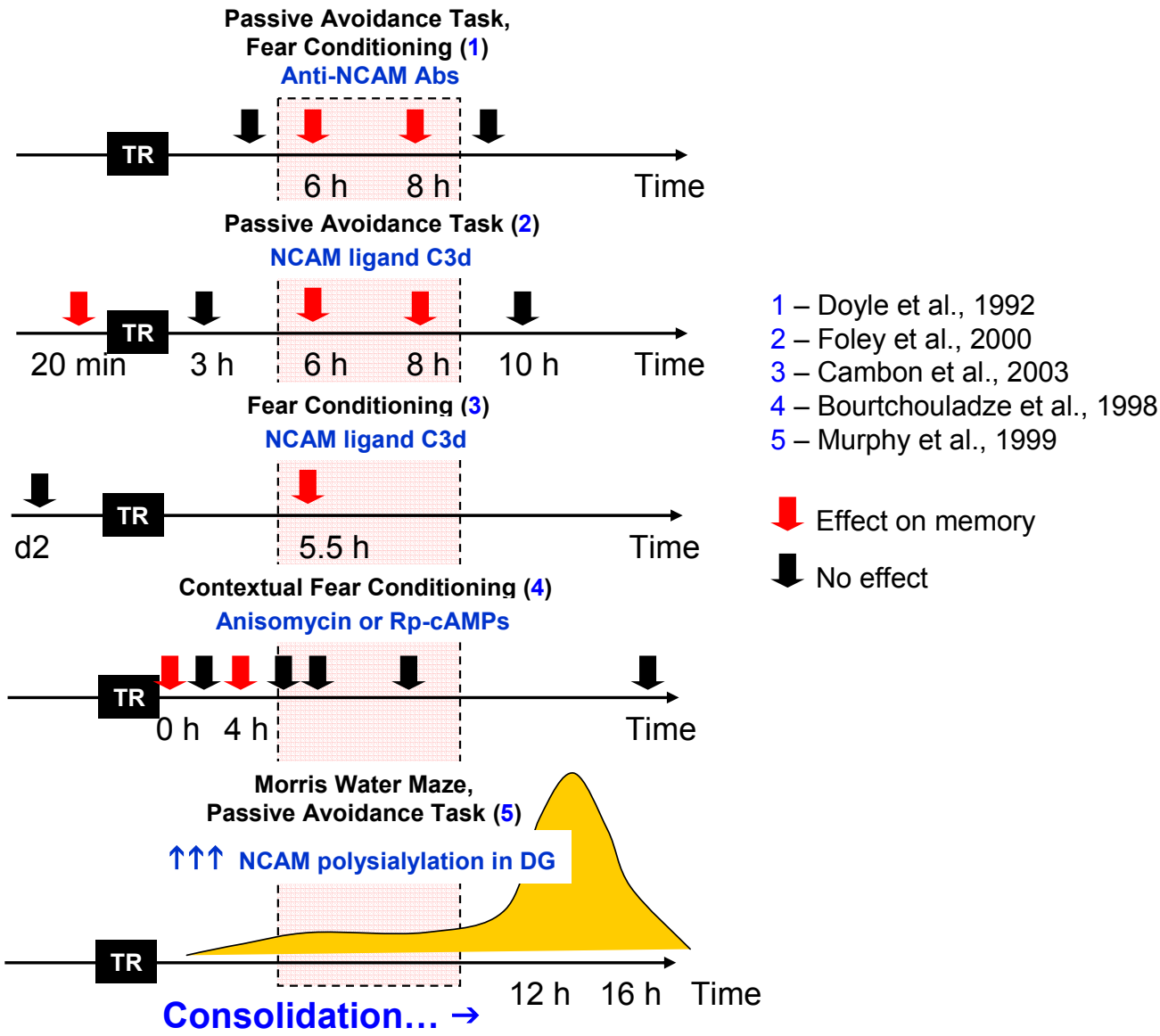
Fear responses pathways

It is widely accepted that after fear conditioning, for example, auditory CS associated with US becomes capable alone to elicit aversive responses such as defensive behavior, freezing, autonomic arousal, hypoalgesia, reflex potentiation, stress hormones, etc. All these fear responses (see Fig. 10-I) are thought to be mediated through CE nuclei of amygdala which project to the certain brainstem areas CS-US and/or context-US associations, since a damage of this structure interferes with the expression of these responses. In contrast, damage to areas to which CE projects selectively interrupts the expression of individual responses; for example, disruption of the lateral hypothalamus affects blood pressure but not freezing conditioned responses, and damage to the peraqueductal gray abolishes freezing but not blood pressure responses (LeDoux, 2000; Dityatev and Bolshakov, 2005).

Plasticity in the amygdala

There have been a number of experiments conducted showing that amygdala, like the hippocampus, can undergo synaptic plasticity (LTP and LTD) induced either artificially, through stimulation of main amygdaloid inputs or by fear conditioning. For example, Quirk et al., (1995, 1997) demonstrated that CS-responsive neurones in LA after fear conditioning (several pairings CS, a tone, with US) increase their firing rates and synchrony whenever CS is presented alone, and such plasticity can be erased by extinction trials, which suggests that long-term memory maybe in part encoded by a synchronous action of certain neuronal networks within LA rather than just in rate of firing. Amygdaloid (LA) AEPs elicited by CS and recorded in freely moving mice become potentiated after fear conditioning (Tang et al., 2001; 2003). Plasticity induced by conditioning also could occur in the auditory cortex (Quirk et al., 1997), however it happens later in time and more rarely than in thalamo-amygdaloid pathway (MGm, see Fig. 10-I), suggesting that cortico-amygdaloid plasticity is not essential. Conditioning-triggered plasticity has also been observed in the basal amygdala (Maren et al., 1991) and CE nuclei of amygdala (Pascoe and Kapp, 1985); however, their latencies of auditory-evoked potentials are longer, suggesting that they are secondary in the LA-B-CE transmission chain. Parallels between artificially-induced LTP (via electrical stimulation) and LTP-evoked by learning have been demonstrated in thalamo-amygdaloid and hippocampo-amygdaloid pathways *in vivo*, providing the strongest evidence of a relation between natural learning and LTP (Barnes 1995; Eichenbaum 1995; Stevens 1998; Maren and Fanselow, 1995).

Fig. 12-I Amnesic effects on consolidation of memory during different manipulations on NCAM in vivo. Injections have been made at given pre- and/or post-training times (3 h, 6 h, d2 etc) and memory retention was tested 24 hr or later. Anti-NCAM Abs – Polyclonal anti-NCAM antibodies which disrupt NCAM-NCAM interactions; NCAM ligand C3d – a synthetic peptide ligand of NCAM capable to interact with IgI domain of NCAM interfering with its homophilic NCAM-NCAM interaction; Anisomycin – a blocker of protein synthesis; Rp-cAMPs – a PKA inhibitor. DG – Dentate Gyrus of the hippocampus.



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Different stages of memory consolidation

Synaptic model of memory declares that the memory formation is based on the strengthening or weakening of transmission at specific synapses of a neuronal network. After formation, memory is thought to be transformed from an initial short-lasting and labile phase (short-term memory) into a long-lasting stable form (long-term memory). This transformation of memory is called consolidation. It has been proved in many experiments that initial phase of memory formation is dependent on the modification of pre-existing synaptic molecules (receptors, channels, enzymes), which rapidly affects the efficiency of synaptic transmission (Kandel, 2001), whereas long-term memory depends on *de novo* synthesis of proteins and structural modifications at synaptic level. For CAMs it is particularly true, since they function like interfacing molecules, and first react on necessity to reshape certain synapses in response to activity-dependent signals, like those involved in induction of LTP and LTD, or memory formation (for review, see Welzl and Stork, 2003).

Thus, it has been widely shown in many studies (see Fig. 12-I and Table 1-I for refs), that disruption of NCAM-NCAM homophilic interactions at 6-8 hours post-training time-window (via injections made *in vivo* either with anti-NCAM Abs, or a small peptide C3d which are capable to interact with the IgI of NCAM) leads to drastic behavioural responses like amnesia for learned task (tested 24 hours or later after a learning event).

Interestingly, this has been proven for several species (chicks, rats) and several tasks (including passive avoidance learning and fear conditioning); post-training injections made during consolidation before this time-window or after it had no effect on learning. All these experiments suggest that there is a particular time-window in late consolidation phase of memory restricted to 6-8 hours post-training time, which is most sensitive to homophilic NCAM-NCAM intervention. Interestingly that protein synthesis driven by contextual fear conditioning occurs earlier than the phase sensitive to disruption NCAM homophilic interaction. Also, it was demonstrated by Bourtchouladze et al. (1998) there are two time-intervals (see Fig. 12-I) sensitive to protein synthesis blockage, immediately after training and about 4 hours post-training. This probably can reflect a causal time-course of sub-cellular molecular events triggered by learning: first, a synthesis of new NCAM protein at early phase of memory consolidation, and shortly after its translocation and insertion into synaptic

cell membrane at the late consolidation phase, where its adhesive function can be disrupted by NCAM-NCAM homophilic blockers.

Strikingly, learning-induced polysialylation of NCAM occurs later than phase sensitive to disruption NCAM homophilic interaction (see Fig. 12-I): after a spatial task in Morris water maze or passive avoidance task, the level of PSA-positive granular neurons in dentate gyrus of the hippocampus significantly increased at 12-16 hours post-training time-interval (Murphy et al. 1999), suggesting that a wave of massive polysialylation of NCAM occurs at late phase of memory consolidation. However, putative molecular mechanisms that underlie the role of NCAM polysialylation at different stages of learning and memory are mostly unknown.

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AIMS OF THE STUDY

The major aims of this study were to address the following questions:

1. is PSA itself or PSA attached to NCAM (i.e. PSA-NCAM), required for learning in the fear conditioning paradigm;
2. at which stages of learning and memory consolidation do PSA and/or PSA-NCAM act; and
3. is the action of PSA due to modulation of NCAM-NCAM homophilic interactions or there are molecules different from NCAM involved?

To investigate the roles of PSA and NCAM, we injected the recombinantly produced extracellular domains of PSA-NCAM and NCAM fused to the Fc portion of human immunoglobulin (PSA-NCAM-Fc and NCAM-Fc, respectively) as well as free PSA into the hippocampi of wild-type and NCAM-deficient mice before and after induction of learning in a fear conditioning paradigm. As an internal control that injections into the hippocampus would not have unspecific effects on hippocampus-independent forms of learning, we used a fear conditioning paradigm in which an animal was simultaneously subjected to hippocampus-independent learning of tone and hippocampus-dependent learning of context.

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MATERIALS & METHODS

Mice

Constitutive NCAM deficient (NCAM-/-) mice were generously provided by Dr. Harold Cremer (Cremer et al., 1994) and were inbred for at least eight generations onto the C57BL/6J background. ST8SiaIV/PST-deficient (PST-/-) mice and their wild-type controls (PST+/+) were inbred for six generations onto the C57BL/6J background (Eckhardt et al., 2000). For behavioural experiments, 3- to 6-month-old males were used. For recording of LTP in vitro, 6- to 8-week-old mice of both sexes were used. C57BL/6J, NCAM-/- mice and their wild-type littermates (NCAM+/+) were bred in the animal facility of the Universitätsklinikum Eppendorf-Hamburg (Germany), whereas ST8SiaIV/PST-deficient mice and their wild-type controls were delivered from the Medizinische Hochschule Hannover (Germany). At least 1 week before the experiments, mice were transferred to a small vivarium where they were housed individually in type II Macrolon^{TM} cages (25x19x15 cm) with food and water *ad libitum* on a reverse 12h:12h light/dark cycle (lights on at 8:00 pm) under constant temperature (22 $\pm$ 1°C) and humidity (55 $\pm$ 5%). Behavioural experiments were conducted during the dark part of the cycle. All surgical and behavioural procedures were approved by the Committee on Animal Health and Care of the local governmental body.

Surgical procedures

Mice were anesthetized with a Ketamin-Rompun solution (Ketamin 100 mg/kg, Rompun 16 mg/kg, i.p, 0.01 ml/g b.w.) with a dose to provide a surgical level of anaesthesia lasting about 1.5 h without additional reinjections. The skull was shaved and cleaned with 75% ethanol. Mice were placed in a stereotaxic frame (Narishige, Tokyo, Japan) with their eyes being protected from drying by an eye-gel. The scalp was incised and retracted. The skull was levelled to have lambda and bregma positions in the same horizontal plane. Three small holes (two for cannulas and one for a screw, Fig. 13-ID) were drilled using a microsurgery handbore (Technobox, Bien Air, Bienne, Switzerland). The surface of the cranium bone was carefully cleaned with 75% ethanol and dried with a hot air gun HG 2000E (Steinel, Herzebrock-Clarholz, Germany) under a temperature not higher than 40°C. Sterile stainless steel 23 gauge

double guide cannulas (Plastic One, Roanoke, USA) were stereotaxically bilaterally implanted reaching the border of the dorsal hippocampus with the cortex (AP: - 2 mm and ML: ±1.5 mm from the bregma; DV, -1.5 mm from the skull surface, see Fig. 13-ID) according to Franklin and Paxinos (2001). For NCAM -/- mice, AP was -1.5 mm, because their brain is shorter than normal due to abnormal development of olfactory bulbs and ventricles (Cremer et al., 1994). Guide cannulas were fixed on the skull bone by dental kallocryl cement (CPGM gelb, Speiko, Münster, Germany) and a small jeweler's screw (see Fig. 13-ID). After implantation, the incision was closed and the wound was edged by Histoacryl glue (Braun-Aesculap, Tuttlingen, Germany). The body temperature of mice was maintained at 37°C with a regulated homeothermic blanket control unit (HSE-Harvard, March-Hugstetten, Germany) throughout the surgery. After the operation, mice were placed back to their home cages and kept in a warm (~37°C) room until awakening. Metamizol (50 mg/kg b.w., i.p.) was used as a post-operative analgetic. All following behavioural investigations were performed after mice had fully recovered during 5-7 days after surgery. Special care was taken to minimize both the suffering and the number of animals used in this study.

Conditions for behavioral tests

Before training, all animals were handled and habituated to the experimental conditions in an unpaired context (CC–) of a tone cage (see Fig. 13-IEb) for 5 min during 2 consecutive days prior to training. The unpaired context for a cued memory test was represented by a transparent Plexiglas cage (185x150x210 mm) cleaned with 1% acetic acid and equipped with two loudspeakers (M10, 8Ω, Visaton, Haan, Germany) in opposite sites of the box, a dark (3 Lux) experimental room (a white noise of 40 dB) lighted with a red lamp (0.2 Lux). Conditioning of mice was performed in a gray-brown aluminium conditioning chamber (ENV-307A; MED Associates, St. Albans, VT, USA) that was tooled with a shock floor consisting of 3.5 mm stainless steel rods spaced 7 mm apart and one loudspeaker on the back wall. The conditioned context (CC+) was represented by the above described conditioning chamber (see Fig. 13-IEa) that was cleaned with 75% ethanol before training of each animal, the day-lighted experimental room (630 Lux) and the isolation cubicle (40 Lux) and a noise (52 dB) produced by a fan. All experiments were performed in a double-walled sound-attenuated isolation cubicle. The conditioned stimulus CS+ was

a patterned tone, i.e. series of 20 brief tones (50 ms, 7.5 kHz, 75 dB) presented every second. The neutral stimulus (CS–), that was not paired to a foot shock, was a 7.5 kHz continuous pure tone (75 dB, 20 s). Mice show a good discrimination between patterned and continuous tones in a fear conditioning paradigm (Tang et al., 2003). A self-customized acoustic stimulator (Tang et al., 2001, 2003) produced both stimuli. The unconditioned stimulus (US) was a 1 s, 0.7 mA (AC) scrambled middle intensity shock delivered to the rod floor. Delivery of tones and shocks was controlled by customized software (Signal 1.88, CED, Cambridge, UK) using a PC computer (Pentium-II, 340 MHz, 128 MB RAM) through an AD/DA converter (Micro 1401 board, CED, Cambridge, UK).

Fear conditioning paradigm

The procedure of fear conditioning was the following (Fig. 13-IB): (i) a naive mouse was placed into the conditioning chamber for 100 s to record its baseline behaviour before a footshock is delivered, abbreviated as a trial (B) (stands for baseline); (ii) then, the animal received 2 CS–US pairings spaced apart by 60 s. For CS-US pairing, a 20-s patterned tone was co-terminated with a 1-s foot shock; the 60 s time period between the shocks was taken to evaluate freezing as measure of the shock sensitivity of all mice. This period is labelled as trial (S) (stands for sensitivity to a shock); (iii) 30 s after the last CS–US pairing the mouse was returned to its home cage.

Freezing behavior

Behaviour of animals was videotaped for each session and analyzed off-line by a trained observer blind to the identity of mice and to the applied tones (conditioned or neutral) for each of the 60-s uniform tone episodes (cued freezing). Freezing time, which served as a measure of fear-related memory, was quantified as described (Tang et al., 2001). Briefly, freezing was assigned if the animals remained motionless, except for respiratory movements, in a tense posture that was characterized by horizontal positioning of the head, a stretched state of the body and stiffening of the tail.

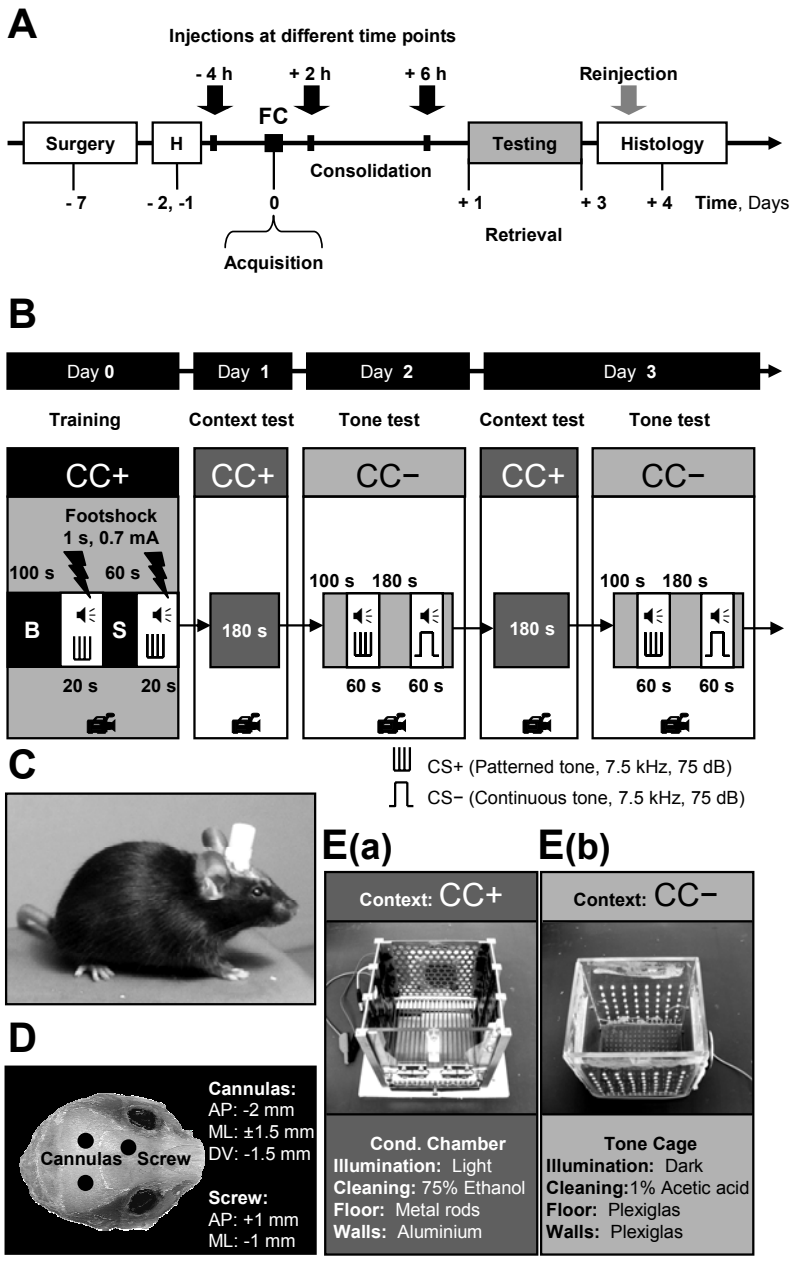


Figure 13-I. Description of the fear conditioning paradigm. (A) Scheme with the time course of all manipulations performed with mice. After surgery mice were allowed to recover for 5 days, next 2 days they were handled and habituated for 5 min to a non-conditioned context, CC–. Fear conditioning (FC) started on the day "0" followed by series of tests, lasting 3 consecutive days (+1 to +3). Reinjection of recombinant proteins to mice was done on the day + 4, followed by immunohistological verification of their distribution and analysis of cannula placement. (B) Fear conditioning paradigm and following retention tests. On day 0, mice underwent training in the conditional context CC+, receiving 2 x CS-US pairings. US was 1-s long middle strength (0.7 mA) foot shock delivered to the conditioning chamber rods floor. Next day, mice were tested for 180 s in the same context for contextual memory retrieval. On day 2, mice were tested for cued memory by presentation of CS+ and CS– in a non-conditioned context CC–. On day 3, both contextual and cued memory tests were performed with a 3-hour interval. (C) An image of a cannulated mouse. (D) Stereotaxic coordinates used for implantations. (E) Images and detailed description of conditioned CC+ (a) and non-conditioned CC– (b) contexts.

Injections

To evaluate the effects of the infused compounds at the chosen two phases of learning (acquisition of task and consolidation of memory), mice were divided into three groups: with infusion made 4 h before fear conditioning and 2 or 6 h after training. Compounds were injected into the hippocampus during very short CO_2 anaesthesia (~2 min), with the same injection volume (0.4 µl per side) and infusion rate (0.4 µl/min) for all animals. The injection bilateral cannula (#C235, Plastic One, Bilaney, Germany) was connected to a pump (TSE Systems, Bad Homburg, Germany) through a Tygon tubing system, filled by 2/3 of the tube length with a sterile sesame oil (Sigma-Aldrich, Deisenhofen, Germany) as a pressure conductor, and by 1/3 with the injected compound. The injection cannula was gently inserted into the bilateral guide cannula ~ 10 s before injection, extending the tip of the guide cannula by 1 mm. The site of the injection was aimed to the hilus of both hippocampi. The injection cannula was left within the guiding cannula for at least 30 s before removing. Then, the guide cannula was closed by a dummy cannula and with a screw cap. All animals recovered from this procedure within 2-3 min after transferral to their home cage.

Injected compounds

Recombinantly produced proteins NCAM-Fc and PSA-NCAM-Fc, containing the extracellular portion of NCAM fused with a human Fc-fragment of IgGs, were used for injections. Murine PSA-NCAM-Fc was produced according to (Vutskits et al., 2001) using a stably transfected TE671 cell line kindly provided by Genevieve Rougon. Mouse NCAM-Fc was produced using stably transfected CHO cells as described (Chen et al., 1999) and had been tested for stimulation of neurite outgrowth in vitro. Polysialylation of PSA-NCAM-Fc and absence of PSA on NCAM-Fc were checked by Western blotting (Vaithianathan et al., 2004) using a monoclonal antibody to PSA (clone 735; Frosch et al., 1985). As a control for NCAM-Fc and PSA-NCAM-Fc, we used the human Fc-fragment (Dianova, Hamburg, Germany). Concentration of all injected proteins was 0.5 mg/ml, corresponding approximately to 15µM for Fc and 2 µM for NCAM-Fc and PSA-NCAM-Fc, which is similar to the effective concentration of NCAM antibodies and NCAM ligand peptide C3d in disruption of memory formation in different behavioural paradigms (Roullet et al., 1997; Cambon et al., 2003; Foley et al., 2000). PSA was administered as the bacterially produced PSA, known as

colominic acid (#27698; Sigma-Aldrich). Another negatively charged carbohydrate, chondroitin sulfate C (CHS) (C-4384; Sigma-Aldrich), served as a control in the

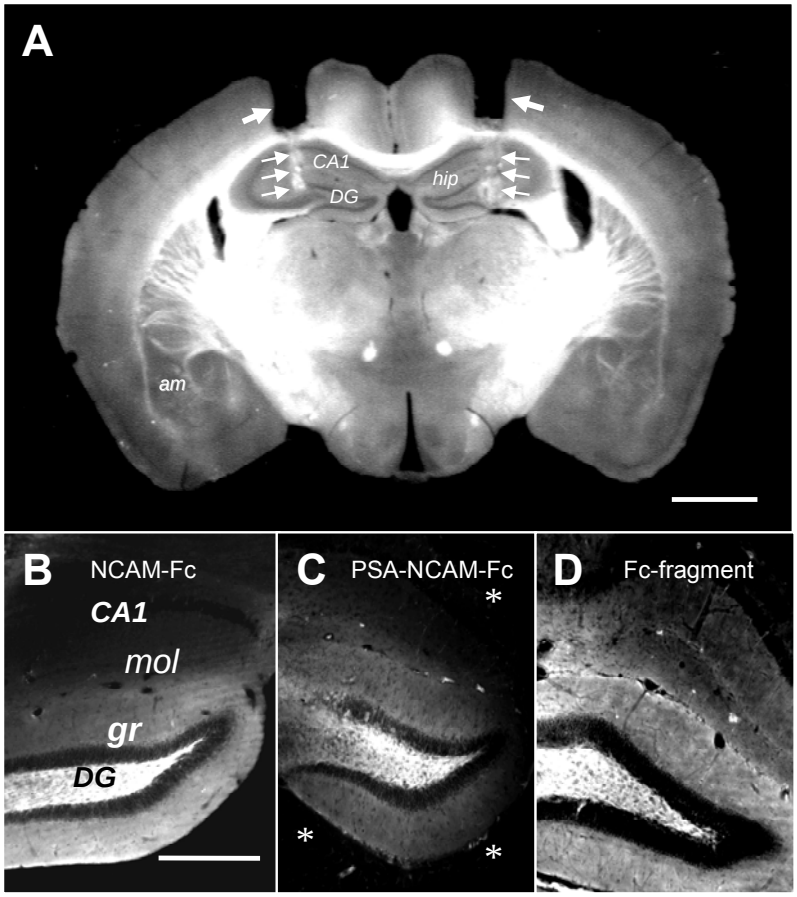


Figure 14-I. Histological verification of cannula position and distribution of injected recombinant proteins. (A) An image of a mouse brain coronal section showing traces from guide cannulas (big arrows) aimed to the border of cortex and dorsal hippocampus (hip) and injection cannulas (small arrows) targeted to the hilus of the dentate gyrus (DG). (B) An example of NCAM-Fc, PSA-NCAM-Fc (C) and control Fc fragment. (D) Fc immunoreactivity within the mouse brain 4 h after injection into the dorsal hippocampus. Note that the maximal signal is within the hilus and molecular layer, an intermediate intensity signal is in the CA1 and CA3 regions of hippocampus, and no signal (see asterisks) is outside of the hippocampus; *am* – amygdala; *mol* (molecular) and *gr* (granular) layers of hippocampus. Scale bars, (A): 2 mm, (B): 300 μm.

behavioural experiments. Both carbohydrates were applied at a concentration of 1 mg/ml, corresponding approximately to 50 μM. All compounds were freshly prepared in a sterile artificial cerebrospinal fluid, ACSF (for composition, see recordings of LTP in acute hippocampal slices). Implantations, injections and behavioural tests were performed in groups of 15-20 mice. Control mice receiving compounds such as CHS

or Fc were always tested in parallel to mice receiving PSA and Fc-fused proteins, respectively.

Histology

To verify the injection sites and distribution of the injected Fc-fused recombinant proteins on a per animal basis, mouse brains were investigated histologically on the 4th day (after behavioural tests). For evaluation of the distribution patterns of Fc-injected compounds in the living brain (Fig. 14-I), as well as for verification of exact place of injection, mice were reinjected with a given compound using the same injection procedure as on day "0". Reinjection has to be used since on the 4th day after the first injection initially injected compound was no longer detectable. Four hours (that is the time interval between the first injection and fear conditioning) after reinjection mice were deeply anesthetized with pentobarbital (100 mg/kg) and transcardially perfused with 4% paraformaldehyde in PBS (pH 7.4) during 20 min, and brains were postfixed overnight in the same fixative at 4°C. Brains were then washed in PBS and embedded in 5% agar. Several mice were perfused 24 or 48 h after reinjection to assess the availability and distribution of injected compounds at these time points, corresponding to the 1st and 2nd test days after the first injection. Coronal sections (50 μm thick, taken every 100 μm) were cut using a vibratome VT 1000S (Leica, Bensheim, Germany). Freely floating slices were treated for 1 hour with blocking solution containing 1% BSA in PBS, followed by detection of the Fc portion by anti-human IgG Cy3-coupled antibody (Dianova, Hamburg, Germany) diluted 1:200 in 0.1% BSA/PBS solution and incubated for 60 min at room temperature. Sections were then washed three times for 10 min each in PBS and finally embedded in Aqua-Poly/Mount (Polyscience, Eppelheim, Germany) for further visualization using Axiophot 2 (Zeiss, Jena, Germany). Injections of NCAM-Fc, PSA-NCAM-Fc or Fc-fragment alone (Fig. 14-I, B, C, and D, respectively) resulted in prominent Fc immunoreactivity signal in the hilus and the molecular layer of the dentate gyrus when tested 4 h after injection. Diffusion of the injected proteins at this time point was quite extensive and the immunohistochemical signal was detectable across most of the dorsal hippocampus, covering also the CA1 and CA3 regions, although less prominently than the dentate gyrus. The immunohistochemical signal was limited to the hippocampus and was detectable at only very low levels in adjacent cortical and thalamic areas (asterisks in Fig. 14-I, C). In ACSF injected mice

no signal was detectable anywhere (data not shown), confirming the specificity of the immunofluorescence reaction. The Fc-fragment was detectable in a larger area than NCAM-Fc and PSA-NCAM-Fc.

However, also the Fc-fragment was never observed outside of the hippocampus. Immunoreactivity for the Fc-fragment was detectable in the dorsal hippocampus at 24 but not at 48 h after injection (data not shown).

To evaluate the positions of injection cannulas in the hippocampi injected with ACSF, PSA and CHS, mice were reinjected on the 4th test day with methylene blue (0.4 µl per side with an infusion rate of 0.4 µl/min) and were killed by an overdose of CO_2 4 h later. Brains were rapidly removed and frozen in the cryoprotecive embedding medium Tissue-Tek (Sakura Finetek Europe, Netherlands) in liquid nitrogen. Frozen 20-µm frontal sections were cut with a cryostat CM3050 (Leica, Bensheim, Germany), dried and assessed at the light microscopic level to verify the positions of injection cannulas. Only animals that had received bilateral injections into dorsal hippocampi were included in the analysis. Among the total of 212 animals, which were taken for the behavioural experiments, 199 were included in the analysis presented in this study, while 13 mice had to be excluded because of misplacement of cannulas or damage of guide cannulas after implantations during animal housing.

Statistics

All data are expressed as mean ±SEM. To analyze the effects of the injected compounds on contextual memory we used two-way ANOVA having *Treatment* as a between groups factor and *Trial* as within groups factor, followed by the *post hoc* Fisher's Least Standard Mean (FLSM) test if significant effects of factors were detected. To analyze the effects of the injected compounds on cued memory we used three-way ANOVA for *Treatment* and repeated measurements *Trial* and *Tone*. Additionally, statistical comparison between freezing responses to CS+ and CS– on a given day within each group was performed by using the nonparametric Wilcoxon signed-rank test. Significant differences between groups were detected by the Wilcoxon test and the paired t-test for the same groups, and therefore we refer in the text only to p-values provided by the Wilcoxon test. Statistical significance of differences was accepted if $p < 0.05$.

***

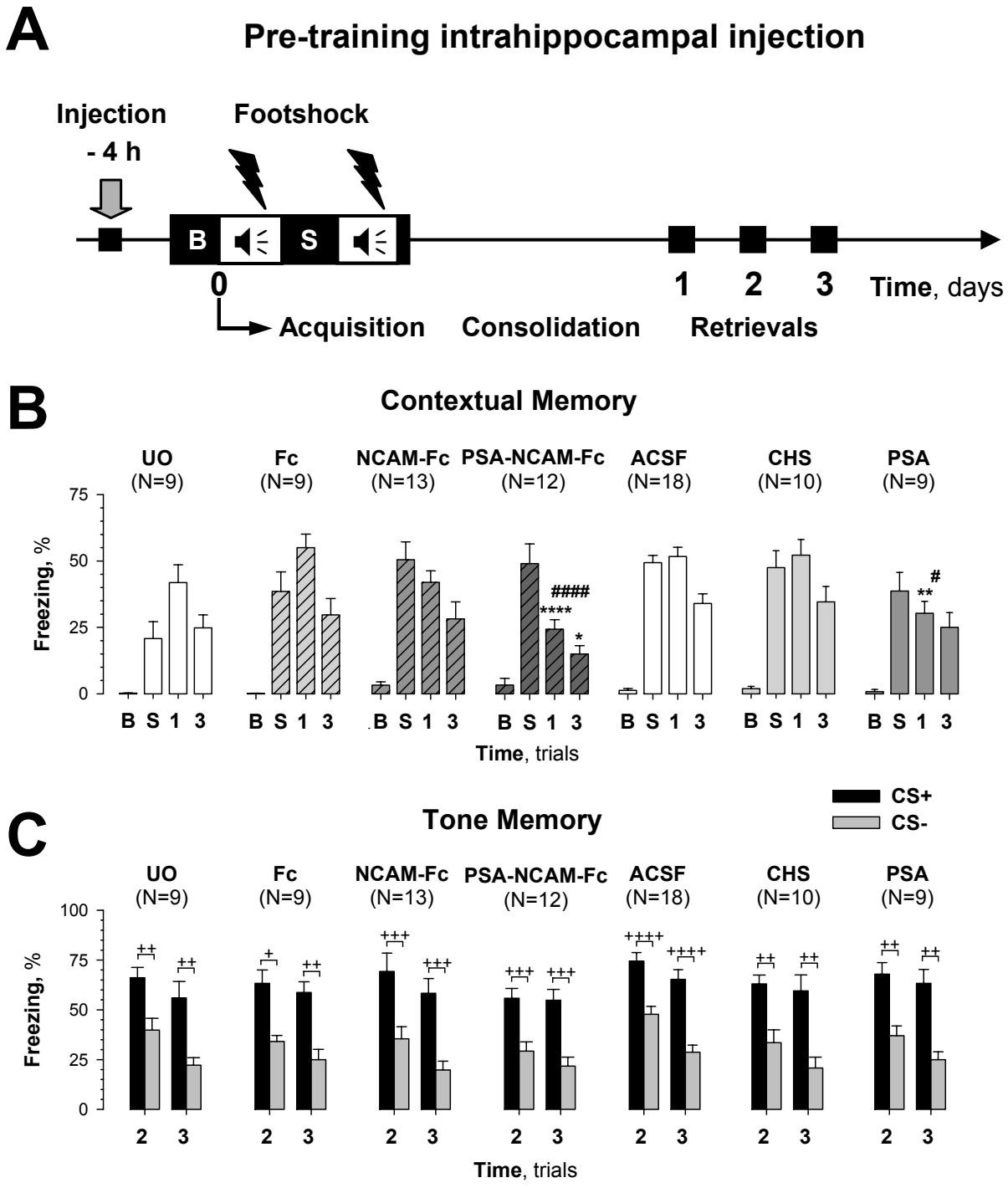
RESULTS

PSA and NCAM in acquisition of fear conditioning

To test whether PSA-NCAM, non-polysialylated NCAM and PSA would affect learning in a fear conditioning paradigm (Fig. 13-I), as a first step, we addressed the question whether injection of control compounds (vehicle ACSF and Fc-fragment) before training (- 4 h, Fig. 15-I, A) would have an effect on acquisition of contextual memory and its subsequent retention as compared to unoperated C57BL/6J mice. As shown in Fig. 15-I, B, the level of freezing in the conditioned context CC+ was ~ 50% of the recorded times on the 1st day and ~ 30% on the 3rd test day in control groups of mice that were unoperated or injected with ACSF or Fc. There was no statistically significant difference in freezing between unoperated and ACSF or Fc injected mice (see Table 2-I, lines 1, 2 for statistics).

Also the behaviour of ACSF or Fc injected mice was normal, i.e. not different from unoperated mice, in the CC+ context before they received a shock (columns “B” in Fig. 15-I): mice showed no signs of anxiety or hyperactivity and the episodes of freezing were not longer than 1-2 seconds and few in number, covering just 2-3% of the recorded times, which is normal for mice in novel contexts (Tang et al., 2003). These results verify our injection procedure and the Fc-fragment as controls.

Figure 15-I. Effects of pre-training (– 4 h) intrahippocampal injection of compounds on fear conditioning. (A) A scheme showing all manipulations performed in this series of experiments. A 100-s trial “B” was used to evaluate a baseline response of mice to the context CC+ prior training. A 60-s trial “S” was used to assess sensitivity of mice to the foot shock. (B) Pre-training injection of PSA-NCAM-Fc or PSA, but not NCAM-Fc, impaired contextual memory when tested on the 1st and 3rd days after fear conditioning (trials “1” and “3”, respectively). Mice injected with the Fc-fragment and chondroitin sulfate (CHS) served as controls. Fc and CHS injected mice did not differ in contextual memory from unoperated and ACSF injected mice, respectively. Fear memory was measured as percentage of time that mice spent in a freezing state. Analysis of freezing during trials “S” and “B” showed no difference between injected groups. (C) Pre-training intrahippocampal injection of all tested compounds did not affect freezing time in response to paired (CS+) and unpaired (CS–) during trials “2” and “3”. Discrimination between CS+ and CS– tones was also not different between injected groups. *p<0.05, **p<0.01, ***p<0.005 (*post hoc* FLSD test as compared to corresponding controls, Fc or CHS); #p<0.05, ##p<0.01 (the effect of treatment by two-way ANOVA); +p<0.05, ++p<0.01, +++p<0.005, ++++p<0.001 (Wilcoxon test).



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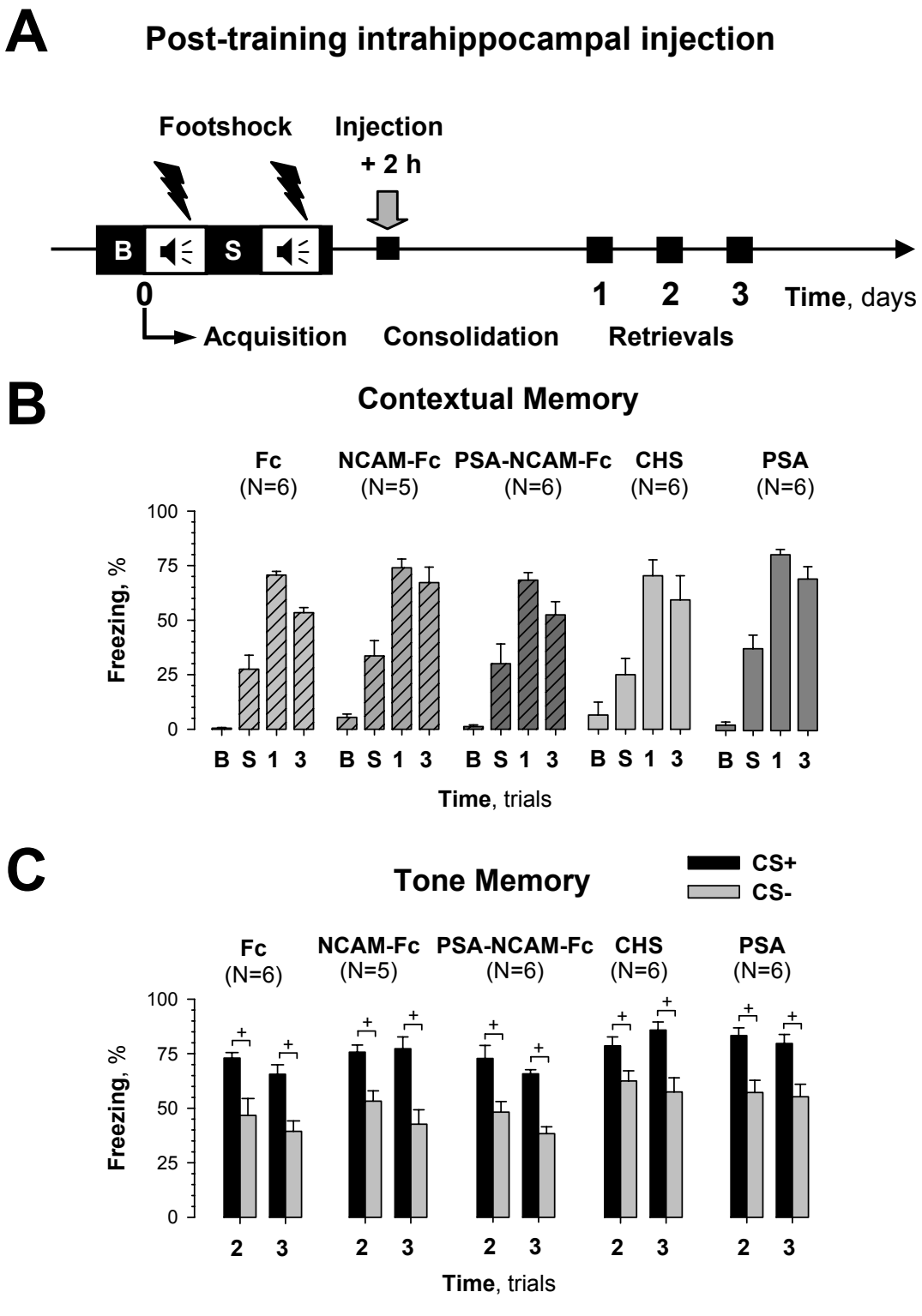
Injection of C57BL/6J mice with PSA-NCAM-Fc resulted in a strong impairment in contextual learning on both the 1st and 3rd test days after training as compared to the control Fc injected group (Fig. 15-I, B and Table 2-I, line 3). In contrast to PSA-NCAM-Fc, injection of NCAM-Fc affected contextual memory retrieval neither on the 1st nor 3rd test days (Table 2-I, line 4). Acquisition of the contextual task was also disrupted in C57BL/6J mice injected with PSA, as compared to ACSF (Fig. 15-I, B and Table 2-I, line 5). Injection of another negatively charged sugar, chondroitin sulfate (CHS), did not influence processing of contextual information or baseline activity (Table 2-I, line 6), showing that the effect of PSA was not only due to its negative charge (Table 2-I, line 7).

Figure 16-I. Effects of post-training (+ 2 h) intrahippocampal injection of compounds on fear conditioning. (**A**) A scheme showing all manipulations performed in this series of experiments. A 100-s trial "B" was used to evaluate a baseline response of mice to the context CC+ prior training. A 60-s trial "S" was used to assess sensitivity of mice to the foot shock. (**B**) None of injected mice showed any impairment in contextual memory when tested on the 1st and 3rd days after fear conditioning (trials "1" and "3", respectively) and during trials "S" and "B". (**C**) Post-training intrahippocampal injection of tested compounds did not affect cued memory during trials "2" and "3". Discrimination of tones, paired (CS+) versus unpaired (CS–), also did not differ between injected groups. +$p<0.05$ (Wilcoxon test).



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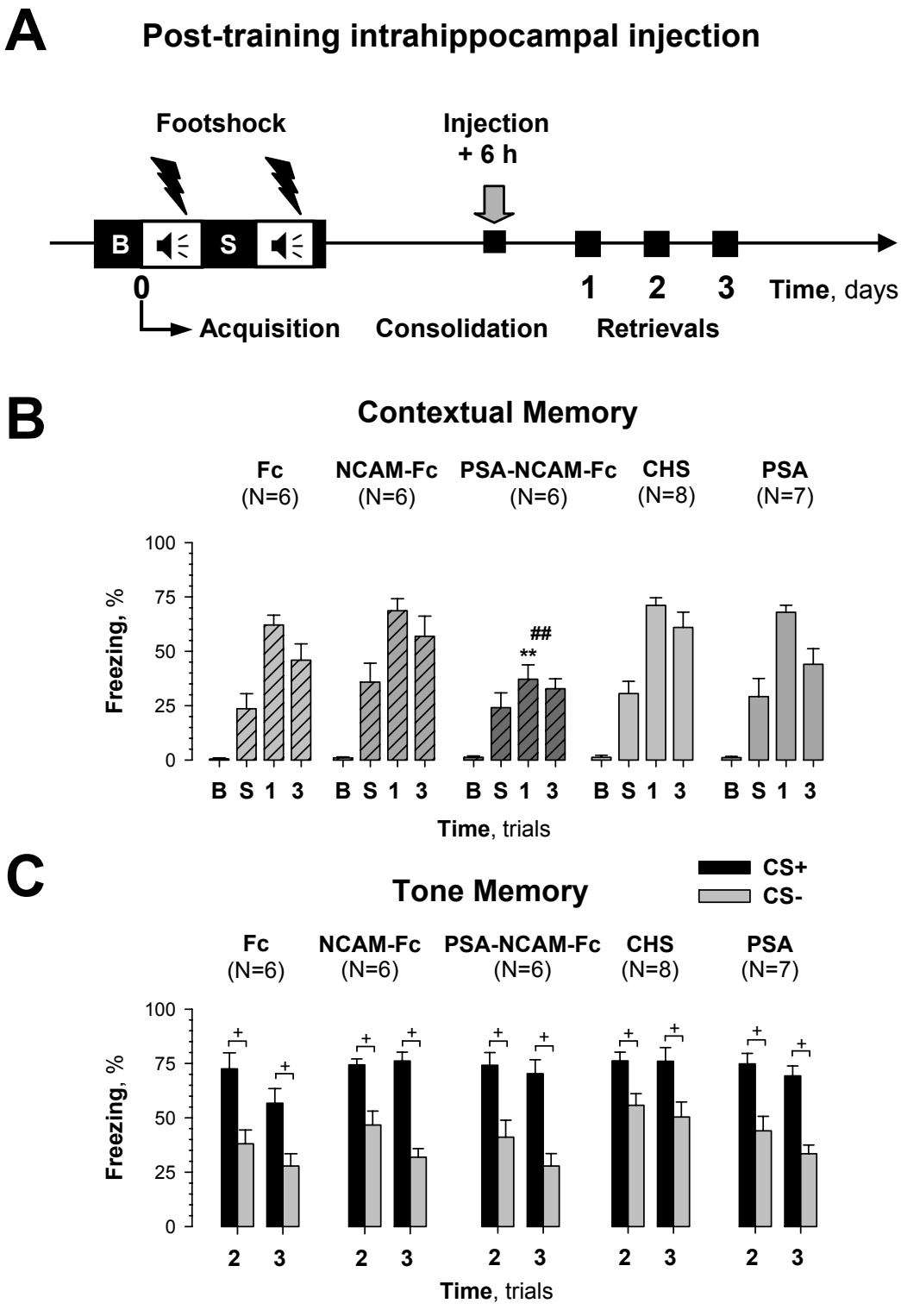
Since contextual learning depends on both hippocampus and amygdala, our next question was whether the compounds injected into the dorsal hippocampi acted exclusively there, or also affected the amygdala, or had unspecific effects on anxiety of injected mice. To address this issue we took advantage of the fact that mice were simultaneously exposed to both contextual and auditory stimuli during conditioning (Fig. 13-I), and analyzed hippocampus-independent amygdala-dependent cued (tone) memory of conditioned mice. We found that intrahippocampal injection of either PSA-NCAM-Fc, NCAM-Fc, or PSA before acquisition of fear conditioning (- 4 h, see Fig. 15-I, C) did not affect ability of mice to learn a cued task: three-way ANOVA did not reveal any differences between mice that received these injections and controls (Table 2-I, lines 1-7). Injection of PSA-NCAM-Fc, NCAM-Fc, or PSA also did not affect ability of mice to differentiate the conditioned tone CS+ from unconditioned CS– on the 2nd and 3rd test days after training: three-way ANOVA did not reveal any significant interaction between treatment and tone (Table 2-I, lines 1-7) and all groups showed significant discrimination of tones (at least $p<0.05$, Wilcoxon test). Thus, hippocampus-targeted injections did not interfere with amygdala-dependent form of learning in our experiments and mice injected with PSA-NCAM-Fc and PSA had normal cued memory, although showed robust impairment in contextual memory.

Figure 17-I. Effects of post-training (+ 6 h) intrahippocampal injections of tested compounds on fear conditioning. (A) A scheme showing all manipulations performed in this series of experiments. A 100-s trial "B" was used to evaluate a baseline response of mice to the context CC+ prior training. A 60-s trial "S" was used to assess sensitivity of mice to the foot shock. (B) Among injected compounds, only PSA-NCAM-Fc impaired contextual memory tested in CC+ on the 1st and 3rd days after fear conditioning (trials "1" and "3", respectively). There was no difference between injected groups during trials "B" and "S". (C) Post-training (+ 6 h) intrahippocampal injection of tested compounds did not affect cued memory and discrimination of tones, paired (CS+) versus unpaired (CS–). **p*<0.05 (*post hoc* FLSD test); #*p*<0.05 (ANOVA); +*p*<0.05 (Wilcoxon test).



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PSA and NCAM in consolidation of fear memories

Since pre-training injections of PSA-NCAM-Fc and PSA could affect either acquisition or consolidation of fear memories, we decided to differentiate between the two phases of learning by injection of compounds during the consolidation phase of learning. Injections were performed 6 h after training, a time-window considered to be sensitive to interference with NCAM functions (see e.g. Cambon et al., 2003) and 2 h after training, a time point previously used to study the early phase of consolidation (Doyle et al., 1992; Scholey et al., 1995).

As shown on Fig. 17-I, B, PSA-NCAM-Fc significantly impaired contextual memory compared to Fc when administered 6 h after training, but had no effect when injected 2 h after training (Fig. 16-I, B). Post-training NCAM-Fc injections, similar to the pre-training ones, had no effect on contextual memory when performed either 6 h or 2 h after training (Table 2-I, lines 8, 9 and 11, 12). Although pre-training injection of PSA impaired contextual memory, it was as ineffective as CHS when injected either 2

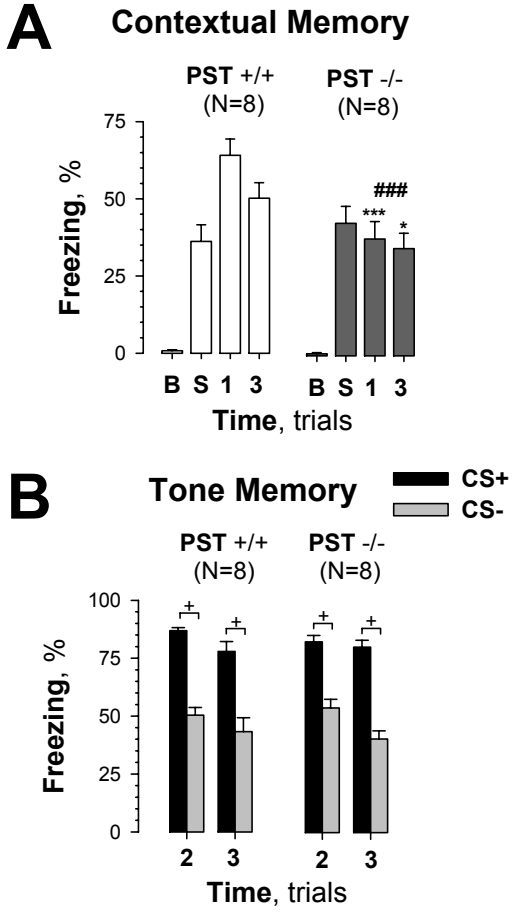


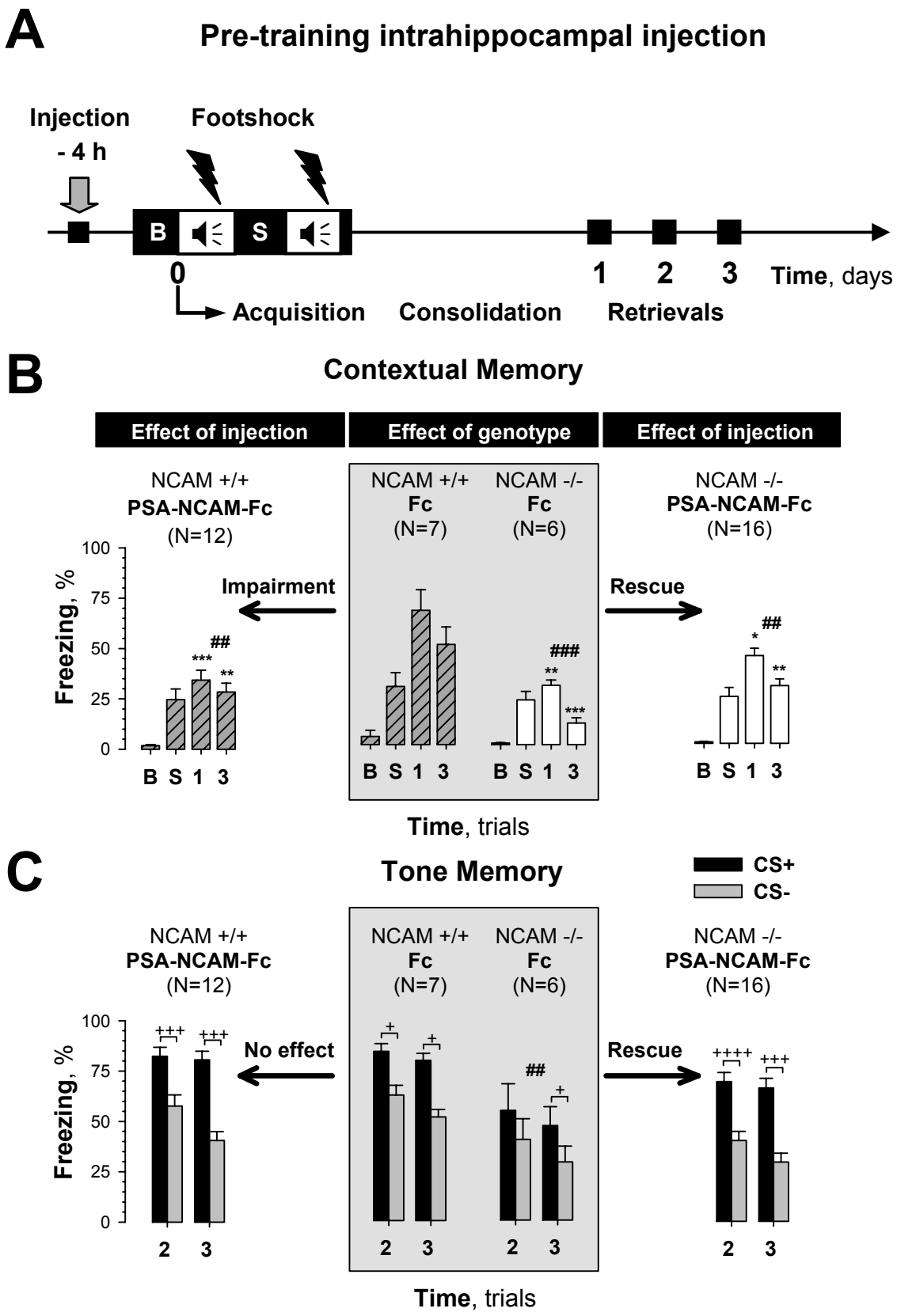
Figure 18-I. Fear conditioning of PST deficient mice (PST-/-) and corresponding wild-type controls (PST+/+). (A) PST-/- mice showed significantly less freezing in conditioned context CC+ compared to PST+/+ mice. Analysis of responses to the first foot shock (trial "S" that served as a measure of pain responsiveness of mice) as well as the baseline values of freezing to not-yet-conditioned context CC+ (trial "B"), showed no difference between PST-/- and PST+/+ mice. (B) Cued memory was normal on the 2nd and 3rd days after fear conditioning (trials "2" and "3") in PST-/- mice. Discrimination of tones, paired (CS+) versus unpaired (CS–), also did not differ between genotypes. *p<0.05, ***p<0.005 (*post hoc* FLSD test); ##p<0.01 (ANOVA); +p<0.05 (Wilcoxon test).

or 6 h after fear conditioning. Similar to the pre-training injection experiments, PSA-NCAM-Fc, NCAM-Fc and PSA altered cued memory neither 2 h (Fig. 16-I, C) nor 6 h after training (Fig. 17-I, C). Three-way ANOVA (Table 2-I, lines 8, 9 and 11, 12) did not reveal any differences between PSA-NCAM-Fc and Fc, NCAM-Fc and Fc, and PSA versus CHS injected groups. Discrimination of CS+ and CS– tones in all mice which were injected after training was also normal (at least $p<0.05$, Wilcoxon test, see Figs. 16-I, C and 17-I, C). In summary, only post-training injection of PSA-NCAM-Fc at 6 h but not 2 h reduced contextual memory, whereas PSA and NCAM-Fc were ineffective.

Impaired fear conditioning in PST deficient mice

To obtain genetic evidence for the role of PSA in fear conditioning, we tested ST8SiaIV/PST deficient (PST-/-) mice that lack a polysialyltransferase important for polysialylation of NCAM in adult mice. PST-/- mice showed impaired contextual memory on all test days (Fig. 18-I, A), as compared to their wild-type PST+/+ controls (Table 2-I, line 14). However, cued memory in PST-/- mice was normal as well as discrimination between CS+ versus CS– (at least $p<0.05$, Wilcoxon test; Fig. 18-I, B).

Figure 19-I. Effects of pre-training intrahippocampal injection of compounds on fear conditioning in NCAM deficient (NCAM-/-) mice. (A) A scheme showing all manipulations performed in this series of experiments. A 100-s trial "B" was used to evaluate a baseline response of mice to the context CC+ prior training. A 60-s trial "S" was used to assess sensitivity of mice to the foot shock. (B) NCAM-/- mice showed robust impairment of contextual memory tested on the 1st and 3rd days after fear conditioning (trials "1" and "3" respectively) compared to their wild-type littermates, NCAM+/+ (compared mice were injected with Fc-fragment). The deficit in contextual memory in NCAM-/- mice could be partially rescued by pre-training injections of PSA-NCAM-Fc, as compared to Fc-injected NCAM-/- mice. Injections of PSA-NCAM-Fc into NCAM+/+ mice could significantly impair contextual memory. Fc injected NCAM-/- and PSA-NCAM-Fc injected NCAM+/+ mice showed similar freezing responses during the baseline period (trial "B") and after the first foot shock (trial "S"). (C) NCAM-/- mice showed impairment in cued memory, including tone discrimination failure: Fc injected MCAM-/- mice froze less to CS+ and to CS– tones as compared with Fc injected NCAM+/+ mice. NCAM-/- mice were not able to discriminate CS+ from CS- tone on the 2nd day (trial "2") after FC, although they did so later, on the 3rd day (trial "3"). This deficit in NCAM-/- mice could be rescued by the pre-training intrahippocampal injection of PSA-NCAM-Fc. Other injected groups showed no difference in cued memory processing as well as in discrimination of CS+ and CS-. **p*<0.05, ***p*<0.01, ****p*<0.005 (*post hoc* FLSD test); #*p*<0.05, ##*p*<0.01, ###*p*<0.005 (ANOVA); +*p*<0.05, +++*p*<0.005, ++++*p*<0.001 (Wilcoxon test).



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Impaired fear conditioning in NCAM deficient mice and its rescue by injection of PSA-NCAM-Fc

A previous study reported that NCAM-/- mice are impaired in both contextual and cued fear memories (Stork et al., 2000). In agreement we found a difference in the freezing response between conditioned NCAM-/- and NCAM+/+ mice injected with the control Fc-fragment 4 h before training. We used Fc-injected rather than unoperated animals as has been done before (Stork et al., 2000) because i) Fc was found not to affect fear memory and ii) we could use Fc-injected NCAM-/- mice as controls for injection of other compounds. A clear deficit in contextual memory in NCAM-/- mice was seen on the 1st and on the 3rd post-training days (Fig. 19-I, B, middle panel; Table 2-I, line 15). NCAM-/- mice were also deficient in cued memory (Fig. 19-I, C, middle panel; Table 2-I, line 15) and did not discriminate well between CS+ and CS– on the 2nd test day (Wilcoxon test, $p=0.06$), although showed significant discrimination on the 3rd test day.

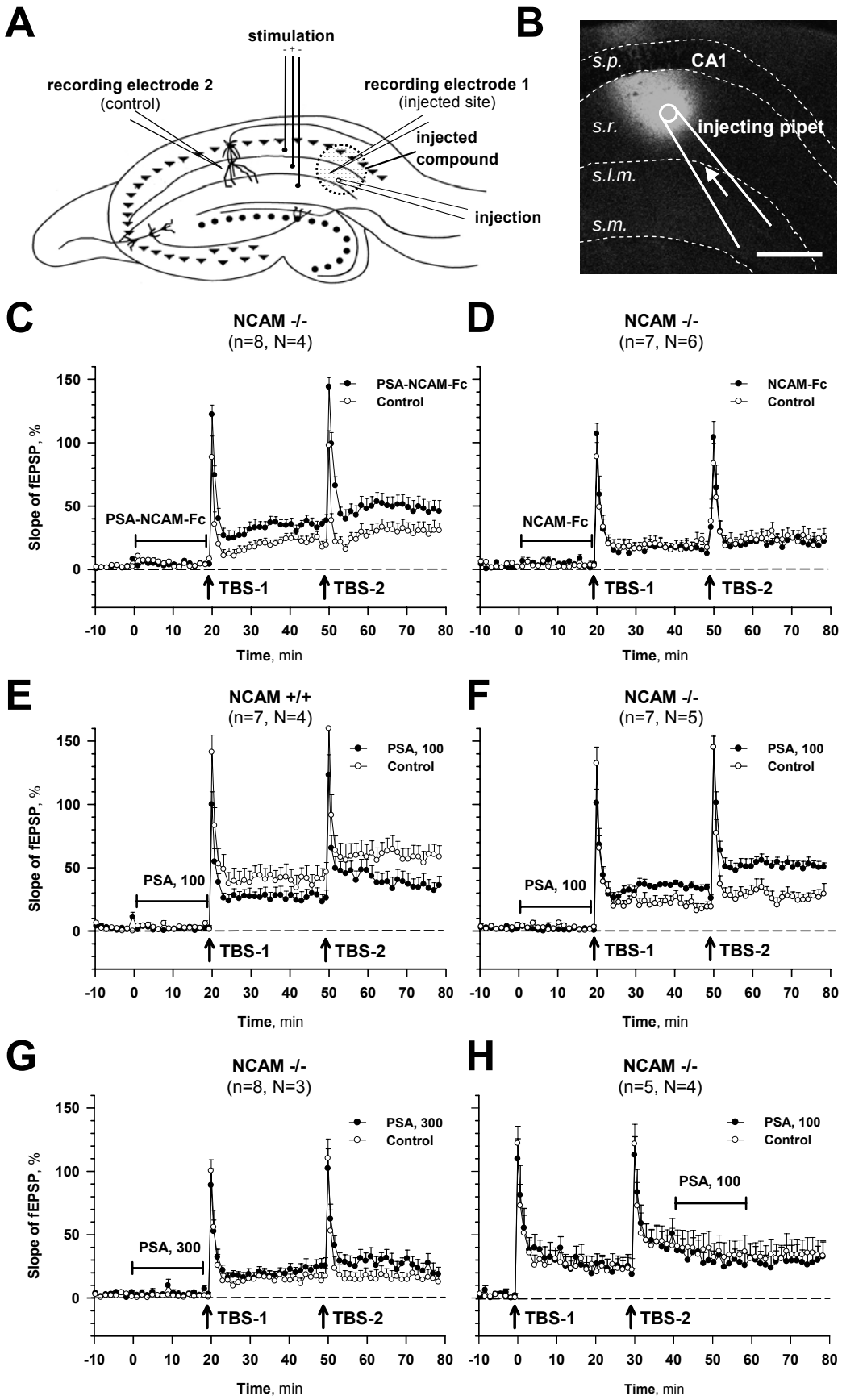
Next, we asked whether PSA-NCAM-Fc could rescue the deficit in contextual memory in NCAM-/- mice. Indeed, pre-training injection of PSA-NCAM-Fc into NCAM-/- mice (Fig. 19-I, B, right panel) resulted in a significant improvement of contextual learning as compared to Fc injected NCAM-/- mice (Table 2-I, line 16). This rescue, however, was not complete since there remained a significant difference between PSA-NCAM-Fc injected NCAM-/- mice and Fc injected NCAM+/+ mice (Table 2-I, line 17). Intrahippocampal injection of PSA-NCAM-Fc into NCAM-/- mice also tended to improve cued learning as compared to Fc injected NCAM-/- mice. Furthermore, injection of PSA-NCAM-Fc into NCAM-/- mice completely recovered discrimination of CS+ versus CS– tones, as compared to Fc injected NCAM-/- mice ($p<0.001$ for the 2nd day; $p=0.001$ for the 3rd day, Wilcoxon test).

To investigate whether the effect of injected PSA-NCAM-Fc depends on the presence of endogenous NCAM, PSA-NCAM-Fc was injected into NCAM+/+ mice (rather than C57BL/6J as before) in parallel with injections into NCAM-/- mice (Fig. 19-I, B, left panel). In agreement with our results for C57BL/6J mice, injection of PSA-NCAM-Fc impaired contextual memory in NCAM+/+ mice, as compared to Fc injected NCAM+/+ mice. Injection of PSA-NCAM-Fc into NCAM+/+ mice, however, did not affect cued memory (Fig. 19-I, C, left panel, Table 2-I, line 18) or discrimination between CS+ and CS– (at least $p<0.05$, Wilcoxon test).

Figure 20-I. *In vitro* LTP recording in the CA1 hippocampal region of NCAM deficient (NCAM-/-) and wild-type (NCAM+/+) mice. (A) A scheme showing placement of two recording electrodes, one triple stimulation electrode and an injection pipette over the hippocampal slice. Note that all recordings were made simultaneously from two distinct sites in the CA1 *stratum radiatum,* adjacent to and distant from the injection place, in response to stimulation of one of two independent Schaffer collateral/commissural pathways. (B) An image of hippocampal slice fixed and stained against the Fc-fragment immediately after the end of 20-min injection of NCAM-Fc in the CA1 *stratum radiatum* (*s.r.*). *S.p., stratum pyramidale; s.l.m., stratum lacunosum-moleculare; s.m., stratum moleculare.* Scale bar, 200 μm. (C) Local 20-min injection of PSA-NCAM-Fc (100 μg/ml) into slice from NCAM-/- mice prior first theta-bust stimulation (TBS1) could significantly increase LTP at the recording site adjacent to injection place as compared to LTP recorded at the control site in the same slice. Also LTP induced by second TBS2 after PSA-NCAM-Fc injection was higher at the injected than at the control site. (D) Injection of NCAM-Fc (100 μg/ml) failed to rescue LTP induced either TBS1 or TBS2 in NCAM-/- mice. (F) Injection of PSA (100 μg/ml) had the same restoring effect as injection of PSA-NCAM-Fc. (E) Injection of PSA could impair LTP in NCAM+/+ mice. (G) PSA injected at 300 μg/ml could not rescue LTP in NCAM-/- mice. (H) Injection of PSA at 100 μg/ml starting 10 minutes after second TBS did not affect LTP in NCAM+/+ mice. All data are represented as a mean slope ± SEM of field excitatory postsynaptic potentials (fEPSPs) normalized to the baseline values.

Recordings of long-term potentiation (Methods).

Slices was transferred to a submerged recording chamber and perfused with carbogen-bubbled ACSF at the rate of 2 ml/min. Recordings of field excitatory postsynaptic potentials (fEPSPs) were simultaneously performed using glass pipettes filled with ACSF and having a resistance of 2 MOhm at two sites in the *stratum radiatum* of the CA1 region of the hippocampus: near the injection pipette and in a control non-injected site, being approximately 400 μm apart from the injection pipette (Strekalova et al., 2002). The recombinant proteins and PSA were diluted in ACSF and delivered using a nanoliter injector (World Precision Instruments, Berlin, Germany) at a rate of 2.3 nl every 3 s for 20 min at concentrations of 100 μg/ml. Two independent pathways were stimulated by a tripolar electrode made from three Teflon-insulated platinum wires (World Precision Instruments). Theta-burst stimulation (TBS) was applied to one of these pathways, whereas the second pathway was used to control for the stability of recordings at both recording sites. TBS consisted of 4 trains of 10 bursts of 4 stimuli; 20 s, 200 ms and 10 ms intervals between trains, burst and stimuli, correspondingly (Eckhardt et al., 2000). The paired-pulse test was used to control the independence of the two pathways. The ratio S_2/S_1 was used as a measure of independence, where S_1 and S_2 denotes slopes of fEPSPs evoked by a single-pulse stimulation of the control pathway and by stimulation of the control pathway which was followed a single-pulse stimulation of the TBS pathway with an interval of 50 ms. Only experiments without slow-developing changes in the control pathway (deviation from initial values <15%) and a ratio of S_2/S_1 smaller than 115% were taken for analysis. Results are presented as relative changes produced in the TBS-applied pathway above the control pathway. Two TBSs were applied to measure immediate and later functional consequences of the compound injections: one immediately following the injection and a second one 30 min after the injection. Data acquisition and analysis were performed using AxoClamp 2B (Axon Instruments, Union City, CA, USA), EPC9, and the PULSE software (HEKA, Lambrecht, Germany). (These recordings have been done by Dr. Mu Sun, ZMNH, Hamburg)



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#	Comparison: Mice (Treatment)	Contextual Memory		Cued Memory	
		ANOVA statistics	Effect	ANOVA statistics	Effect
	C57BL/6J mice				
	Pretraining (- 4 hr) injection group				
1.	- (ACSF) - (UO)	*Treat:* $F_{(1,25)}$=2.77, p=0.1 *Treat x Trial:* $F_{(1,25)}$=0.006, p=0.94	–	*Treat*: $F_{(1,25)}$=1.64, p=0.21 *Treat x Trial*: $F_{(1,25)}$=0.002, p=0.96 *Treat x Tone*: $F_{(1,25)}$=0.11, p=0.73	–
2.	- (Fc) - (UO)	*Treat:* $F_{(1,16)}$=1.71, p=0.2 *Treat x Trial:* $F_{(1,16)}$=0.78, p=0.38	–	*Treat*: $F_{(1,16)}$=0.014, p=0.9 *Treat x Trial*: $F_{(1,16)}$=1.21, p=0.28 *Treat x Tone*: $F_{(1,16)}$=0.065, p=0.80	–
3.	- (PSA-NCAM-Fc) - (Fc)	*Treat:* $F_{(1,19)}$=18.49, p<0.001 *Treat x Trial:* $F_{(1,19)}$=5.56, p=0.029 FLSD 1st day p<0.001, 3rd day p=0.035	++++	*Treat*: $F_{(1,19)}$=0.69, p=0.41 *Treat x Trial*: $F_{(1,19)}$=0.18, p=0.66 *Treat x Tone*: $F_{(1,19)}$=0.096, p=0.76	–
4.	- (NCAM-Fc) - (Fc)	*Treat:* $F_{(1,20)}$=1.0, p=0.32 *Treat x Trial:* $F_{(1,20)}$=2.57, p=0.12	–	*Treat*: $F_{(1,20)}$=0.003, p=0.95 *Treat x Trial*: $F_{(1,20)}$=1.07, p=0.31 *Treat x Tone*: $F_{(1,20)}$=0.45, p=0.50	–
5.	- (PSA) - (ACSF)	*Treat:* $F_{(1,25)}$=7.42, p=0.01 *Treat x Trial:* $F_{(1,25)}$=5.2, p=0.03 FLSD 1st day p=0.001, 3rd day p=0.17	++	*Treat*: $F_{(1,25)}$=0.83, p=0.37 *Treat x Trial*: $F_{(1,25)}$=2.49, p=0.12 *Treat x Tone*: $F_{(1,25)}$=0.40, p=0.52	–
6.	- (PSA) - (ASCF)	*Treat:* $F_{(1,26)}$=0.008, p=0.92 *Treat x Trial:* $F_{(1,26)}$=0.0001, p=0.99	–	*Treat*: $F_{(1,26)}$=2.34, p=0.13 *Treat x Trial*: $F_{(1,26)}$=3.26, p=0.08 *Treat x Tone*: $F_{(1,26)}$=0.26, p=0.61	–
7.	- (PSA) - (CHS)	*Treat:* $F_{(1,17)}$=4.74, p=0.04 *Treat x Trial:* $F_{(1,17)}$=3.96, p=0.06 FLSD 1st day p=0.01, 3rd day p=0.25	+	*Treat*: $F_{(1,17)}$=0.29, p=0.59 *Treat x Trial*: $F_{(1,17)}$=0.006, p=0.94 *Treat x Tone*: $F_{(1,17)}$=0.011, p=0.91	–
	Post-training (+ 2 hr) injection group				
8.	- (PSA-NCAM-Fc) - (Fc)	*Treat*: $F_{(1,10)}$=0.14, p=0.71 *Treat x Trial*: $F_{(1,10)}$=0.049, p=0.82	–	*Treat*: $F_{(1,10)}$=0.001, p=0.97 *Treat x Trial*: $F_{(1,10)}$=0.04, p=0.84 *Treat x Tone*: $F_{(1,10)}$=0.002, p=0.96	–
9.	- (NCAM-Fc) - (Fc)	*Treat*: $F_{(1,9)}$=2.84, p=0.12 *Treat x Trial*: $F_{(1,9)}$=3.95, p=0.07	–	*Treat*: $F_{(1,9)}$=1.32, p=0.28 *Treat x Trial*: $F_{(1,9)}$=0.25, p=0.62 *Treat x Tone*: $F_{(1,9)}$=0.27, p=0.61	–
10.	- (PSA) - (CHS)	*Treat*: $F_{(1,10)}$=1.17, p=0.3 *Treat x Trial*: $F_{(1,10)}$=0.0001, p=0.98	–	*Treat*: $F_{(1,10)}$=1.19, p=0.66 *Treat x Trial*: $F_{(1,10)}$=0.64, p=0.44 *Treat x Tone*: $F_{(1,10)}$=0.17, p=0.68	–
	Post-training (+ 6 hr) injection group				
11.	- (PSA-NCAM-Fc) - (Fc)	*Treat*: $F_{(1,10)}$=9.22, p=0.01 *Treat x Trial*: $F_{(1,10)}$=1.10, p=0.31 FLSD 1st day p=0.01, 3rd day p=0.16	++	*Treat*: $F_{(1,10)}$=0.35, p=0.56 *Treat x Trial*: $F_{(1,10)}$=0.99, p=0.34 *Treat x Tone*: $F_{(1,10)}$=0.64, p=0.44	–
12.	- (NCAM-Fc) - (Fc)	*Treat*: $F_{(1,10)}$=0.97, p=0.34 *Treat x Trial*: $F_{(1,10)}$=0.28, p=0.6	–	*Treat*: $F_{(1,10)}$=1.81, p=0.2 *Treat x Trial*: $F_{(1,10)}$=1.47, p=0.25 *Treat x Tone*: $F_{(1,10)}$=0.38, p=0.54	–
13.	- (PSA) - (CHS)	*Treat*: $F_{(1,13)}$=2.04, p=0.17 *Treat x Trial*: $F_{(1,13)}$=3.58, p=0.08	–	*Treat*: $F_{(1,13)}$=1.81, p=0.2 *Treat x Trial*: $F_{(1,13)}$=0.89, p=0.36 *Treat x Tone*: $F_{(1,13)}$=4.33, p=0.06	–
	PST deficient mice				
14.	- PST+/+ - PST-/-	*Gene*: $F_{(1,14)}$=11.02, p=0.005 *Gene x Trial*: $F_{(1,14)}$=1.9, p=0.18 FLSD 1st day p=0.004, 3rd day p=0.045	+++	*Gene*: $F_{(1,14)}$=0.02, p=0.89 *Gene x Trial*: $F_{(1,14)}$=0.002, p=0.96 *Gene x Tone*: $F_{(1,14)}$=0.12, p=0.72	–
	NCAM deficient mice				
	Pretraining (- 4 hr) injection group				
15.	- NCAM-/- (Fc), - NCAM+/+ (Fc)	*Gene*: $F_{(1,11)}$=13.96, p=0.003 *Gene x Trial*: $F_{(1,11)}$=0.08, p=0.77 FLSD 1st day p=0.007, 3rd day p=0.002	+++	*Gene*: $F_{(1,11)}$=8.5, p=0.01 *Gene x Trial*: $F_{(1,11)}$=0.08, p=0.77 *Gene x Tone*: $F_{(1,11)}$=1.41, p=0.25	++
16.	- NCAM-/- (PSA-NCAM-Fc) - NCAM-/- (Fc)	*Treat*: $F_{(1,20)}$=7.8, p=0.01 *Treat x Trial*: $F_{(1,20)}$=0.72, p=0.40 FLSD 1st day p=0.035, 3rd day p=0.007	++	*Treat*: $F_{(1,20)}$=1.0, p=0.32 *Treat x Trial*: $F_{(1,20)}$=0.24, p=0.62 *Treat x Tone*: $F_{(1,20)}$=6.57, p=0.019	+
17.	- NCAM-/- (PSA-NCAM-Fc) - NCAM+/+ (Fc)	*Treat*: $F_{(1,21)}$=8.02, p=0.01 *Treat x Trial*: $F_{(1,21)}$=0.17, p=0.68 FLSD 1st day p=0.01, 3rd day p=0.01	++	*Treat*: $F_{(1,21)}$=7.53, p=0.012 *Treat x Trial*: $F_{(1,21)}$=0.043, p=0.83 *Treat x Tone*: $F_{(1,21)}$=3.01, p=0.097	+
18.	- NCAM+/+ (PSA-NCAM-Fc) - NCAM+/+ (Fc)	*Treat*: $F_{(1,17)}$=9.10, p=0.008 *Treat x Trial*: $F_{(1,17)}$=2.99, p=0.1 FLSD 1st day p=0.005, 3rd day p=0.02	++	*Treat*: $F_{(1,17)}$=0.45, p=0.5 *Treat x Trial*: $F_{(1,17)}$=0.19, p=0.66 *Treat x Tone*: $F_{(1,17)}$=2.12, p=0.16	–

Table 2-I. A statistical evaluation of freezing after fear conditioning. Repetitive two-way ANOVA was used to estimate significance of effects produced by different treatments (Treat). The columns "Effect" indicates by "+" a level of significance if any difference has been found, "-" stands for no significant effects.

Measurements of LTP in the hippocampal CA1 region of NCAM deficient mice in vitro (the recordings have been done by Dr. Mu Sun, ZMNH, Hamburg)

Previous studies had revealed impairment of LTP in the CA1 region of the hippocampus after enzymatic removal of PSA and in ST8SiaIV/PST deficient mice (Muller et al., 1996, Eckhardt et al., 2000), suggesting that polysialylated NCAM is required for CA1 LTP. Here we aimed to verify whether the extracellular domain of PSA-NCAM or PSA alone is responsible for normal induction of LTP in CA1. To address this question, we introduced these molecules in hippocampal slices derived from NCAM-/- mice.

In agreement with previous data (Muller et al., 1996) we found that LTP is reduced in NCAM-/- mice (for comparison of the two genotypes, see Figs. 20-I,C, D and E, control pathways). The mean level of LTP recorded in NCAM-/- mice 20-30 min after the first TBS was 117.9±4.0%, whereas it was 132.7±3.6% in NCAM+/+ controls. Mean levels of LTP recorded in NCAM-/- mice 20-30 min after the second TBS were also significantly lower than in slices from NCAM+/+ mice (122.6±3.8% versus 139.2±2.2%).

The deficit in LTP in NCAM-/- mice could be rescued by injection of recombinant PSA-NCAM-Fc (100 mg/ml; Fig. 20-I,C), pointing to the possibility that the polysialylated extracellular domain of NCAM is responsible for LTP. Injection of NCAM-Fc without PSA did not significantly increase LTP in NCAM-/- mice (Fig. 20-I,D). Injection of PSA (100 mg/ml) into slices of NCAM+/+ mice decreased levels of LTP measured 30 min after the first and second TBS (Fig. 20-I,E), in agreement with data of Muller and co-workers (2000). Application of the same concentration of PSA to slices of NCAM-/- hippocampi significantly increased LTP as compared to the control site and reached levels comparable with those measured in NCAM+/+ hippocampi (Fig. 20-I,F): mean levels of LTP recorded at the injected site after the first and second TBS were 130.9±4.2% and 143.1±5.9%, respectively. However, at a higher concentration (300 mg/ml), PSA did not normalize the levels of LTP in NCAM mutants: After the second TBS, LTP from both recording sites were only 117.4±6.8% and 113.6±7.6% (Fig. 20-I,G). To test the effect of PSA on consolidation of LTP, we injected PSA after the second TBS. There was no LTP promoting effect at the injection site, where the level of LTP was 126.4±9.0% versus 126.2±13.0% at the control site (Fig. 20-I,H).

These results together with previously published data demonstrate that 1) PSA is the functional entity of the PSA-NCAM modulating induction of LTP and 2) there is an optimal PSA concentration which supports induction of LTP – absence or high concentrations of PSA are prohibitive for LTP.

Auditory and pain sensitivity

Since acquisition of cued (tone) memory depends on how well mice process sound as CS, we inspected the auditory abilities of mice. We observed that after the 1st series of tones has been paired with a foot shock, all mice exhibited a startle response to the second application of the tones, a phenomenon known as sensitization (Kamprath and Wotjak, 2004). We took this parameter as a confirmation of normal auditory sensitivity in all mice tested (data not shown).

Since the freezing response is a function of the footshock intensity, it was necessary to control whether all mice received foot shocks of equal intensity. We thus measured the level of freezing which mice exhibited immediately after the 1st foot shock during training ("S" trial in Figs. 15-I, B-19-I, B). This parameter reflects both pain sensitivity and reaction to it. All C57BL/6J mice injected before fear conditioning (Fig. 15-I, B) showed similar freezing during the "S" trail at the level of 46.5±2.4%. The one-way ANOVA evaluation did not detect any statistically significant difference among mice injected with Fc, NCAM-Fc, PSA-NCAM-Fc, ACSF, CHS, and PSA (Treatment: $F_{(5,65)}=0.73$, $p=0.6$).

The C57BL/6J mice that were injected after fear conditioning (regardless of the post-training time) and the unoperated C57BL/6J mice also exhibited comparable reactions to the first footshock. The levels of freezing were 30.6±3.2% and 28.8±3.2% when injections were performed 2 and 6 h after conditioning (Figs. 16-I, B and 17-I, B, respectively) and in unoperated mice freezing time was 20.8±6.3% (Fig. 15-I, B). By ANOVA, no statistically significant difference was detected among mice injected with Fc, NCAM-Fc, PSA-NCAM-Fc, CHS, PSA and unoperated mice (Treatment: $F_{(5,32)}=0.76$, $p=0.57$; $F_{(5,36)}=0.6$, $p=0.69$, for 2 and 6 h, respectively). It is noteworthy that freezing in mice injected after fear conditioning was significantly lower than in mice injected before training.

This difference probably reflects that the procedure of injection is unpleasant and increases susceptibility to further anxious stimulus such as the foot shock. This difference, however, is not of importance since we did not compare freezing in mice injected before versus after memory acquisition but rather evaluated effects of

different injections performed at one time point. PST-/- mice had similar reaction to the foot shock compared to PST+/+ mice (Genotype: $F_{(1,14)}$=0.73, p=0.4, Fig. 18-I, B). Also NCAM-/- mice did not differ from NCAM+/+ mice in their reaction to the first foot shock (Genotype: $F_{(1,37)}$=0.46, p=0.4; Fig. 19-I, B). This analysis confirms that mice from all compared experimental groups equally perceived the foot shock.

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DISCUSSION

Effects of intrahippocampal injections of PSA-NCAM and PSA on contextual memory in wild-type mice

One of the major findings in the present work is that apparently only injections of PSA (alone or attached to the extracellular domain of NCAM) were potent enough to disrupt formation of contextual memory in the fear conditioning paradigm in wild-type mice. On the other hand, soluble NCAM-Fc is known to have biological functions, the most widely studied of which is stimulation of neurite outgrowth of cerebellar and cerebral cortical neurons via homophilic binding (Saffell et al., 1997; Niethammer et al., 2002). In our experiments, however, NCAM-Fc showed significant effects neither on formation nor consolidation of fear memories, nor on CA1 LTP. Thus, mechanisms underlying modulation of cognitive functions by NCAM may be less dependent of homophilic NCAM-NCAM interactions than, for example, neurite outgrowth. Our results are supported by an observation that another modulator of NCAM homophilic binding - peptide C3d, which binds to the first Ig domain of NCAM and stimulates neurite outgrowth (Ronn et al., 1999) also could not affect the acquisition phase of contextual learning in rats (Cambon et al., 2003). However, C3d did work as an amnesic agent in a passive avoidance task (Foley et al., 2000) or after training in fear conditioning paradigm (Cambon et al., 2003), suggesting differential roles of NCAM-mediated homophilic interactions in different forms and stages of learning. This idea is supported by our observation that both PSA-NCAM-Fc and PSA disrupted acquisition of learning but only PSA-NCAM-Fc impaired consolidation, thus highlighting the role of the NCAM glycopeptide backbone in association with PSA in consolidation of fear memory.

The interference of PSA-NCAM-Fc and PSA with acquisition of learning could be explained by several possible mechanisms. First, they may work as competitive antagonists abolishing PSA-NCAM driven synaptogenesis (Dityatev et al., 2004) at loci where PSA-NCAM is endogenously upregulated by a learning event (Fox et al., 1995; Murphy et al., 1996; O'Connell et al., 1997). Second, since PSA was found to inhibit NR2B-containing NMDA glutamate receptor activity (Martin Hammond, Melitta Schachner, Alexander Dityatev, unpublished data), the effect of injected PSA on formation and maintenance of contextual memory in our study can be at least

partially attributed to inhibition of these NMDA receptors (see project III in this thesis). Systemic injection of antagonists to NR2B-containing NMDA receptors is known to inhibit acquisition of cued and contextual fear memories (Rodrigues et al., 2001). Also, intrahippocampal injection of antagonists to all subtypes of NMDA receptors leads to similar memory deficits as observed in our experiments (Stiedl et al., 2000).

PSA-NCAM in late consolidation phase of fear memory

Previous experiments with injection of NCAM ligands during different phases of learning and memory (see Fig. 12-I and Table 1-I) revealed the existence of a short post-training time-window in which consolidation can be disrupted via alterations in NCAM functions. For example, NCAM antibodies and C3d peptides caused amnesia in a passive avoidance task (Doyle et al., 1992) and in fear conditioning (Cambon et al., 2003) when injected within the 5.5-8 h post-training time-window, but not earlier or later. In addition, during memory consolidation, a transient, time-dependent learning-induced increase in polysialylation of NCAM has been observed in the hippocampus, entorhinal and perirhinal cortices throughout different behavioral paradigms and species (Fox et al., 1995; Murphy et al., 1996; O'Connell et al., 1997). These changes start within 10-12 h post-training time and last for several hours. Also, the peak of NCAM expression identified in both chick and rat after passive avoidance task occurs within a 6-8 h period post-training (Doyle et al., 1992; Scholey et al., 1993; Arami et al., 1996).

Considering these data together with our results, we suggest that there is a time window at 6 h post-training that is PSA-NCAM dependent. Indeed, none of the substances, including PSA-NCAM-Fc, NCAM-Fc and PSA, injected at 2 h post-training disrupted fear memory. However, at 6 h post-training PSA-NCAM-Fc, although not PSA or NCAM-Fc, was enough potent to do this. Since immunoreactivity against the Fc-fragment is detected in the hippocampus even 24 h after injection, the question is why PSA-NCAM-Fc did not impair contextual memory when injected at 2 h post-training, although it was effective when injected at 6 h post-training. We suppose that this phenomenon related to changes in concentration of functionally active injected molecules and expression of their receptors after fear conditioning. Since only injection of PSA-NCAM-Fc at 6 h but not at 2 h post-training was effective, we have to expect that expression/sensitivity of molecules interacting with/dependent on PSA-NCAM is increased at 6 h in comparison to the early phase. The

concentration of injected molecules evidently depends on their rates of diffusion, degradation and inactivation via endocytosis or binding to numerous extracellular molecules known to interact with extracellular part of NCAM. Immunostaining against the Fc portion of fusion proteins does not distinguish between partially degraded (but Fc carrying) and full-size molecules or between extracellular and internalized or matrix-bound molecules. Thus, the concentration of functionally active PSA-NCAM-Fc may decline in the scale of hours and a 4 hour-difference in time of injection may explain why 2 h post-training injection could not affect later consolidation phase by the unavailability of the compound under study.

Recovery of contextual fear memory in NCAM deficient mice by PSA-NCAM

In this study for the first time we show that deficits in CA1 LTP and memory in NCAM-/- mice can be restored via delivery of exogenous PSA-NCAM and PSA, thus highlighting the importance of PSA and its receptors in learning and memory. Since there is no NCAM in NCAM deficient mice, effects of PSA and PSA-NCAM-Fc in restoration of contextual memory are not mediated by modulation of NCAM-NCAM homophilic interactions.

In this context, it is noteworthy, that the heavily hydrated “cloud” of PSA may independently of NCAM modulate adhesion of other CAMs, such as cadherins, L1 and integrins (Fujimoto et al., 2001). Another mechanism may involve signaling via receptors to FGF, platelet derived neurotrophic factor (PDNF) and brain derived neurotrophic factor (BDNF), which is enhanced in the presence of PSA (Dityatev et al., 2004; Zhang et al., 2004; Muller et al., 2000). The latter pathway may be of particular importance, since application of BDNF has been shown to restore the impaired CA1 LTP in both NCAM deficient and PSA-removed wild-type slices (Muller et al., 2000). Hence, exogenously added PSA or PSA-NCAM may potentiate BDNF signaling, thus restoring LTP and contextual memory in NCAM deficient mice. PSA may also potentiate a subset of AMPA glutamate receptors (Vaithianathan et al., 2004), which may elevate depolarization of cells during learning and, thus, promote synaptic plasticity. PSA also inhibits NMDA receptors containing the NR2B subunit (Martin Hammond, Melitta Schachner, Alexander Dityatev, unpublished observation), known to be involved in synaptic plasticity.

Recovery of cued memory in NCAM deficient mice by injection of PSA-NCAM-Fc

It is widely accepted that contextual memory in the fear conditioning paradigm is hippocampus-dependent, whereas cued memory is stored extrahippocampally, and relies mostly on of the amygdala, thalamus and auditory cortex (for reviews see LeDoux, 2000; Dityatev and Bolshakov, 2005). However, some recent studies yielded strong evidence that the hippocampus, at least its ventral part, is not only involved in contextual fear conditioning, but also in a cued fear conditioning to a tone (see refs in Bast et al., 2003). Furthermore, activation/inhibition of the dorsal hippocampus, through intrahippocampal connections can lead to alterations in its ventral part, altering processing of auditory information. For example, lesions of the dorsal hippocampus or injections of NMDA or the $GABA_A$ agonist muscimol into the dorsal hippocampus before fear conditioning led to a deficit in cued (tone) memory (Bast et al., 2003). Interestingly, after auditory fear conditioning neuronal activity in the CA1 area of the hippocampus and in the basolateral amygdala in mice becomes rhythmically synchronized, coupled at theta (4–12 Hz) frequencies, providing a neuronal correlate of fear during subsequent fear memory retrieval episodes (Seidenbecher et al., 2003).

Moreover, after fear conditioning there is a long-lasting increase in the amplitude/slope of auditory-evoked potentials elicited by the conditioned tone in both the lateral amygdala and dorsal hippocampus (Tang et al., 2003). All these data suggest that there is an exchange of fear-related information between amygdala and the hippocampus. Thus, the rescue effect of intrahippocampal PSA-NCAM-Fc injection on cued memory in NCAM-/- mice can be explained in a way that a weak deficit in the amygdala in NCAM-/- mutants can via hippocampus-amygdala cross-talk be compensated by improvement of hippocampal function, evidently reached via hippocampal injection of PSA-NCAM-Fc. Another noteworthy observation is that cued memory was impaired in NCAM-/- but not PST-/- mice, suggesting that polysialylation of NCAM by PST in the amygdala is less important than in the hippocampus.

In vitro restoration of PSA-dependent hippocampal LTP

Despite the great progress of advanced mouse genetic techniques, in vitro acute perturbation/rescue experiments remain to be irreplaceable, particularly in light of multiple roles that NCAM plays in development and function of the hippocampus. Here, we performed parallel injections of PSA, NCAM and PSA-NCAM in the

hippocampal slices and intact brains and compared effects of these compounds on LTP in the CA1 region versus contextual memory.

Correlations between CA1 LTP and contextual and spatial learning with regard to NCAM functions have been shown in several studies using other approaches, including 1) application of NCAM antibodies that impaired CA1 LTP in slices and caused amnesia in a passive avoidance task and spatial learning in rats (Luthi et al., 1994; Arami et al., 1996); 2) analysis of NCAM deficient mice revealed a deficit in CA1 LTP and contextual and spatial learning (Muller et al., 1996, 2000; Cremer et al., 1998; Stork et al., 2000; Bukalo et al., 2004); 3) enzymatic removal of PSA impaired CA1 LTP and spatial learning (Becker et al., 1996; Muller et al., 1996); and 4) analysis of PST deficient mice showed a deficit in CA1 LTP (Eckhardt et al., 2000) and contextual learning (the present study). All these experiments suggest that PSA carried by NCAM is necessary for CA1 LTP and contextual/spatial learning.

On the other hand, Muller and colleagues (2000) reported that application of PSA-NCAM-Fc or PSA may inhibit CA1 LTP. In our present study, we confirmed this result, showing that PSA decreased CA1 LTP in wild-type hippocampal slices. Also pre-training injections of either PSA or PSA-NCAM-Fc in hippocampi of wild-type mice reduced freezing time, suggesting that too much of PSA is inhibitory for learning. Moreover, we found that either PSA-NCAM-Fc or PSA could rescue impaired CA1 LTP in NCAM deficient mice, as was previously shown for BDNF (Muller et al., 2000). Rescue of LTP by PSA-NCAM-Fc correlated with partial recovery of contextual memory after injection of PSA-NCAM-Fc. In summary, these data suggest that optimal levels of PSA are responsible for induction of LTP and hippocampal learning. It is currently not known whether this concentration-dependence is related to binding of PSA to different high- and low-affinity receptors or a concentration-dependent effect of PSA on a single receptor.

Dysfunctions mediated by abnormalities in PSA and NCAM

A recent study demonstrates that biosynthesis of sialic acid regulates and limits the synthesis of polysialic acid (Bork et al., 2005). Thus, one can expect to find abnormalities in polysialylation of NCAM in sialuria, a disease in which patients overproduce sialic acid, and lysosomal free sialic acid storage diseases that include Salla disease and infantile sialic acid storage disease. If confirmed, abnormalities in

polysialylation of NCAM may be related to cognitive deficits found in patients suffering from these disorders.

Previous studies have shown abnormally increased levels of soluble NCAM in the cerebrospinal fluid of people suffering from schizophrenia (Poltorak et al., 1995) and dementia (Strekalova et al., 2006). Also, there is an increase in levels of soluble NCAM, whereas total amount of NCAM is normal and number of polysialylated cells is reduced in brains of patients with schizophrenia (Barbeau et al., 1995; Vawter et al., 2001). The potential mechanisms of generation of different soluble NCAM isoforms in the extracellular space and CSF involve shedding of membrane NCAM or secretion of intact isoforms (see refs in Kalus et al., 2006).

All these alterations in soluble NCAM and PSA-NCAM expression are noteworthy in light of cognitive deficits in schizophrenia patients (Goldberg et al., 1993). In our experimental model, using injection of compounds into the mouse hippocampus, we showed that elevated levels of soluble PSA and PSA-NCAM in the hippocampus of wild-type mice may result in a deficit in contextual memory. Furthermore, recently generated transgenic mice (NCAM-EC) which overexpress soluble NCAM in the brain also exhibit deficits in contextual fear conditioning (Pillai-Nair et al., 2005) as our PSA-NCAM-Fc injected mice. These and our results strongly suggest that upregulation of soluble NCAM, not yet studied with regard to its polysialylation pattern, as found under different pathological conditions, may lead to cognitive deficits. Thus, elucidation of mechanisms by which soluble PSA and NCAM affects synaptic functions may guide in development of therapeutic strategies to prevent or compensate these deficits.

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PROJECT II: "The effects of tamoxifen-inducible ablation of NCAM gene on fear conditioning in mice"

REVIEW OF THE LITERATURE

Transgenic models

The generation of mouse mutants (see Fig. 1-II) through homologous recombination (the recombining of an exogenous piece of DNA with its endogenous homologous sequence) in embryonic stem (ES) cells (the pluripotent derivatives of the inner cell mass of the blastocyst) and transgenesis is a powerful tool to study complex brain functions such as learning and memory, highly dynamic processes such as synaptic plasticity, and to produce animal models for neurodegenerative diseases (for review see Elgersma and Silva, 1999; Mayford and Kandel, 1999). However, germline mutations often result in lethal phenotypes, thus precluding the analysis of the

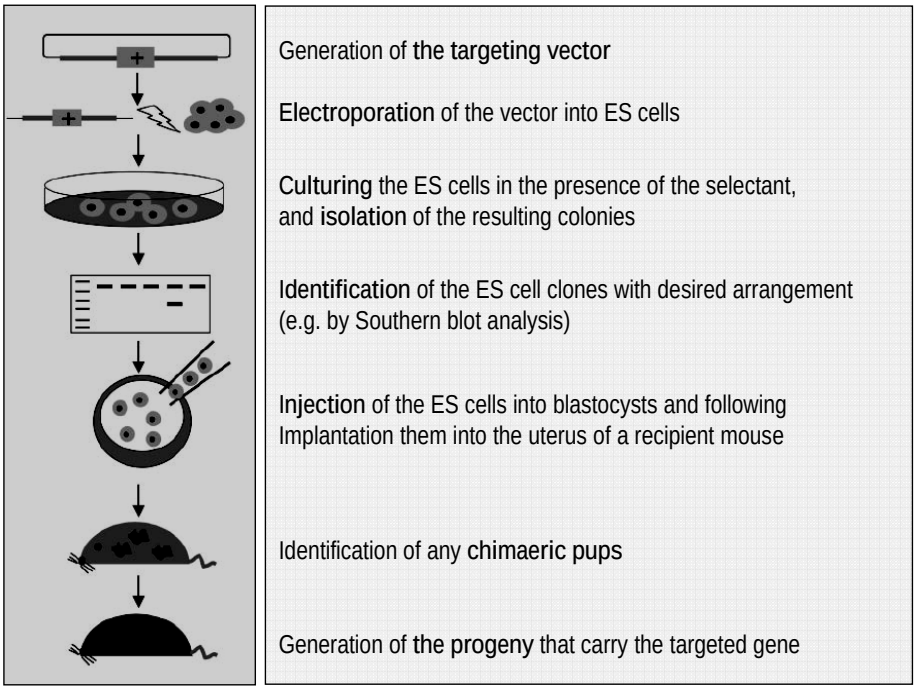


Fig. 1-II. Procedure for the construction of genetically modified mice generated by gene targeting strategies (modified from review of Van der Weyden et al., 2002)

physiological function of many genes. The introduction of spatio-temporally controlled somatic mutations in the CNS would allow the study of a gene function in the brain of adult mice independently not only such a lethality, but also of possible effects of the mutated genes during development. Furthermore, particularly in the field of learning

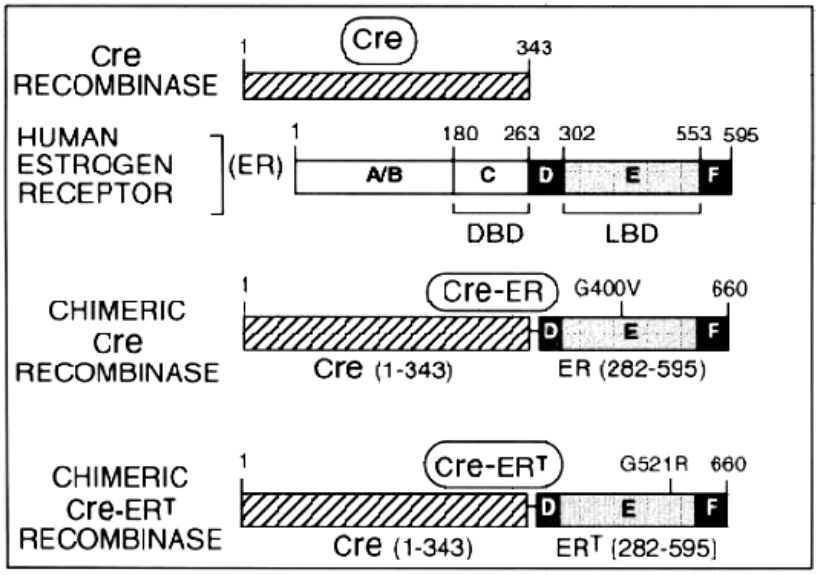


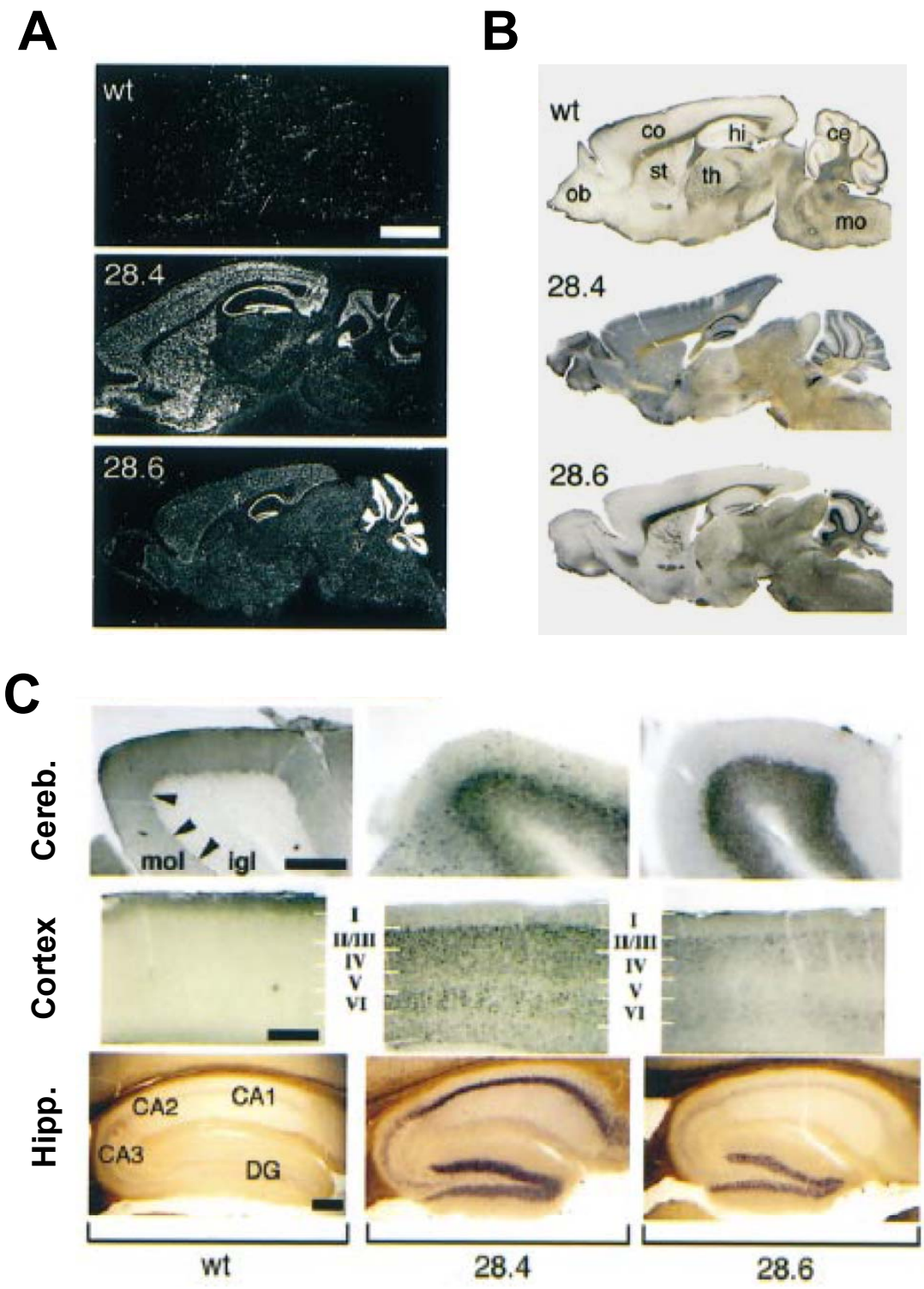
Fig. 2-II. Conditional site-specific recombination in mammals cells using ligand-dependent chimeric Cre-recombinases. Schematic representation of Cre-recombinase, the human estrogen receptor (ER), and the Cre-ER and Cre-ER^T fusion proteins. Amino acid sequences of Cre and LBDs of ER are represented by hatched and gray boxes, respectively. Numbers refer to amino acids positions. The ER A7B, C, D, E and F regions (36), the DNA binding domain (DBD), and the LBD, as well as the G400V and G521R mutations, are indicated. (Modified from Metzger and Chambon, 2001.)

and memory, problems linked to genetic background, maternal effects, and interindividual variations could be minimized, as such an animal could be analyzed before and after inactivation of the gene of interest (Lipp and Wolfer, 1998; Silva et al., 1997).

The combination of gene targeting techniques in mouse ES cells and Cre-loxP recombination system has resulted in the emergence of chromosomal engineering technology in mice. Genetically modified mice can be generated (see Fig. 1-II) either by direct pronuclear injection of exogenous DNA into fertilized zygotes (Palmiter and Brinster, 1986) or by injection of genetically modified mouse ES cells into a blastocyst (Kuehn et al., 1987). In contrast, ES cells have the advantage in that they can be

genetically modified by means of homologous recombination prior to being injected into the blastocyst. This process is known as “gene targeting” and was first reported in mammalian cells in 1985 (Smithies et al., 1985; Lin et al., 1985). Strategies for conditional gene targeting in mice, based on cell type-specific or inducible expression of the bacteriophage P1 site specific Cre recombinase have been developed (see Fig. 2-II). The Cre recombinase can efficiently excise a DNA segment flanked by two loxP sites (floxed DNA, for review see Nagy, 2000) in animal cells. Spatially or temporally controlled somatic mutations can be obtained by placing the Cre gene under the control of either a cell-specific or an inducible promoter, respectively. However, these conditional gene targeting systems have also a number of limitations, as they are either spatially or temporally controlled. Ideally, one would like to have a system that allows generation of somatic mutations in at a given time in the life of animal and in a specific cell type. To obtain a spatio-temporal control of floxed DNA excision, several systems have been recently developed to regulate Cre recombinase activity in a ligand-dependent manner in the adults. In these approaches, the Cre recombinase (see Fig. 2-II) was fused to a mutated ligand binding domain (LBD) of the steroid hormone receptor.

Fig. 3-II *In situ* hybridization (A) and immunohistochemistry (B) of Cre-ER^T expression patterns in the brain of PrP-Cre-ER^T transgenic adult mice of lines 28.4 and 28.6 and wt, wild-type controls. Olfactory bulbs (ob), cortex (co), striatum (st), hippocampus (hi), thalamus (th), cerebellum (ce), and medulla oblongata (mo) are indicated. Scale bar: 2 mm. C Detailed immunohistochemical analysis of Cre-ER^T expression patterns in different brain areas of adult transgenic mice of lines 28.4 and 28.6, and wt, wild-type controls. Upper panel: sagittal sections through the cerebellum; molecular layer (mol), internal granular layer (igl), arrowheads point to the Purkinje cell layer. Middle panel: sagittal sections of the neocortex; layers are indicated by roman numerals. Bottom panel: sagittal sections of the hippocampus; dentate gyrus (DG), and CA1, CA2, and CA3 are indicated. Scale bars: Upper and bottom panels, 200 µm, middle panel, 500 µm (modified from Weber et al., 2001).



← Fig. 3-II (the figure legend is on the previous page)

Cre-Tamoxifen transgenic system

It has been shown that activity of a chimeric recombinase generated by the fusion of Cre to the LBD of estrogen receptor (Cre-ER) is dependent on the presence of estrogen agonist (e.g., 17β-estradiol, E2). To achieve tight control (see Fig. 2-II) of the activity of such a chimeric protein in the presence of endogenous estradiol, it has been made a mutation in the LBD of the human ER (Gly521Arg), resulting in the chimeric protein Cre-ER^T which binds only to the synthetic ligands like *tamoxifen* (further, *Tamo*) and 4-hydroxytamoxifen (OHT), but does not bind endogenous E2 (Danielian et al., 1993).

In line 28.4 the Cre-ER^T transgene was expressed under the mouse prion protein (PrP) promoter in almost all brain areas (see Fig. 3-II), with highest expression in olfactory bulbs, hypothalamus, striatum, hippocampus, cortex, cerebellum, and spinal cord. In contrast, line 28.6 showed more restricted expression in the hippocampus and prominently in the granular layer of the cerebellum, whereas lower levels were detected in other brain regions. In both lines, expression of the Cre-ER^T transgene was also detected in the neural retina. To translocate effectively Cre-ER^T complex into the nucleus it has been necessary to inject *Tamo* for five consecutive days.

As it was shown before, short-term *Tamo* treatment has very low acute toxicity and causes no severe abnormalities in mice (Metzger and Chambon, 2001). Cre-ER^T protein is essentially cytoplasmic in the absence of *Tamo* treatment, whereas it becomes almost completely nuclear after 3 days of *Tamo* treatment (Metzger and Chambon, 2001). Similar results were obtained when either *Tamo* or OHT was used, and *Tamo* is about 40 times cheaper than OHT. The DNA excision after *Tamo* administration can occur at least in some part of cells as fast as 24 hr (Metzger and Chambon, 2001). Expression pattern of endogenous PrP promoter visualized by Southern blot analysis was similar in wild type, and PrP-Cre-ER^T transgenic mouse lines (28.4 and 28.6). Moreover, expression of the transgenic PrP promoter in the complex PrP-Cre-ER^T transgene (see Fig. 4-II,A) in 28.4 and 28.6 lines was equal between these two lines (See Fig. 4-II,C, Weber et al., 2001) Expression of a floxed reporter gene LacZ which was wired with PrP-gene as one transgenic construct (see Fig. 4-II,A) was highly profound in the cortex, the hippocampus and the cerebellum; and it correlates with expression pattern of endogenous PrP in pyramidal and dentate gyrus granular cells of the hippocampus, neurons of neocortex and cerebellum

(Kretzschmar et al., 1986; Manson et al., 1992; Chishti et al., 1997), proving that transgenic PrP gene in the construct is correctly expressed in brain (Weber et al., 2001).

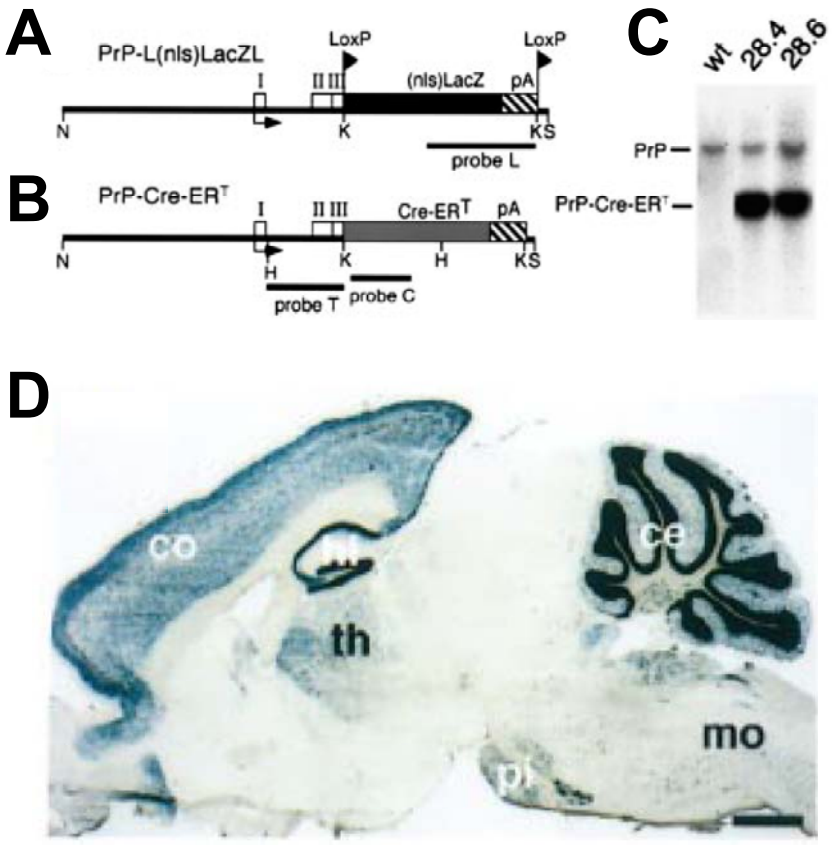


Fig. 4-II Schematic representation of PrP-L(nls)LacZL and PrP-Cre-ER^T transgenes (A and B, respectively). Exones I-III of the prp gene are indicated by open boxes; the coding region for (nls)LacZ is indicated by a closed box and loxP-sites are indicated by flags; the Cre-ER^T coding sequence is indicated by a grey box. C – Estimation of PrP- Cre-ER^T transgene copy number in two transgenic mouse lines (28.4 and 28.6). Genomic DNA was extracted from tail biopsies of wt-type (wt), 28.4, and 28.6 mice and analyzed by Southern blotting. D – LacZ expression pattern in brain of PrP-L(nls)LacZL transgenic mice performed by X-Gal staining of a 50 μm thick brain sagittal sections. Note the intense staining of cells in the hippocampus (hi), thalamus (th), pituitary gland (pi), the cortex (co), the cerebellum (ce), and medulla oblongata (mo) (modified from Weber et al., 2001).

AIMS OF THE STUDY

The aims of this study were:

1. To investigate whether tamoxifen may induce ablation of NCAM in adult mice with floxed NCAM gene and expressing Cre-ER^T recombinase (NCAMff, cre+ mice), and if so, to which degree, and in which brain areas and time-scale it occurs;
2. To test NCAMff, cre+ mice in auditory fear conditioning paradigm, and verify whether inducible elimination of NCAM gene in adult leads to the same cognitive deficits as seen in mice where NCAM has been ablated prenatally;
3. To develop a repetitive fear conditioning paradigm to be able to explore learning and memory functions before and after inducible ablation of a gene of interest using the same animals.

***

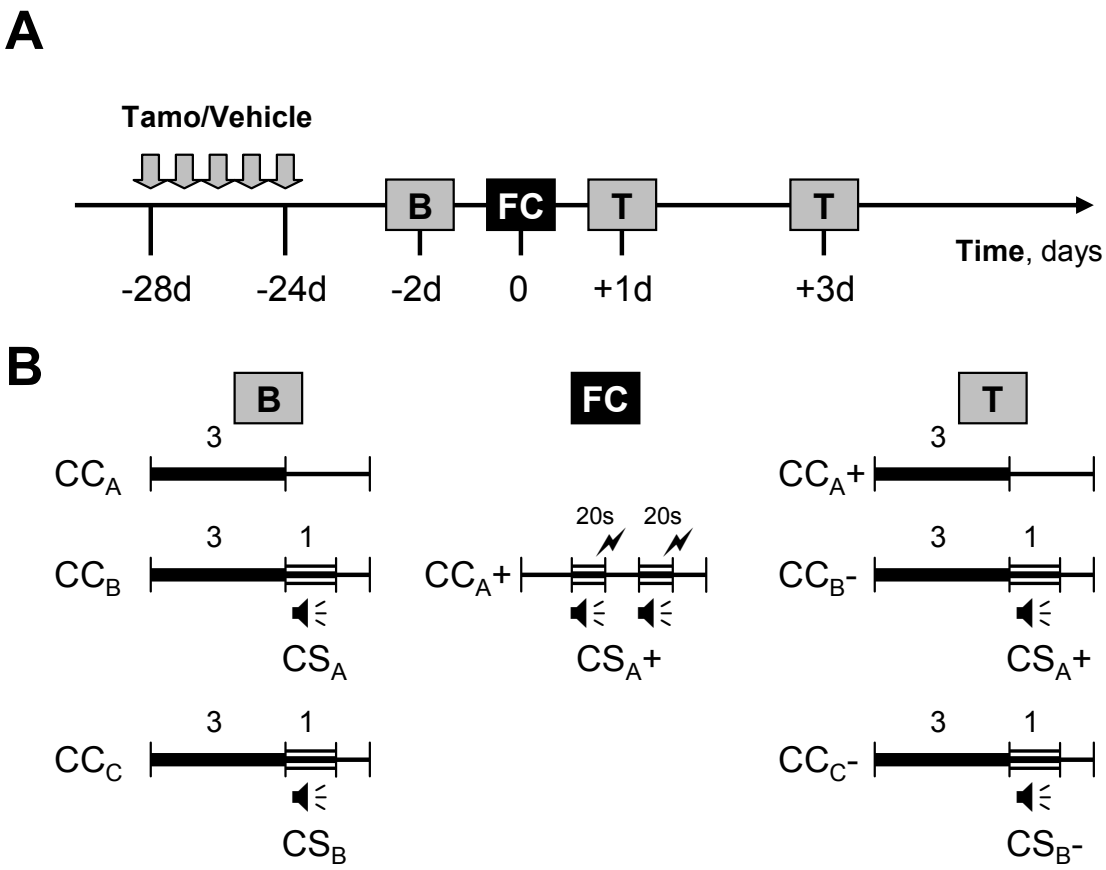
MATERIALS & METHODS

Mice

For the experiment 1 in this project, 24 tamoxifen-inducible NCAM transgenic mice (males, 2-3 month old) have been used (further abbreviated like “NCAMff”): 16 mice carried Cre-recombinase (NCAMff+) and 8 mice had no Cre-recombinase in their genome (NCAMff-). Genetically modified NCAM floxed (ff) mice (mice in which NCAM gene is flanked by special DNA LoxP-sequences, recognizable only by the Cre-recombinase, were crossbred with mice carrying Cre-recombinase or not under the control of the mouse prion protein (PrP) promoter (Weber et al., 2001). The resulting progenies of these mice were all homozygous for the NCAM-floxed allele (NCAMff) and either heterozygous for the PrP-Cre-ER^T transgene (+) or had not it (-). After their generation mice were crossed with 129/Sv and C57BL/6 mice and then backcrossed at least 4 times with C57BL/6 mice. These NCAMff mice, the line ERT4 (ERT stays for Estrogen Receptor Tamoxifen) were bred in the UKE animal facility. One week before starting experiments mice were delivered to a small vivarium in the laboratory and housed in separate cages with *ad libitum* water and food and 12 h shifted day/night cycle (light on at 8 p.m.). For the experiment 2 with a double repetitive fear conditioning 14 C57BL/6J adult (males 2-3 month old) mice have been used. All behavioural procedures were approved by the Committee on Animal Health and Care of the local governmental body.

Fig. 5-II Detailed description of the experimental design. A – Time-course of all manipulations conducted to tested mice. Baseline (B), fear conditioning (FC), testing (T). B – Design of contextual and cued tests. Three different contexts (CC_A, CC_B, CC_C) and two distinct cued stimuli (CS_A and CS_B) were used to evaluate levels of retention of contextual and tone memories, respectively. A sign (+) means a paired CC or CS with US; a sign (-) means unpaired ones. Numbers indicate time intervals in minutes. C – Photographs and descriptions of three different contexts (A, B, C), and cued stimuli which have been used in the experiments.

Design of experements with tamoxifen inducible NCAMff ERT4 mice



CS_A – patterned tone of 7 kHz, 80 dB
CS_B – patterned tone of 3 kHz, 80 dB

← Fig. 5-II (the figure legend is on the previous page)

Injection of tamoxifen

For the experiments 1 and 2, intraperitoneal (i.p.) injection of *Tamo* was performed for 5 consecutive days, with 1 mg per animal daily, as it was previously shown to be sufficient to activate Cre-ERT-complex and translocate it into the cell nuclei (Weber et al., 2001). *Tamo* was prepared by the following protocol: 200 mg of tamoxifen free base (Sigma) was dissolved in 99% ethanol and added to 20 ml sesame autoclaved sterile oil (Sigma) ended by 30 min sonication with an ultrasonicator (USC 100T, VWR International, Darmstadt, Germany) and stored either at 4 °C for a week or – 20 °C for months. The prepared *Tamo* and vehicle (further, *Veh*, the above mixture of the oil and ethanol without *Tamo*) solutions were sonicated every time just before usage. Injections were made through i.p. route, 100 µl per animal, using a short (~ 2 min) sedative CO_2 anaesthesia to restrain animals without a stress.

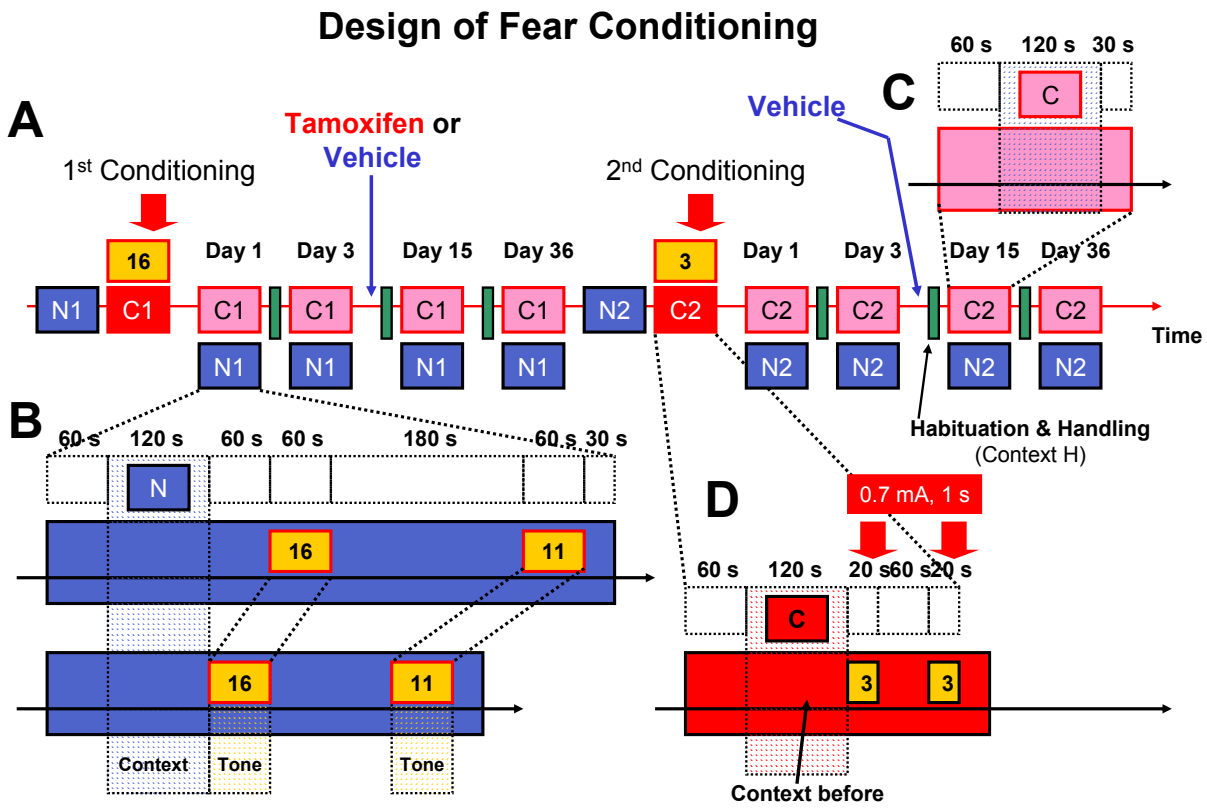
Fear conditioning

Experiment 1. After a week of accommodation in a small laboratory vivarium, the mice were injected with tamoxifen or vehicle in a distinct from the experimental room area to avoid any possible unpleasant associations of injections with the experimental conditions. Training of animals in auditory fear conditioning started exactly 4 weeks after the first injection had been made. This time was chosen on the basis of immunoblotting analysis showing that 4 weeks is enough to remove most of NCAM from the hippocampus (see Results). Before starting behaviour experiments, mice were carefully handled and habituated to experimental conditions (those which have to be not associated with a footshock).

Two days before training the mice were subjected for baseline recordings in three (CC_A, CC_B, CC_C) different contexts (see description of contexts in Fig. 5-II, C) as well as to two distinct auditory stimuli (CS_A, CS_B, see Fig. 5-II, C). It has to be mentioned that in these experiments we introduced two dissimilar contexts in order to reduce potential interference between cued and contextual stimuli. The procedure of fear conditioning was as following (Fig. 5-II, A and B): (i) a naive mouse was placed into the conditioning chamber (CC_A) and after 60 s subjected to 2 CS–US pairings spaced apart by 60 s; the CS–US pairing consisted of a 20-s patterned tone (either 3 or 7 kHz, 80 dB) co-terminated with a 1-s footshock; (ii) 30 s after the last CS–US pairing the mouse was returned to its home cage. The CSs were counterbalanced;

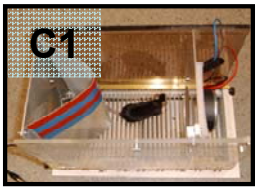
pairing was done with one of the tones, either CS_A or CS_B, while another served as a control. Testing of mice was performed at days 1 and 3 after fear conditioning (+d1 and +d3 in graphs) to test short and long-term memory, respectively. For each test day mice were exposed to three different tests in a pseudorandom order. These tree tests were: (i) contextual memory test: a naive mouse was placed into the context CC_A previously associated with a footshock for 5 min and only the first 3 min has been taken for further analysis; (ii) two tone memory tests in neutral contexts CC_B and CC_C, in each context a tone has been presented once: either CS_A or CS_B for 60 sec at the end of 3-min time interval that served as a control for contextual memory test to measure generalized anxious behaviour of mice.

Fig. 6-II Detailed description of the double repetitive fear conditioning. A – Time-course of all manipulations conducted to the C57BL/6J mice. C1 and C2 – two conditioned contexts, N1 and N2 – two neutral contexts; in red depicted two trainings with either from two different tones (here 16 and 3 kHz). B – Temporal design of the tone memory tests performed in one of two neutral contexts (N) with shifted presentations of CS. C – Temporal design of contextual memory test. Note that only 120 sec in both tests were used for counting freezing responses, after mice accommodated for first 60 s. D – Temporal design of each training; two fear conditionings were absolutely symmetrical; they were distinct only in contexts and tones; Note that 120 seconds after 60-s accommodation time served like baseline recording before the first US was applied. E – Photographs and descriptions of 4 different contexts (two conditional C1 and C2; and two neutral N1 and N2).

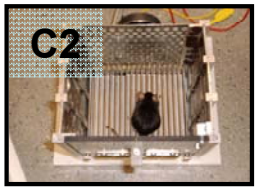


E

Conditional contexts



Cond. Chamber
Illumination: Bright light
Cleaning: 75% Ethanol
Floor: Metal rods
Walls: Plexiglas
Way: long



Cond. Chamber
Illumination: Bright light
Cleaning: 1% acetic acid
Floor: Metal rods
Walls: Aluminum
Way: long

Neutral contexts

Tone Cage
Illumination: Dark light
Cleaning: Vanilla
Floor: Soft Bedding
Walls: Celled
Way: short

Tone Cage
Illumination: Dark light
Cleaning: Citron
Floor: Normal Bedding
Walls: Circled
Way: short

← Fig. 6-II (the figure legend is on the previous page)

Experiment 2. After at least a week of accommodation in a small laboratory vivarium, C57Bl mice were carefully handled and habituated to experimental conditions (which have to be not associated with a footshock). For this double repetitive fear conditioning paradigm we introduced 4 different contexts (C1, C2, N1, N2; "C" stays for "Conditioned", "N" – "neutral", numbers mean the order of training, 1st or 2nd, see descriptions in Fig 6-II, E) and 4 distinct auditory stimuli as a cued unimodal stimuli that were patterned with 1 Hz loud clicks of 4 different frequencies pure tones (3, 7, 11 and 16 kHz), each adjusted to produce 75 dB (SPL). We took these particular frequencies in order (i) to make tone clicks sounded as different as possible to facilitate discrimination of conditioned and neutral tones after conditioning, (ii) to use audible range of frequencies (1-20 kHz) to allow us an easy control of their presentations.

Two days before each of training session baseline recordings were made for each animal, where one of two different pairs of tones (either 3 and 7 kHz, or 11 and 16 kHz) in one of the neutral contexts (N1 or N2) was presented. Pairs of tones and two neutral contexts were carefully counterbalanced among two injected groups of animals and between two trainings. After the baseline recording, on the next day mice were trained according to the following procedure (Fig. 6-II, D): (i) a mouse was place into one of two conditioning chambers (either C1 or C2, counterbalanced) for 180 seconds to record baseline behaviour; (ii) the first 20-s episode of CS was presented coterminating with 1-s footshock of 0.7 mA, as a US; (iii) after 60 seconds of resting time, the second pairing of CS with US was made; (iv) 30 seconds after pairing the mouse was returned to its home cage. During first or second conditioning only one tone from the pair (3 and 7 kHz) or (11 and 16 kHz) was paired with a footshock, another always served as a neutral control tone.

Tests were performed at 4 different days after each training; on 1st, 3rd, 15th and 36th days. On each testing day we ran two different tests for tone memory and for contextual memory, which were counterbalanced in order. To test contextual memory, a mouse was placed into context previously associated with a footshock (either C1 or C2) for 210 seconds (see Fig 6-II, C). Only 120 seconds after first 60 seconds of accommodation time were used for calculations of freezing. The test for tone memory was more complicated and included: (i) placing a mouse into one of two neutral contexts (either N1 or N2) in a tone cage (see Fig 6-II, E) and to present there two series of tones; (ii) each series of tones lasted for 60 seconds; (iii) between these

two presentations of tones there was resting time of either 180 or 120 seconds to make their appearance less predictable for mice; (iv) the order of tones was carefully counterbalanced between animal groups and within every subject (e.g., if a tone of 3 kHz was first presented to a mouse, followed by a tone of 7kHz on one of test days, next day the mouse received tones in the opposite order, i.e., first 7 kHz and then 3 kHz); (v) before presentation of the first tone, we took either 180 or 240 seconds to record free exploratory behaviour of mice in neutral context (see Fig 6-II, B). 120 seconds from this time, immediately after first 60 seconds of accommodation time, we considered for contextual memory test as a stay in a neutral context. All shifts in time of tone presentations were also carefully counterbalanced between two injecting groups and within each animal. Additionally, between every test day we habituated all animals for 3 min in a context distinct from 4 experimental ones – a grey home cage – to reduce anxiety of mice.

Injection with *Tamo/Veh* was performed for 5 consecutive days, between 3rd and 15th test days, in an area distinct from the experimental room to avoid any possible unpleasant associations of injections with the experimental conditions.

Freezing behavior

Behaviour of animals was videotaped for each session and analyzed off-line by a trained observer blind to the identity of mice and to the applied tones (paired or unpaired) for each of the 60-s uniform tone episodes (cued freezing). Freezing time, which served as a measure of fear-related memory, was quantified as described (Tang et al., 2001). Briefly, freezing state was assigned if an animal remained motionless, except for respiratory movements, in a tense posture that was characterized by horizontal positioning of the mouse head, a stretched state of the body and stiffening of the tail.

***

RESULTS

Experiment 1.

Spatiotemporal profile and degree of tamoxifen-inducible NCAM ablation in the brains of NCAMff+ mice from the line 28.4

As was shown previously (Weber et al., 2001) by in situ hybridization, the activity of the transgenic Cre-ERT recombinase in the mice of the line 28.4 is prominent almost in all brain areas, with the highest expression in the hippocampus, cortex, thalamus and cerebellum (see Fig. 3-II). Our immunoblotting analysis revealed the same pattern of NCAM ablation by the Cre-ERT recombinase confirming that the transgenic recombinase is correctly expressed, can be activated by tamoxifen and efficiently deletes floxed NCAM gene via recombination. As depicted in Fig.7-II, Bb, the amount of total NCAM in the brain of NCAMff+ mice 2 weeks after the beginning of *Tamo* injection (below we will refer to these as NCAMff+*Tamo* mice) significantly reduced in all probed areas, including the hippocampus, cortex, cerebellum and the midbrain. Ablation of NCAM, however, was not complete since there were still some traces of all three NCAM isoforms detectable after 2 weeks of *Tamo* treatment. It is noteworthy that postsynaptic isoform of NCAM, NCAM180, was nearly fully eliminated from the hippocampus, cortex, and cerebellum whereas NCAM140 and NCAM120 were still present in the brain although greatly reduced in comparison to NCAMff- mice injected with *Tamo* (NCAMff-*Tamo*) (Fig. 7-II, Ba).

The same result has been found using immunohistochemical analysis (Fig. 7-II, A). NCAMff+ mice injected with vehicle (NCAMff+*Veh*) showed the characteristic for wild-type mice (Bukalo et al., 2004) pattern of NCAM expression in the hippocampus, which appears as highly immunoreactive NCAM signal in the hilus, inner granular cell layer of the dentate gyrus, mossy fibers of the CA3 area and the stratum lacunosum moleculare, as well as in the cortex (superficial layers are far less positive that dipper ones). The NCAM immunoreactivity in NCAMff+ mice was strongly abolished on the 21st day after tamoxifen treatment (Fig. 7-II, Ab).

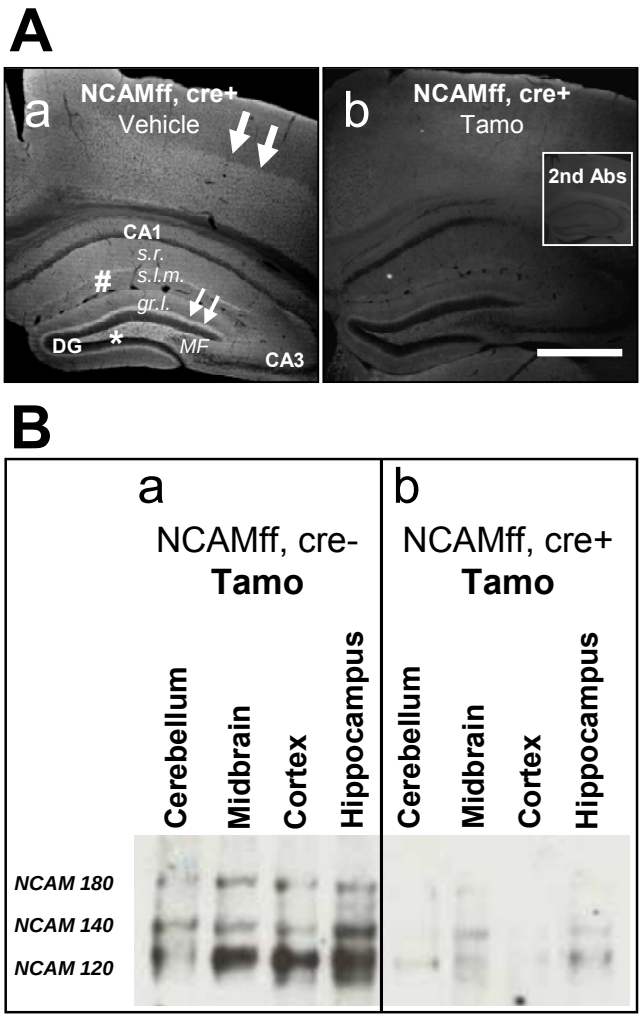


Fig. 7-II Immunohistochemical staining for NCAM in the hippocampus (A) and immunoblotting analysis of the four main brain areas (B) after tamoxifen-inducible ablation of NCAM gene in adult *NCAMff* ERT4 mice. Brain tissues have been taken 3 (A) and 2 (B) weeks after the first injection from 5 days series has begun. NCAM staining of the hippocampus after injection of vehicle A(a) and tamoxifen A(b) revealed that the most of NCAM for 3 weeks has been ablated by the Cre-recombinase. The characteristic NCAM signal in the cortex (big ↙↙), the granular cell layer (*gr.l.*) (small ↙↙), the hilus (*) and stratum lacunosum-moleculare (s.l.m.) (#) completely disappeared after the tamo treatment. The amount of total NCAM left in the brain was significantly reduced in *NCAMff*+ mice B(b) compared to *NCAMff*- mice B(a) after tamoxifen injection. The scale bar in A(b) is about 1 mm. The immunoblotting results have been generously provided by Tatjana Makhina (ZMNH, Hamburg, Germany).

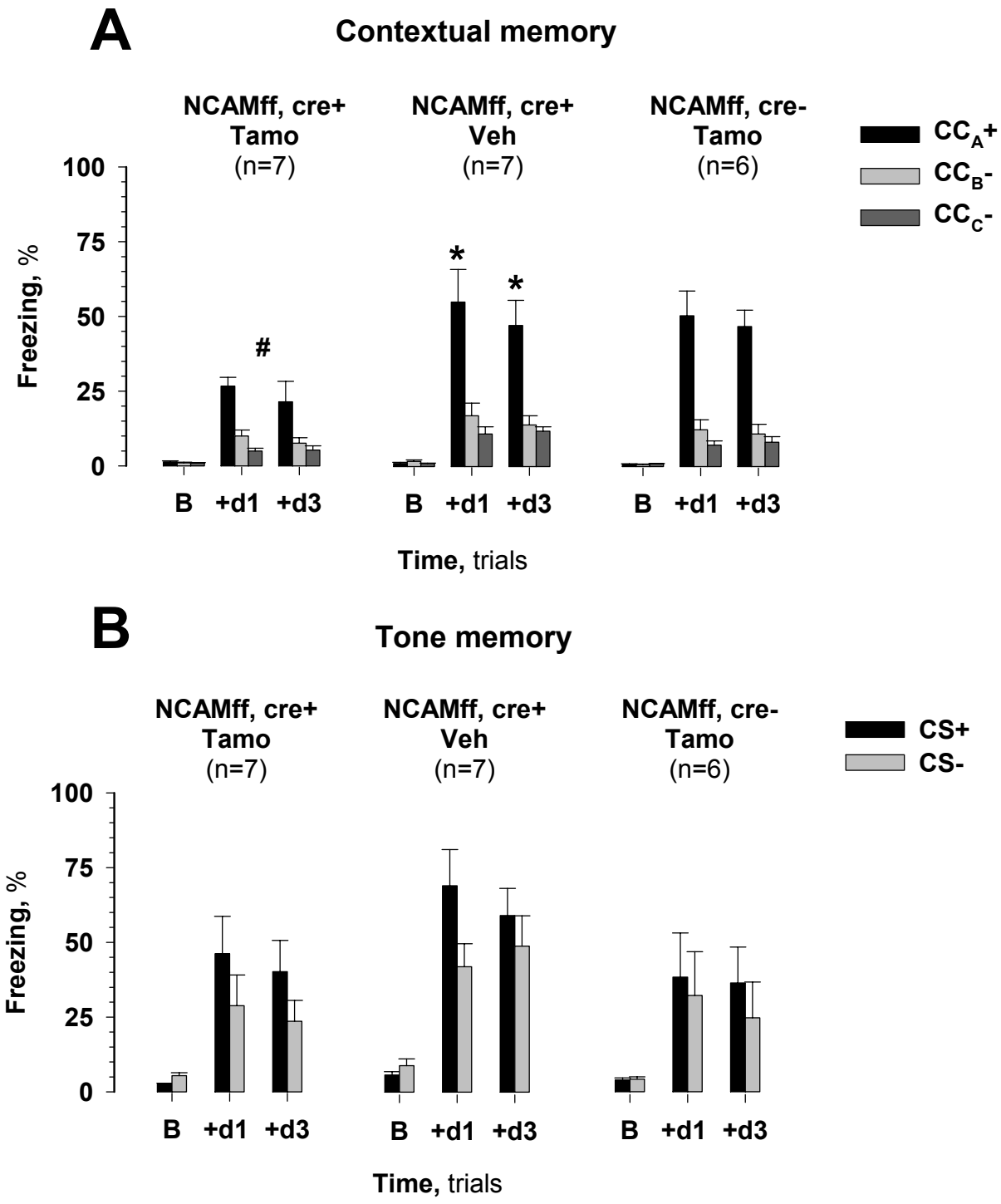
Inducible ablation of NCAM from the brain of the adult NCAMff+ mice resulted in impairment of short- and long-term contextual memory.

Analysis of contextual memory in control groups.

To investigate effects of inducible ablation of NCAM on behaviour of NCAMff+*Tamo* mice we used two controls. One control (NCAMff-*Tamo*) was necessary to reveal effects mediated by Cre. However, it does not allow one to distinguish effects mediated by *Tamo*-independent (so-called "background" or "leaky") activity of Cre, which potentially may ablate NCAM expression on yearly developmental stages, from effects induced via injection of *Tamo* in adult mice. Therefore, the second control (NCAMff+*Veh*) was necessary to reveal effects induced by *Tamo*. This control, however, by itself is insufficient to distinguish the effects mediated by *Tamo*-activated Cre-mediated ablation of NCAM from other unknown "side" effects of *Tamo*.

If there is absence of unspecific effects of *Tamo* and no background activity of Cre, NCAMff+*Veh* and NCAMff-*Tamo* should be not different in all measured parameters. As is depicted in Fig 8-II, there are no any significant differences between NCAMff+*Veh* and NCAMff-*Tamo* in retention of contextual information over the course of trials: freezing responses in paired context CC_A+ were similar in two control groups (*Group:* $F_{1.11}$=0.04, p=0.83; *Day/Trail x Group:* $F_{1.11}$=0.32, p=0.58, ANOVA). Also no difference between groups was found in freezing responses to unpaired contexts (CC_B- and CC_C-) on either test day (*Group:* $F_{1.11}$=1.91, p=0.194; *Day/Trail x Group:* $F_{1.11}$=0.082, p=0.77; *Context x Group:* $F_{1.11}$=0.001, p=0.97). Discrimination between two unpaired (CC_B- and CC_C-) and one paired (CC_A+) contexts was also similarly good in both groups, since there was an effect of context (*Context:* $F_{2.22}$=33.59, p<0.001), independent on the group identity (*Context x Group:* $F_{2.22}$=0.15, p=0.85).

Fig. 8-II Tamoxifen-inducible ablation of NCAM gene in adult mice resulted in impaired fear conditioning. A – Contextual memory is significantly decreased during retrieval trials (day 1, day 3) in Cre-expressing (+) mice after treatment with tamoxifen (*Tamo*) compared to *NCAMff*+ vehicle (*Veh*)-injected mice or *NCAMff-Tamo*-injected mice. B – Neither of treatments influenced amygdala-dependent tone memory.
# p<0.05, two-way repetitive ANOVA; * p<0.05, *post hoc* Fisher's LSD analysis.



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Effects of inducible NCAM ablation on contextual memory

By immunoblotting and immunohistological analysis we proved that ablation of NCAM can be induced in NCAMff Cre-ER^T system by *Tamo* injection in vivo. As described in the previous section, to reveal effects of inducible ablation of NCAM on behavioural parameters, we need to compare NCAMff+*Tamo* mice with two control groups, NCAMff+*Veh* and NCAMff-*Tamo*.

NCAMff+*Tamo* mice showed significant two-fold decrease in retention of contextual memory over the course of tests (see Fig. 8-II), as compared to both control groups (*Group:* $F_{2.17}$=4.63, p=0.025). Repetitive two-way ANOVA followed by *post hoc* Fisher's LSD test revealed significant difference between NCAMff+*Tamo* and NCAMff+*Veh* in freezing responses to conditioned context CC_A+ (*Group*: $F_{1.12}$=6.95, p=0.022), which was trial-independent (*Day/Trail x Group:* $F_{1.12}$=0.08, p=0.77) and had similar significance for both test days (p<0.05, LSD test).

Freezing responses to both neutral contexts (CC_B- and CC_C-) were significantly smaller than those to conditioned one (CC_A+) for all tested groups of animals, thus showing that significant amount of freezing in the conditioned context reflects learned fear rather than general changes in anxiety after fear conditioning. Repetitive two-way ANOVA revealed that NCAMff+*Tamo* mice, despite the fact that they had decreased CC+ responses in comparison to control groups, still froze significantly less in unpaired contexts compared to CC+ context, suggesting that they still could discriminate between three different contexts (*Context:* $F_{2.12}$=17.71, p<0.001; *Day/Trail x Context:* $F_{2.12}$=0.73, p=0.49). Also Wilcoxon test found that freezing was larger in the conditioned context than in unpaired contexts during two testing days (p<0.05). Control NCAMff+*Veh* and NCAMff-*Tamo* mice also had highly significant discrimination between these three contexts for all trials (*Context:* $F_{2.12}$=19.41, p<0.001; *Day/Trail x Context:* $F_{2.12}$=1.0, p=0.39 and *Context:* $F_{2.10}$=48.02, p<0.001; *Day/Trail x Context:* $F_{2.10}$=0.84, p=0.46, respectively), and Wilcoxon test found that it was significant for both unpaired contexts for both groups during two testing days (p<0.05).

Tone memory was not changed by Tamo injection in NCAMff+ mice

Analysis of tone memory in control groups. If there are no unspecific effects of *Tamo* and no background activity of Cre, NCAMff+*Veh* and NCAMff-*Tamo* should not be different in tone memory. Indeed, two-way repetitive ANOVA found no difference between control groups in freezing responses to CS+ and CS- tones (*Group:* $F_{1.11}$=2.96, *p*=0.11; *Group x Day/Trail:* $F_{1.11}$=0.34, *p*=0.55 for CS+ and *Group:* $F_{1.11}$=1.25, *p*=0.28; *Group x Day/Trail:* $F_{1.11}$=2.88, *p*=0.11, for CS-, respectively). Since freezing to both CS+ and CS- was significantly higher on days 1 and 3 after fear conditioning than that during baseline recordings, however, discrimination between CS+ versus CS- was not significant in both control groups (*CS:* $F_{1.11}$=0.55, p=0.47; *CS x Group:* $F_{1.11}$=0.14, *p*=0.71; *CS x Group x Day/Trail:* $F_{1.11}$=2.09, *p*=0.17). This is probably related to relatively small sample size and relatively high level of sensitization to auditory stimuli in both control groups in the training conditions used in this study.

Effects of inducible NCAM ablation on tone memory. A repetitive two-way ANOVA test confirmed that all three groups of animals, regardless of injection (see Fig. 8-II), showed similar responses on CS+ and CS- during two test days (*Group:* $F_{2.17}$=1.62, *p*=0.22; *Group x Day/Trail:* $F_{1.12}$=0.1, *p*=0.75). The discrimination between two CSs was significant (*CS:* $F_{1.17}$=10.58, *p*=0.005) and similar for all groups (*CS x Group:* $F_{2.17}$=0.1, *p*=0.41, *CS x Group x Day/Trail:* $F_{2.17}$=0.85, *p*=0.44). This is despite there was no significant discrimination between control groups and probably reflects an increase in the sample size after addition of the third group.

Conclusions

The results obtained in frame of this project provided evidence that: (i) tamoxifen induced activity of Cre-recombinase is capable of removing the floxed gene of interest, NCAM; (ii) inducible ablation of NCAM gene in mature brains of NCAMff+*Tamo* mice has profound consequences on following acquisition and retention of hippocampus-dependent contextual memory, as has been previously found in constitutive NCAM knockout mice; (iii) inducible ablation of NCAM gene in NCAMff+*Tamo* mice has no detectible effect on amygdala-dependent tone memory, in contrast to constitutive NCAM mice, suggesting either an important role of NCAM

in this structure during development or smaller levels of NCAM ablation in amygdala than in the hippocampus of NCAMff+*Tamo* mice.

Experiment 2.

Taking into account individual variability of behavioural responses, even in inbred strains of mice, analysis of molecular/pharmacological analysis would greatly benefit from possibility to perform within-subject comparisons of behavioural parameters before and after ablation of a gene of interest, or before and after pharmacological treatment. Thus, repetitive tests of mice are of great interest and below we describe the results of repetitive fear conditioning that was applied to test the effects of tamoxifen on contextual and tone memories.

Contextual memory after double repetitive fear conditioning, test of tamoxifen

As shown in Fig. 9-II, retention of contextual memory after either first or second fear conditioning was very robust and lasted for more than a month in both *Tamo* and *Veh* treated animal groups; the level of freezing on either day after training was around 50% for conditioned context and at least twice less for the neutral one.

Contextual memory during the 1st conditioning was similar in both *Tamo* and *Veh* injected groups. Freezing responses to conditioned context did not differ over the course of testing days (*Injection:* $F_{1.12}$=1.97, p=0.18; *Injection x Trials/Days:* $F_{3.36}$=1.95, p=0.13). As seen in Fig. 9-II, discrimination between conditioned and neutral contexts was significant in both groups after the 1st training; repetitive two-way ANOVA found a highly significant difference in freezing responses among them (*Context:* $F_{1.12}$=86.28, p<0.001), and this difference was similar in both treatment groups (*Context x Injection:* $F_{1.12}$=0.13, p<0.71) and stable over the course of tests (*Context x Injection x Trials/Days:* $F_{3.36}$=0.16, p=0.92). Wilcoxon test found that the discrimination was highly significant (p<0.005) for all test days in both injected groups.

After the second training freezing responses to conditioned contexts in two injected groups were not significantly different (*Injection:* $F_{1.12}$=3.31, p=0.094; *Injection x Trials/Days:* $F_{3.36}$=1.33, p=0.27). Discrimination between two contexts was also robust (*Context:* $F_{1.12}$=78.02, p<0.001), and did not differ in both treatment groups (*Context x Injection:* $F_{1.12}$=1.53, p<0.24) and was stable over all test days (*Context x Injection x Trials/Days:* $F_{3.36}$=1.31, p=0.28). Wilcoxon test proved that the

difference in freezing responses between two contexts was highly significant (p<0.005) for the time-course of tests in both injected groups.

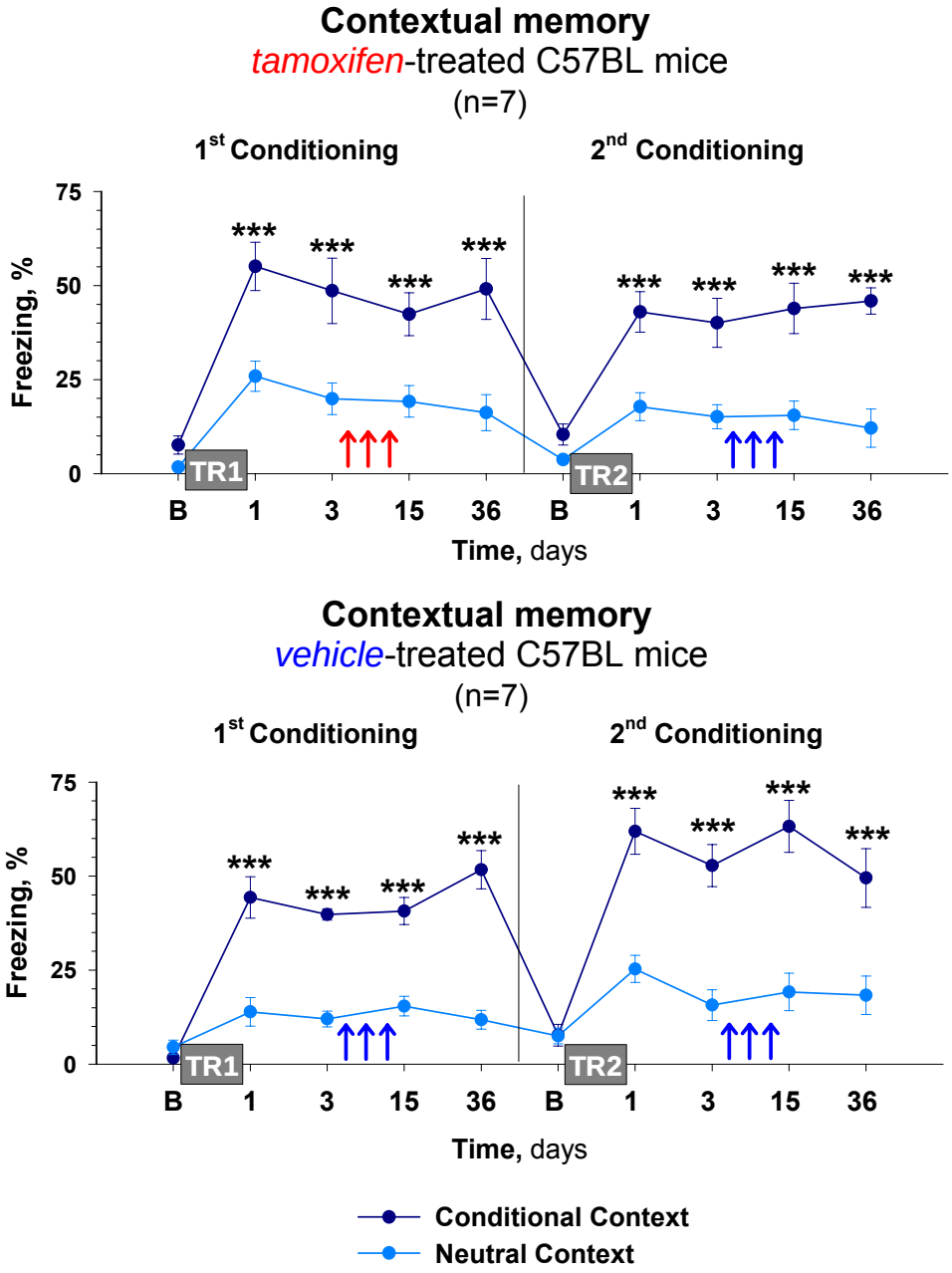

Fig. 9-II Temporal profiles of contextual memory retention in long-time scale (more than a month) during double repetitive fear conditioning in two groups of C57BL mice; one of them received intraperitoneal injection of tamoxifen (marked with red arrows) before second training, the other – a control vehicle (blue arrows). Note that for the design symmetry, additional vehicle injections have been made between retention tests (day 3 and 15, blue arrows) after second training.

*** p<0.005 Wilcoxon test (discrimination between conditioned context CC+ versus neutral context CC-).

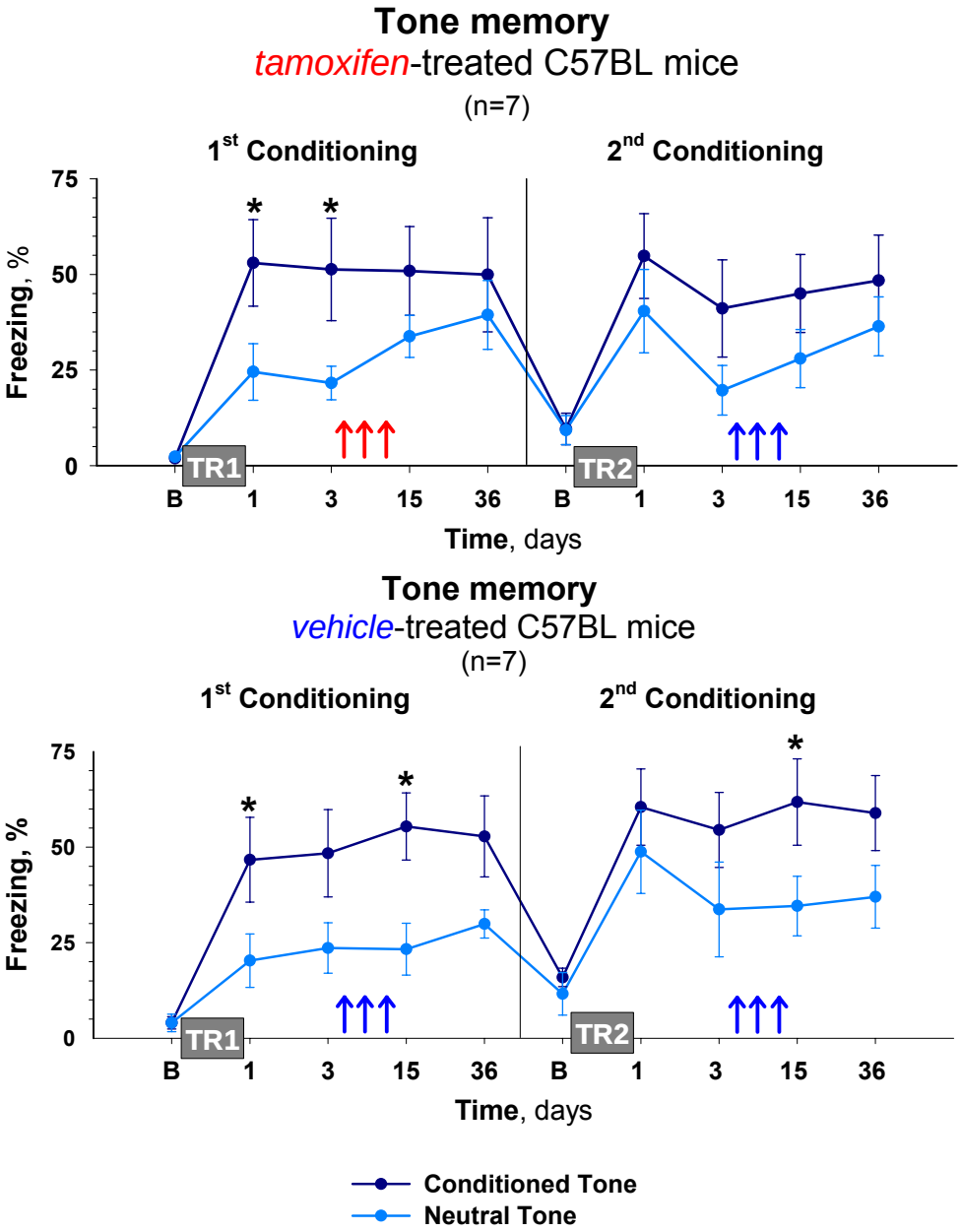

Fig. 10-II Temporal profiles of tone memory retention in long-time scale (more than a month) during double repetitive fear conditioning in two groups of C57BL6 mice; one of them received intraperitoneal injection of tamoxifen (marked with red arrows) before second training, the other – a control vehicle (blue arrows). Note that for the design symmetry, additional vehicle injections have been made between retention tests (day 3 and 15, blue arrows) after second training.

* $p<0.05$ Wilcoxon test (discrimination between conditioned tone versus neutral tone)

Since we injected tamoxifen long after the acquisition of the first fear conditioning (between 3rd and 8th days), it was interesting to test whether tamoxifen can affect already consolidated contextual memory on day 15th and 36th of the same training. Repetitive two-way ANOVA found that *Tamo* did not influence contextual memory consolidated before *Tamo* injection (*Injection:* $F_{1.12}$=0.18, p=0.67; *Injection x Trials/Days:* $F_{1.12}$=0.13, p=0.72). The discrimination between two contexts was also unchanged by *Tamo* (*Context x Injection:* $F_{1.12}$=0.38, p=0.54), and was stable in time (*Context x Injection x Trials/Days:* $F_{1.12}$=0.14, p=0.71).

To evaluate influence of the first training on the next one, we compared freezing responses after first and second fear conditioning. Repetitive three-way ANOVA applied to data from two consecutive trainings found no difference between the first and second training (*Training:* $F_{1.12}$=1.37, p=0.26), and in different injecting groups over the course of trials (*Training x Injection x Trials/Days:* $F_{4.48}$=2.54, p=0.051), although the latter interaction was close to significance. Ability to discriminate between paired and unpaired contexts was also similar between two trainings (*Context x Training x Injection x Trials/Days:* $F_{4.48}$=0.85, p=0.49). Also the baseline levels of freezing in both contexts before the first and second trainings were similar, since ANOVA did not find any significant effects of training (*Training:* $F_{1.12}$=4.31, p=0.06), or injection (*Injection:* $F_{1.12}$=0.11, p=0.73; *Training x Injection:* $F_{1.12}$=0.42, p=0.52) or context (*Context x Injection x Training:* $F_{1.12}$=0.21, p=0.65).

Tone memory after double repetitive fear conditioning, test of tamoxifen

As depicted in Fig. 10-II, mice in two injected groups showed significant tone memory over the time-course of tests, i.e. for more than one month after each training. It is noteworthy that variability of freezing responses to conditioned tone was higher than to conditioned context. Repetitive two-way ANOVA did not reveal any difference between two injecting groups after the 1st training in freezing responses to the tones (*Injection:* $F_{1.12}$=0.08, p=0.77), and it was stable over the testing time (*Injection x Trials/Days:* $F_{3.36}$=0.11, p=0.95), proving that the two injected groups of mice acquired and retained tone association with a footshock equally. Discrimination between two CSs (conditioned versus neutral) was also significant: ANOVA found an effect of CS applied during 60-s test episodes (*CS:* $F_{1.12}$=32.7, p<0.001), and this ability to discriminate tones was equal in both injecting groups (*CS x Injection:* $F_{1.12}$=0.11, p=0.57) and persistent in time (*CS x Injection x Trials/Days:* $F_{3.36}$=0.62,

p=0.6). Nonparametric Wilcoxon test found that significant discrimination between CS+ versus CS- was on 1st and 3rd testing days ($p<0.05$) in tamoxifen-treated group, and on 1st and 15th testing days ($p<0.05$) in vehicle-treated group (see Fig. 10-II).

After the second auditory fear conditioning, there was also no difference between mice injected with *Veh* and *Tamo* (*Injection:* $F_{1.12}=0.71$, $p=0.41$; *Injection x Trials/Days:* $F_{3.36}=0.52$, $p=0.67$). Discrimination between CSs was worse than after the first training, but yet significant, since ANOVA found an effect of CS (*CS:* $F_{1.12}=11.01$, $p=0.006$) and it was similar in two injecting groups (*CS x Injection:* $F_{1.12}=0.14$, $p=0.70$) and independent of trials (*CS x Injection x Trials/Days:* $F_{3.36}=0.33$, $p=0.8$). Wilcoxon test revealed that the significant discrimination ($p<0.05$) of two tones was on 15th day in vehicle-treated group.

Again, to investigate whether tamoxifen has an effect on already consolidated tone memory, we ran the ANOVA analysis just for two last testing days of the first training. There was no any significant influence of injection on retrieval of consolidated tone memory (*Injection:* $F_{1.12}=0.08$, $p=0.78$), which was consistent over the course of two tests (*Injection x Trials/Days:* $F_{1.12}=0.001$, $p=0.97$). Discrimination between CS+ and CS- was significant (*CS:* $F_{1.12}=23.66$, $p<0.001$), stable in time (*CS x Trials/Days:* $F_{1.12}=0.66$, $p=0.43$), and similar between two injected groups (*CS x Injection:* $F_{1.12}=2.59$, $p=0.13$), independently of trials (*CS x Injection x Trials/Days:* $F_{1.12}=0.015$, $p=0.9$).

Comparison between two consecutive trainings revealed that there is no significant difference between the 1st and 2nd trainings (*Training:* $F_{1.12}=0.48$, $p=0.49$; *Training x Injection:* $F_{1.12}=0.78$, $p=0.39$; *Training x Injection x Trials/Days:* $F_{3.36}=0.12$, $p=0.94$). Discrimination between tones was also similar in two trainings since ANOVA could not find any interaction in factors CS and training (*CS x Training:* $F_{1.12}=0.39$, $p=0.54$), independently of injection (*CS x Training x Injection:* $F_{1.12}=0.002$, $p=0.96$) and time (*CS x Training x Injection x Trials/Days:* $F_{3.36}=0.049$, $p=0.98$).

Baseline levels of freezing before each training were similarly low in both injected groups (*Injection:* $F_{1.12}=1.17$, $p=0.3$, ANOVA). Although baseline responses before second training were significantly bigger than before the first one (*Training:* $F_{1.12}=9.47$, $p=0.01$), this effect was the same in both injected groups (*Training x Injection:* $F_{1.12}=1.18$, $p=0.67$). Also CS+ and CS- triggered similar freezing responses during baseline recordings: ANOVA did not find any effect of CS (*CS:* $F_{1.12}=0.54$,

p=0.47), and this was true for both injected groups (*CS x Injection:* $F_{1.12}$=0.63, *p*=0.44) and both trainings (*CS x Injection x Training:* $F_{1.12}$=0.62, *p*=0.44).

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DISCUSSION

Effect of inducible NCAM ablation on learning

Despite the great progress in elucidation of the role of NCAM in learning and memory using constitutive and conditional NCAM deficient mice (Cremer et al., 1994; Bukalo et al., 2004), these models could not provide definitive answer to the question "how important NCAM is for learning in the adult brain", since NCAM is ablated prenatally or early postnatally in these mutants and that may result in developmental or compensatory abnormalities in organization of neural circuitries.

In this aspect, the technique we used in this study, which allows temporally controlled (within 1-2 weeks) inducible ablation of the NCAM gene in adult mice, could avoid pitfalls of other methods. On the other hand, this method does not provide a full ablation of a gene, thus allowing on the other side to investigate effects of different levels of gene expression on behaviour of mice. As shown on Fig 8-II, A, contextual memory of NCAMff+ mice injected with tamoxifen was significantly reduced compared to the control NCAMff- tamoxifen-treated mice and NCAMff+ vehicle-treated mice. This deficit in formation and consecutive retention of contextual information over the time course of tests was similarly apparent for 1st and 3rd testing days. Interestingly, the amount of freezing shown by NCAMff+*Tamo* mice was reduced to the same degree (~ 25%), as it was found for NCAM constitutive mice in our work (see the project I in this thesis) and in the previous study performed by Stork et al. (2000). These results suggest that (i) NCAM has a key role in hippocampal learning since its partial ablation from adult brain leads to serious deficits in formation and retention of contextual associations; (ii) there is a critical level of NCAM expression (>20% of wild-type level that was detected in NCAMff+Tamo mice) necessary for normal contextual learning in the fear conditioning paradigm.

Interestingly, despite the fact that tone memory is also impaired in constitutive NCAM knockout mice (see the project I and Stork et al., 2000), inducible ablation of NCAM gene brought no detectable malfunction in processing, association and retention of cued information, suggesting either that all observed deficits in tone memory of constitutive NCAM deficient mice are due to some developmental abnormalities rather than due to NCAM function during synaptic plasticity, learning

and memory, or that inducible ablation of NCAM in amygdala is not so efficient as in the hippocampus, or that lower amounts of NCAM are sufficient for amygdala than for hippocampus dependent learning.

Treatment with tamoxifen influenced neither contextual nor tone memory in double repetitive fear conditioning paradigm in C57BL6 mice

Here, for the first time we describe a learning paradigm of repetitive auditory fear conditioning, which was developed in order to be able to compare memory formation within a subject before and after disruption of certain genes of interest or before and after pharmacological treatments. We found that mice could not only retain fear association with one of two different contexts/tones in rather long-term scale (36 days), but also could be trained for the second time with similar learning response as at the first time, at least when a care is taken to "diversify" contexts and tones used during the first and second training. This property makes this model very attractive for behavioural analysis after inducible genetic interventions.

Application of this paradigm to study effects of tamoxifen on fear conditioning showed that it has significant effects neither on contextual nor tone memories. This is an important conclusion to interpret data showing that NCAMff-*Tamo* and NCAMff+*Veh* mice have the same levels of freezing after fear conditioning. Two possible explanations of this result are 1) that tamoxifen and background Cre activity have no effects or 2) they influence behaviour in a similar direction. Our additional data showing that tamoxifen has no effects on fear conditioning correspond to results of previous studies indicating that short-term *Tamo* treatment has very low acute toxicity and causes no severe abnormalities in mice (Furr and Jordan, 1984; Metzger and Chambon, 2001) and fully justify the use of NCAMff-*Tamo* mice as appropriate control for NCAMff+*Tamo* mice.

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PROJECT III: "PSA-NCAM & NMDA receptor signaling"

REVIEW OF THE LITERATURE

Glutamate NMDA receptors

Glutamate is the major excitatory neurotransmitter in the mammalian CNS acting through both ligand-gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors (see Fig. 1-III, A). There are three major classes of glutamate ionotropic receptors (for review, see Dingledine et al., 1999) divided by their selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and kainate glutamate receptors. All of these receptors transduce the postsynaptic signal rapidly. Activation of these channels by glutamate mediates basal excitatory synaptic transmission or induces different forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), and is important for learning and memory.

The NMDA receptors (NMDA-Rs) are unique among all known ionotropic receptors since their activation is quite complex, requiring three independent events to simultaneously occur, namely: binding of two molecules of glutamate and two glycine molecules, and depolarization of postsynaptic membrane to a certain level has to be achieved for releasing Mg^{2+} block of these receptors (see Fig. 1-III, B). Thus, the NMDA-Rs act as a coincidence detector; two contacting cells have to be simultaneously activated to produce transmission of a signal. Such activation of NMDA-Rs leads to Na^{+} and Ca^{2+} entry into the postsynaptic terminals. Although the activation of the NMDA receptor requires the binding of both glutamate and glycine, only glutamate has to be released, while ambient glycine appears to be present continuously in the synaptic cleft from low up to saturating NMDA-R concentrations. Sustained NMDA-Rs activation promotes signaling, which activates multiple genes and leads to long-term synaptic plasticity, learning and memory. However, overstimulation of glutamate receptors results in excitotoxic action of intracellular Ca^{2+} leading to degeneration and apoptosis of neuronal tissue, as known in various CNS disorders.

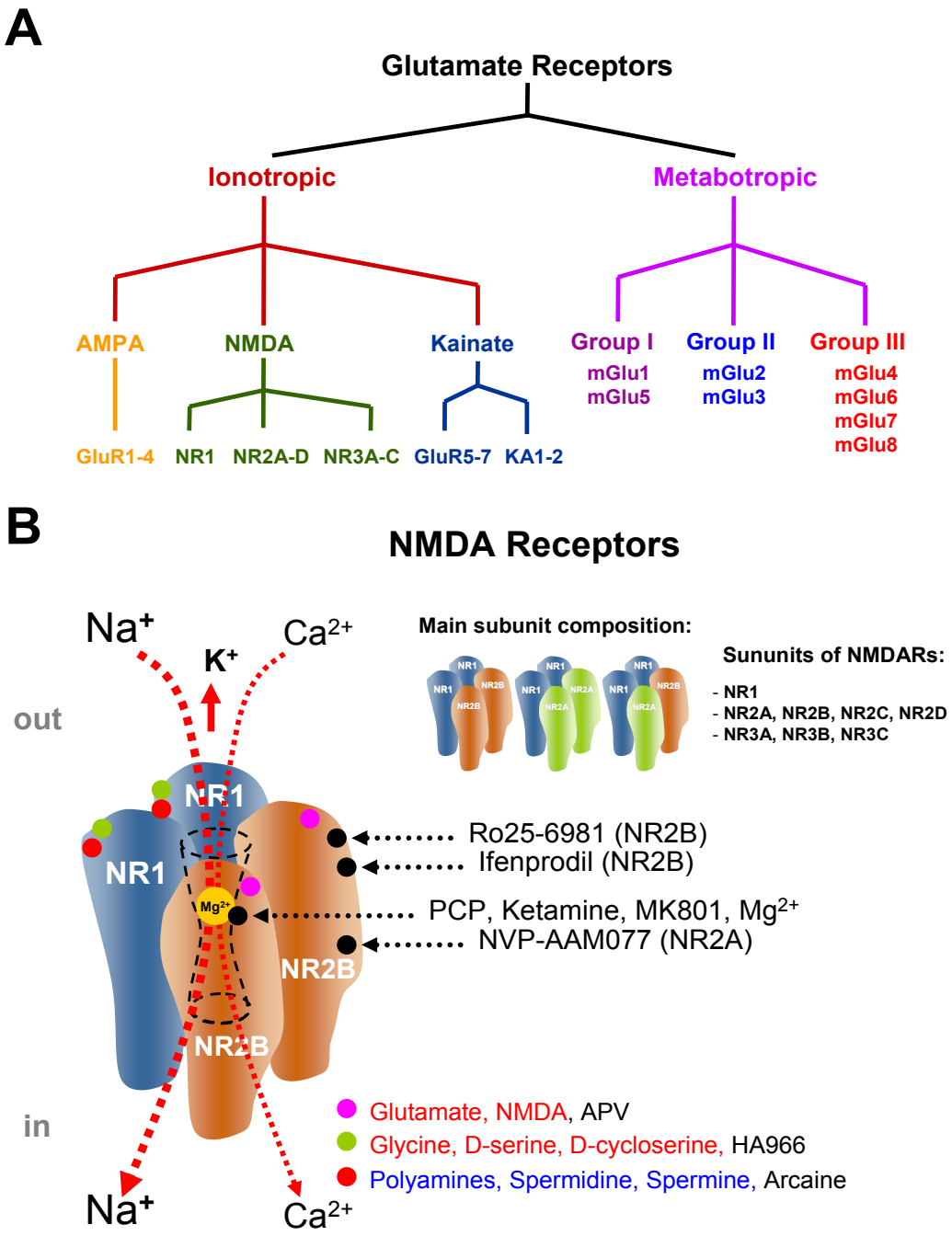

Fig. 1-III. A diagram of the glutamate receptor family (A) and a scheme of NMDA receptors with the binding sites showing where some of their pharmacological modulators act, and their main subunit composition (B). For the "B" scheme, agonists or co-agonists are given in red, modulators – in blue, and antagonists or blockers – in black.

NMDA-Rs are hetero-tetramers, composed of two obligatory NR1 subunits in combination with either two different or the same NR2 (A, B, C, D) or NR3 (A, B, C) subunits (see Fig. 1-III, A). Various heteromeric NMDA-Rs formed by combinations of NR1 and NR2 subunits are known to differ in gating properties, Mg^{2+} sensitivity, and pharmacological profile. For instance, NR1/NR2C complex has lower sensitivity to

Mg^{2+} but increased sensitivity to glycine and very restricted distribution in the brain. Another example, NR1/NR2A receptors are most ubiquitously distributed in the adult brain; with the highest density occurring in the hippocampus, whereas NR1/NR2B combination is maximally expressed in neonatal animals, and thereafter its levels in the brain decline. However, the NR1/NR2B complex is still highly expressed in adults, predominantly in forebrain and in the hippocampus, e.g. in the granule cells of the dentate gyrus, but not in cerebellum, where NR1/NR2C dominates. The NR1/NR2D containing receptors are mainly localized in the brainstem (Moriyoshi et al., 1991; Monyer et al., 1992).

Agonists of the NMDA-Rs glycine site (D-cycloserine, D-serine and glycine) and their effects on synaptic plasticity and cognitive functions

Since the finding by Johnson and Ascher (1987) demonstrating that glycine enhances electrophysiological responses mediated by NMDA, considerable interest has been devoted to this topic. Glycine is a nonpolar amino acid (see Fig. 2-III) which can act either as an inhibitory neurotransmitter in CNS, especially in the spinal cord, through its strychnine-sensitive receptor associated with Cl^- channels or as a co-

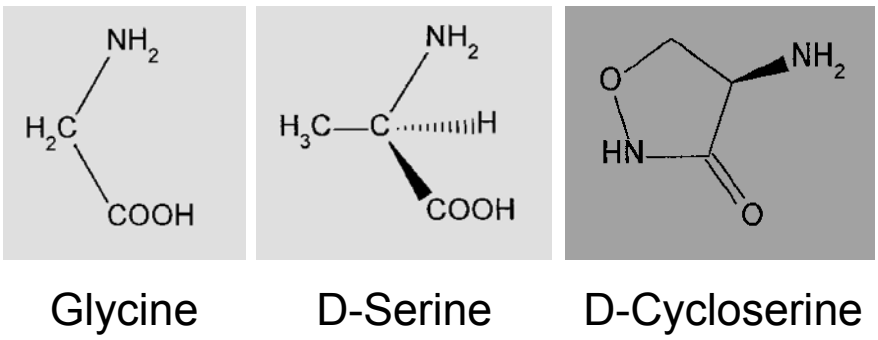

Fig. 2-III. Chemical formulas of the NMDA-Rs glycine site agonists: Glycine, D-serine and D-cycloserine.

agonist of excitatory NMDA-Rs which curry a strychnine-insensitive glycine binding site on NR1 subunits (see Fig. 1-III, B). Glycine site agonists may only increase NMDA-Rs function to the physiological maximum having a greater safety margin, whereas NMDA site agonists exhibit considerable toxicity. Among other modulators of NMDA-Rs at the glycine site is D-serine (see Fig. 2-III), that is mostly secreted by astrocytes in the brain and can facilitate hippocampal CA1 LTP (Yang et al., 2003). Its derivative, D-cycloserine (DCS) is another glycine sit partial agonist (see Fig. 2-III).

DCS has been used in many behavioural studies since it interacts only with strychnine insensitive glycine binding site associated with the NMDA receptor complex (Hood et al., 1989) and has good bioavailability in humans by freely crossing the blood-brain barrier. In mice and rats, only about 20% of DCS cross the barrier (Wlaz et al., 1994; Baran et al., 1995). The half-life of DCS in the mouse is approximately 23 min (Conzelman and Jones, 1956), indicating that the drug is rapidly cleared. DCS has a favourable toxicity profile; the dose as high as up to 320 mg/kg of body weight has no side effects in mice and rats (Millan at al., 1994; Baran et al., 1994). DCS has been reported to have anxiolytic properties in rat and mouse anxiety models; i.p. injections of DCS at doses of 30 – 300 mg/kg could block the fear-potentiated acoustic startle effect in rats (Anthony et al., 1993), whereas only the dose 1 mg/kg but not 5 mg/kg exhibited anxiolytic properties in mice subjected to passive avoidance test (Faiman et al., 1994). DCS may act as a proconvulsant or an anticonvulsant, since it can work as an agonist in some animal models and as an antagonist of the same glycine site in others, depending on its concentration. For example, if endogenous glycine concentration within the particular synaptic cleft is enough to saturate the NMDA-Rs glycine binding sites, then DCS could act as a glycine site antagonist because of its lower intrinsic activity then glycine. However, if the synaptic glycine concentration is at sub-saturating levels, then DCS may act as a glycine site agonist (Parsons and Danysz, 1998).

The cognitive-enhancing (nootropic) effects of DCS have been known for some time. For example, a dose between 0.3 – 10 mg/kg could facilitate retention of memory in passive avoidance test in rats (Monahan et al., 1989), doses 10, 20 and 80 mg/kg injected immediately following training or 3 mg/kg administered 20 min before acquisition improved spatial learning of mice in a thirst-motivated linear maze (Quartermain et al., 1994), and in a foot-shock avoidance task in mice injected s.c. with 20 mg/kg, but not with doses less than 5 mg/kg or greater than 40 mg/kg (Flood et al., 1992).

DCS is also known as a potent rescue agent in some cognitive deficit models. For example, deficits in working memory in rats after bilateral quinolinic acid-induced hippocampal lesions could be reversed by 12 mg/kg DCS injected 30 min prior to testing in a radial arm maze (Schuster and Schmidt, 1992). Deficits in spatial memory caused by scopolamine injection, which blocks the muscarinic cholinergic input into the hippocampus, could be also rescued via intraperitoneal injection of DCS at doses

0.3 – 30 mg/kg (Fishkin et al., 1993; Sirvio et al., 1992), or via acute injection into the rat dorsal hippocampus (Ohno and Watanabe, 1996).

Several studies have shown that facilitation of NMDA-Rs function via their glycine site can modulate synaptic plasticity measured as LTP and LTD. For example, DCS (100 μM) enhanced CA1 LTP in rat hippocampal slices, whereas its concentration (20 – 100 μM) in low-frequency stimulation protocol increased LTD (Rouaud and Billard, 2003). D-serine enhanced LTP in II/III layers of visual cortex of kitten brain slices, whereas 7-chlorokynurenate (7-ClKY) – a blocker of the glycine binding site of NMDA-Rs, inhibited HFS-induced LTP (Ito and Hicks, 2001). 7-CLKY depressed also CA1 LTP in rat hippocampal slices, whereas D-serine reversed this effect (Bashir et al., 1990). In another recent in vitro study (Krasteniakov et al., 2005), D-serine or glycine could enhance rat CA1 LTP evoked by pairing low-frequency afferent pulses with different levels of postsynaptic depolarization, whereas a blockade of the glycine site by its selective antagonist 7-ClKY during the pairing reversed the direction of plasticity from LTP towards long-term depression.

Interestingly, that DSC agonizes different subtypes of NMDA-Rs differently; e.g. on NR1/NR2A receptors it acts as less as 38% of 10μM of glycine activity, and NR1/NR2B tetramers have 56% of its intrinsic activity, whereas NR1/NR2C receptors have in opposite better activation through DCS (130%) than via endogenous glycine (O'Connor et al., 1996). Thus, the DCS affinity to the different NMDA-Rs subtypes could be drawn as following: NR1/NR2C > NR1/NR2D >> NR1/NR2B > NR1/NR2A.

NMDA-Rs and synaptic plasticity, learning and memory

Both, increase and decrease in synaptic efficiency, namely LTP and LTD, can be triggered by activation of NMDA-Rs in the CA1 area of the hippocampus (Bliss and Collingridge, 1993; Bear and Malenka, 1994). However, the plausible mechanism by which the activation of the same class of receptors can produce two opposing forms of synaptic plasticity remains debatable. The most common theory explaining this long puzzled conundrum was that the degree of NMDA-Rs activation, and hence, the level of postsynaptic Ca^{2+} signal, governs the directionality of NMDA-Rs-dependent synaptic modification. There were several studies proving this hypothesis; a partial blockade of NMDA-Rs with low concentration of NMDA-Rs agonist APV could turn out LTP to LTD (Nishiyama et al., 2000; Cummings et al., 1996). However, recently Liu et al. (2004) put forward the hypothesis that NMDA-Rs with different composition

may mediate different forms of synaptic plasticity: NR2A-containing NMDA-Rs are necessary for induction of LTP, whereas NR2B-containing NMDA-Rs are crucial for LTD.

NR2A-containing receptors are located almost exclusively within mature synapses, whereas NR2B-containing receptors are present extrasynaptically (Stocca and Vicini, 1998; Steigerwald et al., 2000). Additionally, the NR2A-containing receptors have lower affinity to glutamate (Kutsuwada et al., 1992), and therefore extra- and cross-synaptic spillover of glutamate is detected mostly by NR2B-containing receptors but not by NR2A ones (Dalby and Mody, 2003; Scimemi et al., 2004; Lozovaya et al., 2004). Recently, Massey et al., (2004) by using inhibitors of glutamate uptake, which enhanced glutamate spillover from the synaptic cleft to extrasynaptic parts, showed that activation of extrasynaptic NR2B-containing NMDA-Rs is crucially required for induction of LTD in the adult cortical slices, whereas intrasynaptic NR2A receptors are exclusively responsible for LTP only. Moreover, they additionally proved that two forms of synaptic depression, LTD and depotentiation, require the activation of different NR2-containing NMDA-Rs, NR2B and NR2A, respectively.

Despite the fact that both hippocampal and amygdaloid NMDA-Rs are required for acquisition and/or expression of fear conditioning (Morris et al., 1986; Young et al., 1994; Stiedl et al., 2000; Bast et al., 2003; Campeau et al, 1992; Fanselow and Kim, 1994; Maren et al., 1996; Lee and Kim, 1998), the role of different NMDA-Rs subunits in this learning model was not yet comprehensively investigated, particularly in the hippocampus. There are just several studies showing importance of the NR2B subunit in fear conditioning. Thus, Rodrigues et al. (2001) - using intra-amygdala infusion of the selective NR2B antagonist *ifenprodil* - could disrupt acquisition but not expression of both contextual and tone fear conditioning. Complementary data were provided by Tang and colleagues (1999), who demonstrated that transgenic mice named *Doogie*, which postnatally two-fold overexpress NR2B subunit in the forebrain, including the hippocampus, have enhanced many cognitive functions: contextual and cued memories in fear conditioning, performance in spatial navigation in Morris water maze and in a novel-object-recognition task; as well as increased hippocampal CA1 LTP but not LTD.

The recombinant NR1/NR2B complex in vitro shows longer excitatory postsynaptic potentials (EPSPs) than does the NR1/NR2A complex (Monyer et al.,

1994). This means that in vivo NR2B-containing NMDA-Rs have an increased time-window for detecting synaptic coincidence. With age, NR2B expression is downregulated, correlating with the gradual shortening of the EPSP duration of NMDA channels (Carmignoto and Vicini, 1992; Hestrin, 1992). This could decrease NMDA-mediated plasticity, and perhaps explain decreased memory performance in the course of lifetime.

NCAM and NMDA-Rs

NCAM and glutamate receptors are long thought to be functionally linked together. A series of the first observations highlighting significance of NCAM in glutamatergic synaptic plasticity and learning and memory were studies published in the mid 90th by several independent groups. It has been shown that either disruption of NCAM functions via applied antibodies against NCAM or genetic ablation of NCAM expression blocks NMDA-dependent long-term potentiation (LTP) in the CA1 region of the hippocampus (Lüthi et al., 1994; Rönn et al., 1995; Muller et al., 1996). The same manipulations with NCAM lead to deficits in NMDA-R-dependent spatial learning in the water maze (Arami et al., 1996; Cremer et al., 1994). Thus, it has been proven for the first time that NCAM is required for NMDA-R-dependent LTP and spatial learning.

Another link between NCAM and NMDA-Rs was established by a study showing that both the postsynaptically expressed largest isoform of NCAM, NCAM-180, and the NMDA-Rs subunit NR2A, are co-localized and co-distributed in spine synapses after induction the dentate gyrus LTP in vivo from the centre of the postsynaptic density to its edges, whereas the AMPA-Rs subunit GluR2/3 and the metabotropic glutamate receptor mGluR1 behaved more independently from NCAM (Fux et al., 2003).

Moreover, it has been shown that induction of LTP in CA1 region of the hippocampus resulted in NMDA-dependent increase in both NCAM mRNA and NCAM protein expression. The number of NCAM mRNA positively labeled neurons no longer could change 1 h after tetanus; however, the NCAM protein level at 1 h after tetanus was higher than that at 10 min after tetanus (Hu et al., 2004). A brief stimulation of NMDA-Rs with NMDA in hippocampal slice cultures resulted in a delayed (2-3 h after 30 sec application of NMDA) increase in the NCAM-180 isoform, but not in NCAM-140. Interestingly, similar selectivity was evident with prolonged

infusions of NMDA where, in contrast to the effect of brief stimulation, NCAM-180 content was reduced to 50% while levels of NCAM-140 were unchanged (Hoffman et al., 2001).

Furthermore, Muller and colleagues (1996) clearly showed that expression of PSA-NCAM in organotypic cultures occurs in activity-dependent manner. Interestingly, in another study (Bouzioukh et al., 2001) regulation of PSA-NCAM expression was found to be bidirectional: electrical stimulation of the dorsal vagal-complex afferent fibers caused a rapid NMDA-R-dependent increase of PSA-NCAM expression both in vivo and in acute slices before postnatal day (P) 14, whereas a similar stimulation induced a decrease after P15. Also chronic NMDA blockade induced a dramatic decrease in PSA-NCAM expression.

It is noteworthy to mention that neuronal activity appeared to regulate not only expression of PSA-NCAM protein, but also its cleavage. Thus, Bliss group demonstrated that 90 minutes after the induction of LTP in vivo, the concentration of soluble extracellular NCAM was increased in the dentate gyrus of the hippocampus in a NMDA-R-dependent manner (Fazeli et al., 1994).

Since NCAM can be expressed on both sites of a synapse, it is important to know whether pre- or postsynaptically expressed NCAM is involved in regulation of glutamate receptor-dependent synaptic plasticity. A study conducted by Dityatev and co-workers (2000), using homo- and heterogenotypic combinations of cultured dissociated hippocampal neurons from NCAM-deficient and wild-type mice combined with double-cell patch clamp recordings from synaptically coupled cells showed that: (i) synaptic strength of excitatory but not inhibitory synapses depends on expression of NCAM post- but not presynaptically; (ii) potentiation of EPSCs and synaptic coverage of NCAM-expressing neurons is higher compared to NCAM-deficient neurons in heterogenotypic cocultures; (iii) glutamate receptors are involved in NCAM-dependent target selection of growing neurites, since their preference to establish synaptic contacts with cells expressing NCAM versus not expressing NCAM is abolished by simultaneous inhibition of AMPA and NMDA glutamate receptors. A follow-up study revealed that synaptogenesis activity of NCAM required attaching of PSA and that PSA-NCAM-driven synaptogenesis was blocked by a specific antagonist of NMDA subtype of glutamate receptors but not by blockers of non-NMDA glutamate receptors and voltage-dependent Na^+ channels. Enzymatic removal of PSA also blocked the increase in the number of perforated spine synapses

associated with NMDA receptor-dependent LTP in the CA1 region of organotypic hippocampal cultures (Dityatev et al., 2004).

Importance of postsynaptic NCAM, which is mainly NCAM-180 isoform, in NMDA-dependent synaptic plasticity was highlighted in another in vitro study using a different approach: impaired CA1 LTP in NCAM deficient mice could be restored via genetic reintroduction of NCAM-180 isoform into NCAM knockout mice. However, transgenic expression of NCAM-180 could not rescue contextual and cued learning and memory deficits of these mutants in fear conditioning paradigm (Stork et al., 2000). Possibly, it is related to the fact that levels of transgenic NCAM-180 expression were lower in such important for fear conditioning brain areas as like the hippocampus, amygdala and neocortex. Another reason could be a lack of activity-dependent regulation in expression of transgenic NCAM-180, which was driven by neurofilament-L rather than NCAM promoter.

In summary, these data suggest that there is a tight relationship in functioning of both PSA-NCAM and NMDA receptors during different synaptic plasticity events.

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AIM OF THE STUDY

On the basis of the numerous data discribed in the introduction, our prime hypothesis was that a deficit in NCAM may lead in a deficit in NMDA-R signaling. Thus, the aim of this study was to check whether facilitation of NMDA-Rs function may rescue cognitive deficits in NCAM knockout mice. To reach this goal we chose a fear conditioning paradigm, a well established in our laboratory learning model, and D-cycloserine, a partial agonist of glycine binding site of NMDA-Rs, as a stimulator of NMDA receptors signaling.

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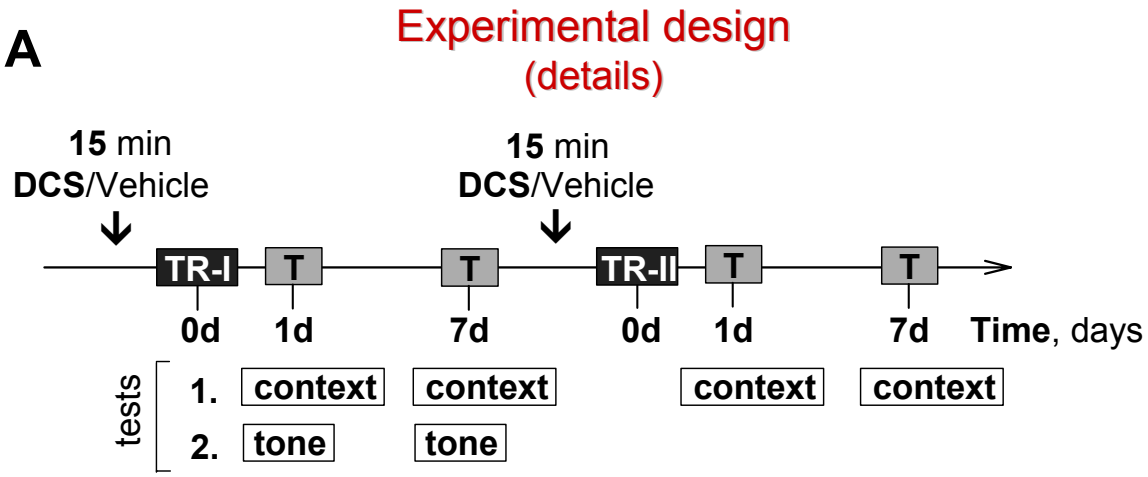
MATERIALS & METHODS

Mice

Constitutive NCAM deficient mice (12 NCAM+/+, 24 NCAM-/-) were bred in the ZMNH animal facility. For behavioural experiments, 3- to 5-month-old males were used. The ages of NCAM+/+ mice match those of NCAM-/- mice. At least 1 week before starting experiments, mice were transferred to a small vivarium in the Institute for Neurophysiology and Pathophysiology, UKE, where they were housed individually with food and water ad libitum on a reversed 12h:12h light/dark cycle (light on at 11:00 pm) under constant temperature (22 ± 1°C) and humidity conditions (55 ± 5%). All behavioural experiments were conducted during the dark part of the cycle when mice are active. All treatments and behavioural procedures were approved by the Committee on Animal Health and Care of the local governmental body.

Fear conditioning

In this study, for the first time, we introduced a new design of fear conditioning paradigm – double repetitive fear conditioning (see Fig. 3-III, A) – in which all mice were subjected to two consecutive trainings spaced apart in approximately one week. The first fear conditioning was an auditory fear conditioning in which both contextual and tone memories were assessed; in the second fear conditioning only a context was associated with a footshock in order to move context from a background (as in the first training) to the foreground in respect to the training scene. The reason to do so was to analyze processing of memory firstly in a more complex situation, when an animal has to create associations of both context and tone with aversive stimulus simultaneously and then in a simpler paradigm, when only a context has to be memorized as a predictor of aversive stimulus.



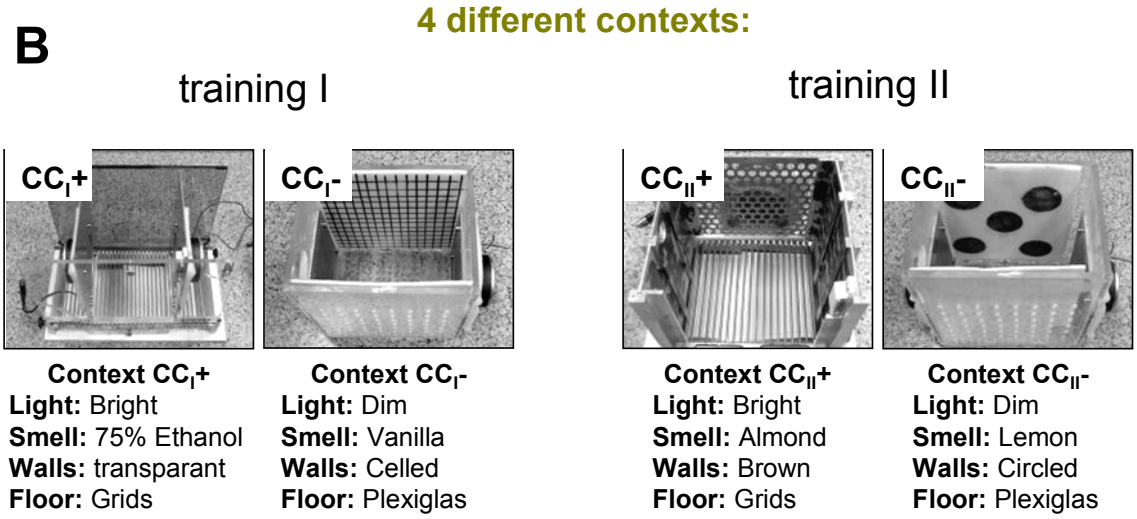


Fig. 3-III Detailed description of experimental design of repetitive fear conditioning. A. Time course of two consecutive trainings (TR-I, TR-II) and following tests at day 1 (d1) and day 7 (d7). Injections of either DCS or vehicle have been made exactly 15 min before each training. After the first auditory fear conditioning mice have been assigned for two tests, context and tone tests, whereas after the second fear conditioning mice were tested only for contextual memory. B. Description of four different contexts utilized in the repetitive fear conditioning; note, only contexts beard sign (+) when paired with US, whereas sign (-) means an opposite, unpaired.

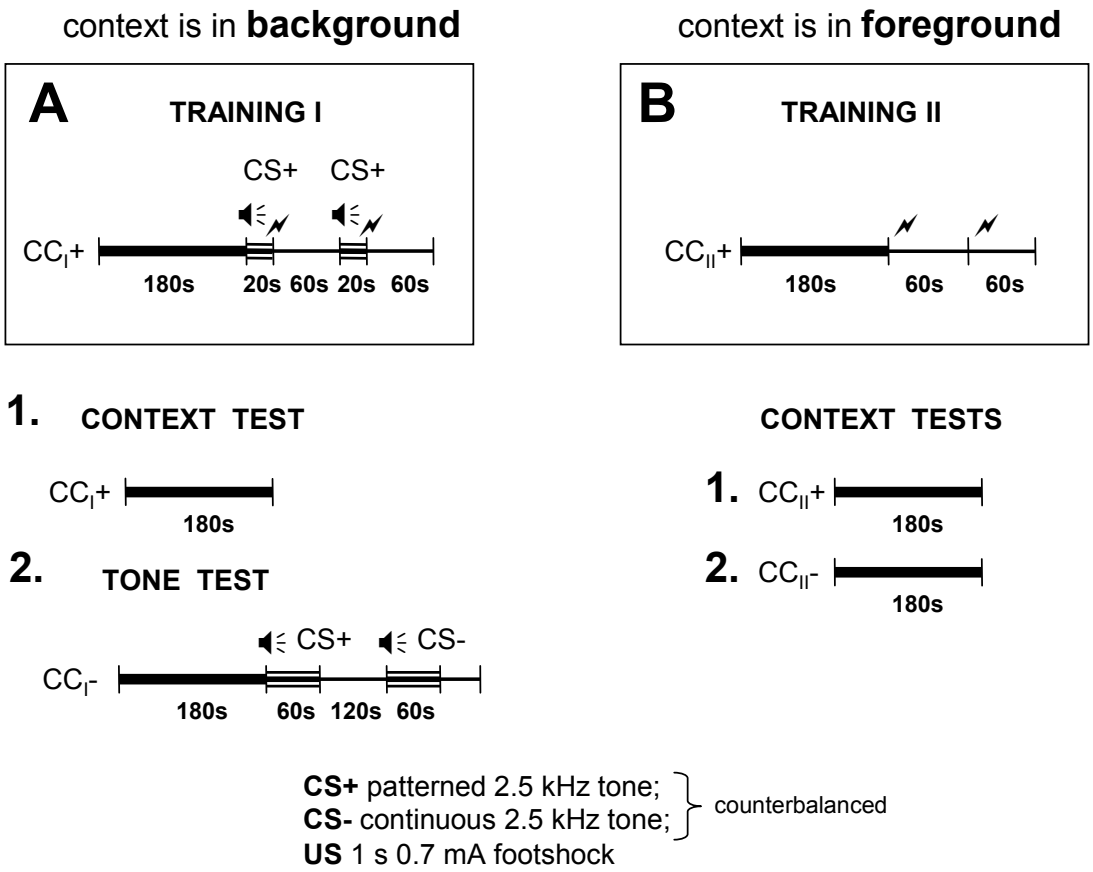


Fig. 4-III Detailed description of two training procedures in repetitive fear conditioning. A. During the first training a context was in background shadowed by two cued (tone) presentations. Two consecutive tests followed the training: 1. context test performed in condition chamber CC+ for 3 min; 2. tone test was performed in a neutral context CC- which served as a negative control for CC+, during which two 60 sec episodes of tones spaced apart for 120 sec have been presented. The first tone was CS+, paired tone, the second was neutral CS-. Both tones were counterbalanced during conditioning. B. The second training, when only particular context CC_{II}+ was associated with a footshock. This is so-called foreground contextual fear conditioning.

First training. First auditory fear conditioning was performed as following: (i) after two days of handling and habituation in a neutral context (which was a clean home cage) for 5 min each day, a naive mouse was intraperitoneally injected with either DCS or vehicle 15 min before training; (ii) then the mouse was placed in a conditioning chamber bearing context CC_I+ (see Fig. 3-III, B for description of context, and Fig. 4-III, A for temporal profile of conditioning) and was allowed to freely explore this context for 180 seconds. Freezing level during this time we took as a baseline ("B" in graphs); (iii) then the mouse was subjected to two pairings of short 20-sec episodes of CS+ (patterned tone of 2.5 kHz was presented to a half of mice, whereas another half received a continuous tone of the same frequency) co-terminated with a 1-s footshock of 0.7 mA as US. Between these two pairings and after the last one, there were 60-sec time-intervals, which are marked in graphs as "S1" and "S2", respectively. Freezing of mice during these intervals was used as a measure of pain sensitivity/response after footshocks; (iv) after completion of fear conditioning, the mouse was returned to its home cage.

Second training. Second contextual fear conditioning was performed as following: (i) after completion of all tests of the first fear conditioning the same mice underwent again two days of handling and habituation in a neutral context (which was a clean home cage) for 5 min each day, and at the day of conditioning, the mice again were intraperitoneally injected with either DCS or vehicle 15 min before training; (ii) then the mice were placed in a conditioning chamber bearing another new context CC_{II}+ (see Fig. 3-III, B and 4-III, B) and were allowed to freely explore this context for 180 seconds, freezing level during this time we took as a baseline ("B" in graphs); (iii) then the mice were subjected to two pairings of this context with a footshock with the same duration and intensity as in the first fear conditioning; between these two pairings and after the last one, there were 60-sec time intervals marked in graphs as "S1" and "S2", respectively, which we used again to measure pain sensitivity after each of footshock; (iv) after completion of fear conditioning, the mouse was returned to its home cage.

Tests after the first training. Tests after the first fear conditioning were as follows: (i) at the day of testing (d1 and d7 after fear conditioning) a mouse was placed into the same conditioning chamber as in the fear conditioning procedure bearing context

CC_I+ for 3 min to assess its retention of contextual memory and then it was returned to its home cage (see Fig 4-III, A); (ii) in approximately 2-3 hours the same mouse was placed in another new, unpaired context (CC_I-, see Fig. 3-III, B), a tone cage, and was allowed to freely explore this control context for 180 seconds. By this way we could measure discrimination between CC+ versus CC- and a level of generalization, as freezing in response to CC-; (iii) next, we applied the first 60 sec episode of CS+ (previously paired with US tone, either patterned or continuous) to assess tone memory; it followed by a 60-sec tone episode when unpaired CS- tone was presented (either patterned or continuous, but different from CS+) to the mouse after 120 sec resting time (see Fig. 4-III, A); (iv) a 60 sec after the last tone presentation the mouse was returned to its home cage.

Tests after the second training. Tests after the second fear conditioning were as follows: (i) at the day of testing (d1 and d7, see Fig. 4-III, B), a mouse was placed into the paired during the second training conditioned context CC_{II}+ (see Fig. 3-III, B) for 180 seconds to assess its retention of contextual memory; (ii) 2-3 hours later the same mouse was introduced into the control unpaired context CC_{II}- (see Fig. 3-III, B) for 180 sec to assess its ability to discriminate between paired and unpaired contexts, and its level of generalization.

All behaviour of mice during conditioning and tests was recorded on a computer Pentium-4, CPU 3 GHz, 2 GB RAM using the PixelSmart video frame-grabber (Pixelsmart, USA, http://www.pixelsmart.com) which digitized video streaming with 3 frames per second and stored it in the computer hard-drive as a set of 8-bit gray images. These images were then converted into avi-standart movies by a freely-available Java-based program ImageJ (http://rsb.info.nih.gov/ij). All recorded movies were analyzed using Microsoft Windows Media Player by a trained observer in a blind fashion in regard to genotype and treatment of a tested mouse.

Drugs & injection

To test whether facilitation of NMDA-Rs function can improve cognitive deficits in contextual and tone memories of NCAM knockout mice, we decided to use a well-known partial agonist of glycine binding site of NMDA-Rs, D-cycloserine (Richardson et al., 2004). D-cycloserine (DCS, Sigma-Aldrich, Deisenhofen, Germany) was dissolved freshly before usage in 0.9% isotonic NaCl solution and injected through

i.p. route at three different concentrations (3, 10 and 20 mg/kg of b.w.) exactly 15 minutes before training procedure. This particular time was taken since the half-life of DCS in mouse blood system is about 23 min (Conzelman and Jones, 1956). A control group of animals were injected just with 0.9% NaCl solution (vehicle group) in the same manner as the DCS groups. Since fear conditioning is very sensitive to stress, especially when it occurs prior to training, a special care has been taken to avoid harmful manipulations like an intraperitoneal injection procedure; all mice were slightly sedated with a mixture of CO_2 gas and room air for a period of injection, approximately 1-2 min. Recovery after such anaesthesia is very fast (2-3 min) and without any side effects on following fear conditioning.

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RESULTS

Training performance

First training. During the first fear conditioning, two control groups (vehicle-injected NCAM+/+ and NCAM-/- mice) and three DCS-injected groups of NCAM-/- mice showed equal freezing responses during either training episodes (see Fig. 5-IIIB), including 180-s baseline (B), two 20-s presentations of CS+ and two 60-s time intervals (S1 and S2) following each footshock. Two-way repetitive ANOVA did not reveal any significant effect of *Group* ($F_{4.33}$=0.29, p=0.87) or any interaction between *Episodes x Group* ($F_{16.132}$=0.40, p=0.98). However, the effect of training was significant: freezing response to unpaired-yet CS+ before the first footshock was significantly lower than freezing response to CS+ before the second footshock (*Training:* $F_{1.33}$=56.45, p<0.001), indicating that CS+ was memorized after the first footshock, serving like a predictor of danger. This training was equally well performed by all tested groups, since their freezing responses to CS+ during training were not different from each other (*CS+ x Group:* $F_{4.33}$=0.52, p=0.72) independently of injection of DCS or genotype. Immediate post-shock reactions (episodes S1, S2) after the first and the second US, were also equal among all tested groups (*S x Group:* $F_{4.33}$=0.45, p=0.77), suggesting that the all compared groups had similar pain sensitivity to US. However, the freezing time after the second US was significantly longer than after the first US (*US Number:* $F_{1.33}$=158.14, p<0.001), demonstrating that freezing is a function of number of USs in this paradigm.

Second training. During the second fear conditioning, the compared groups (see Fig. 5-IIIB) showed similar reactions at 180-s baseline interval (B) preceding the first footshock (*Baseline:* $F_{4.32}$=0.32, p=0.85) and two 60-s S-episodes following footshocks (*Group:* $F_{4.32}$=0.49, p=0.73; *Episodes x Group:* $F_{8.64}$=0.84, p=0.57), despite either genotype or pretraining injections. However, the second immediate post-shock response (S2) was significantly stronger than the first one (S1) (*US Number:* $F_{1.32}$=59.51, p<0.001), again showing that freezing is a function of number of USs.

Comparison in performance between the first training with the second. Interestingly that these two trainings did not differ between each other, at least according to freezing time measured during the two immediate post-shock responses (S1 and S2) (*Training:* $F_{1.32}$=2.83, *p*=0.102) and within each group (*Training x Group:* $F_{4.32}$=0.34, p=0.84), suggesting that "S" is indeed a parameter reflecting immediate post-shock reaction during training, but not some experience-related function, since the mice showed similar S-responses regardless of the number of trainings. However freezing responses during the 180-s baseline (B) episode of the second training were slightly but significantly higher (*Baseline:* $F_{1.32}$=41.02, *p*<0.001) than during the corresponding B-episode of the first training, although there was not any difference between compared groups (*Baseline x Group:* $F_{4.32}$=0.84, p=0.5).

Thus, neither pre-training injection (DCS versus vehicle) nor genotype (NCAM+/+ versus NCAM-/-) changed ability of mice to perceive conditioned and unconditioned stimuli during two repetitive training sessions.

Dose-dependent effects of D-cycloserine on contextual memory in NCAM deficient mice

First training. Repetitive two-way ANOVA analysis revealed a significant effect of genotype between two control groups - NCAM+/+, NCAM-/-, both injected with vehicle (*Veh*) - in freezing responses to conditioned context (CC+) after the first fear conditioning (considering together data collected on the first and seventh test days; d1 and d7 respectively in the Fig. 5-IIIC) (*Genotype:* $F_{1.13}$=11.11, *p*=0.005), and this effect was stable over two test days (*Day/Trail x Genotype:* $F_{1.13}$=0.77, *p*=0.39).

Between all groups of NCAM-/- mice injected with different doses of DCS and *Veh* (here, the dose of DCS=0), repetitive two-way ANOVA found a highly significant effect of dose (*Dose:* $F_{3.26}$=9.31, *p*<0.001), which is persistent over the course of trials (*Day/Trail x Dose:* $F_{3.26}$=1.04, *p*=0.39). Fisher's LSD post-hoc analysis of contextual memory on 1st test day revealed that NCAM-/- mice injected with 3 mg/kg DCS did not differ from NCAM-/- mice injected with *Veh*. However, responses of NCAM-/- mice injected with 10 mg/kg and 20 mg/kg DCS were significantly different (*p*<0.05 and *p*<0.01, respectively) from NCAM-/- mice injected with *Veh*. Moreover, their levels of freezing were not different in comparison to control NCAM+/+ mice injected with Veh, confirming that either of the two DCS concentrations, 10 or 20 mg/kg, was sufficient to normalize contextual memory in NCAM deficient mice.

On the seventh test day, NCAM deficient mice injected with Veh still showed significant impairment of contextual memory compared to NCAM+/+ mice (p<0.005, *post hoc* LSD test). The effect of DCS was significant only at the dose of 20 mg/kg (p<0.01, *post hoc* LSD test), but not at 3 or 10 mg/kg.

Freezing responses of two control groups - NCAM+/+ and NCAM-/-, both injected with *Veh*, were also different (*Day/Trail x Genotype:* $F_{1.13}$=17.39, p=0.001, see Fig. 5-IIIC) in the neutral context that was not paired to a footshock (CC-) on 7th test day (p<0.001, *post hoc* LSD test), but not on 1st day. The freezing responses in this unpaired context were also different in all NCAM-/- groups injected with different concentration of DCS (*Day/Trail x Dose:* $F_{3.26}$=4.34, p=0.013). *Post hoc* LSD analysis revealed that the only two groups, 10 and 20 mg/kg of DCS, were significantly different (p<0.05 both) from NCAM-/- mice injected with *Veh* on the 1st test day, and none of the tested NCAM -/- groups were different on 7th day.

Qualitatively, all five groups showed a similar significant difference in freezing in paired CC+ context versus unpaired CC- context (p<0.05 for all trials of the first training, Wilcoxon nonparametric signed rank test, further, Wilcoxon). However, if the discrimination of contexts was quantified as a difference between freezing values in CC+ and CC- and then subjected to repetitive ANOVA test followed by LSD *post hoc* analysis, some differences in discrimination were detected. Thus, between two genotypes, NCAM+/+ and NCAM-/- injected with Veh, there was a large difference in ability to discriminate CC+ versus CC- (*Genotype:* $F_{1.13}$=9.03, p=0.01), and this effect was dependent on the test day (*Day/Trail x Genotype:* $F_{1.13}$=5.10, p=0.04; p<0.005 for the 1st day, and p>0.05 for the 7th day, *post hoc* LSD test). For the four NCAM-/- groups injected with different DCS concentrations and *Veh*, the discrimination was also highly dependent on a dose of DCS (*Dose:* $F_{3.26}$=13.90, p<0.001) and this dependency was rather stable over the trial course (*Day/Trail x Dose:* $F_{3.26}$=2.86, p=0.056). Fisher's *post hoc* LSD analysis revealed that the discrimination was significantly higher in two groups of mice injected with 10 and 20 mg/kg DCS (p<0.05 and p<0.005, respectively), but not different from mice injected with 3 mg/kg DCS, as compared to NCAM-/- Veh group on the 1st test day. On the 7th day, only one NCAM-/- group injected with highest dose (20 mg/kg) of DCS had significant (p<0.01) improvement of the discrimination.

Second training. After the second training (see Fig. 5-IIIC), the effect of genotype was highly significant: NCAM-/- and NCAM+/+ mice injected with *Veh* showed a big difference in responses to conditioned context CC+ (*Genotype:* $F_{1.12}$=22.23, p<0.001, ANOVA), and this difference was stable over the trials (*Day/Trail x Genotype:* $F_{1.12}$=3.17, p=0.1). Among NCAM-/- mice injected with different DCS concentrations, including *Veh* group, there was a strong effect of dose (*Dose:* $F_{3.25}$=8.44, p<0.001), and this effect was stable over trials (*Day/Trail x Genotype:* $F_{3.25}$=0.61, p=0.61). *Post hoc* Fisher's LSD test revealed that freezing responses in all NCAM-/- DCS injected groups (3, 10 and 20 mg/kg) were significantly higher than in control NCAM-/- *Veh* injected mice (p<0.05, p<0.001, p<0.001, respectively) on 1st test day after the second fear conditioning. The doses 10 and 20 mg/kg of DCS could completely recover contextual memory deficits in NCAM-/- mice since there were no differences between freezing responses of these two DCS injected groups of mice and responses of the control NCAM+/+ *Veh* injected group.

On the 7th day after the second training all mice showed similar responses on exposure to conditioned context CC+ as on the 1st day. DCS at 10 and 20 mg/kg had the strongest effects on contextual memory: *post hoc* Fisher's LSD analysis revealed that freezing responses in these two groups were significantly higher of those in control NCAM-/- *Veh* group (p<0.01 for both) and did not differ from responses of the NCAM+/+ *Veh* group, proving that recovery of freezing in NCAM-/- mice by DCS was complete. In contrast to the 1st test day, DCS at 3 mg/kg had no significant effects on the 7th day (*post hoc* LSD test).

Interestingly, freezing responses to unpaired context CC- after the second training were slightly different from those after the first one. The effect of genotype in NCAM-/- and NCAM+/+ *Veh* injected groups was highly significant (*Genotype:* $F_{1.12}$=11.13, p=0.006) and this effect was very stable over the trials *(Day/Trail x Genotype:* $F_{1.12}$=0.05, p=0.81). *Post hoc* Fisher's LSD analysis found that freezing during exposure in CC- context of NCAM+/+ *Veh* mice was higher on 1st and 7th testing days (p<0.05 and p<0.005, respectively) than in NCAM-/- *Veh* injected group. Among all DCS injected groups (3, 10 and 20 mg/kg plus *Veh*) the responses to this unpaired CC- context were also dose-dependent (*Dose:* $F_{3.25}$=5.4, p=0.005) and trial-independent (*Day/Trail x Genotype:* $F_{3.25}$=0.86, p=0.47). *Post hoc* Fisher's LSD analysis found that significantly differed responses of mice injected with DCS at 3 and 20 mg/kg (p<0.01 and p<0.05, respectively) on the 1st and 7th test days

compared to NCAM-/- *Veh* group. Freezing of 10 mg/kg DCS group during exposure in CC- context did not differ from the aforementioned control.

Discrimination between paired context and unpaired was again quite different of that after the first fear conditioning. First of all, in contrast to the first training, NCAM-/- *Veh* mice lost their ability to distinguish between CC+ and CC- contexts on either day after second training (NS, Wilcoxon test). However, DCS injected NCAM-/- groups and the NCAM+/+ mice showed significant discrimination (p<0.05, Wilcoxon test). As before, we also quantified discrimination as a difference between freezing values in CC+ and CC- and found that there was a strong effect of genotype on discrimination that was higher in NCAM+/+ than NCAM-/- *Veh* injected mice (*Genotype:* $F_{1.12}$=8.49, p=0.013; *Day/Trail x Genotype:* $F_{1.12}$=1.66, p=0.22). *Post hoc* Fisher's LSD analysis found that the difference between genotypes reached significance for the 1st day (p<0.05) but not for the 7th day. Among all DCS injected groups and the NCAM-/- *Veh* group, the discrimination was again dose-dependent (*Dose:* $F_{3.25}$=13.15, p<0.001) and time-independent (*Day/Trail x Dose:* $F_{3.25}$=1.37, p=0.27,). *Post hoc* Fisher's LSD test proved that all DCS injected groups had highly significantly (p<0.001) better discrimination, as compared to the NCAM-/- *Veh* injected mice on the 1st test day. On the 7th day, only mice injected with DCS at 10 and 20 mg/kg, but not at 3 mg/kg, showed better discrimination than NCAM-/- *Veh* controls (p<0.01).

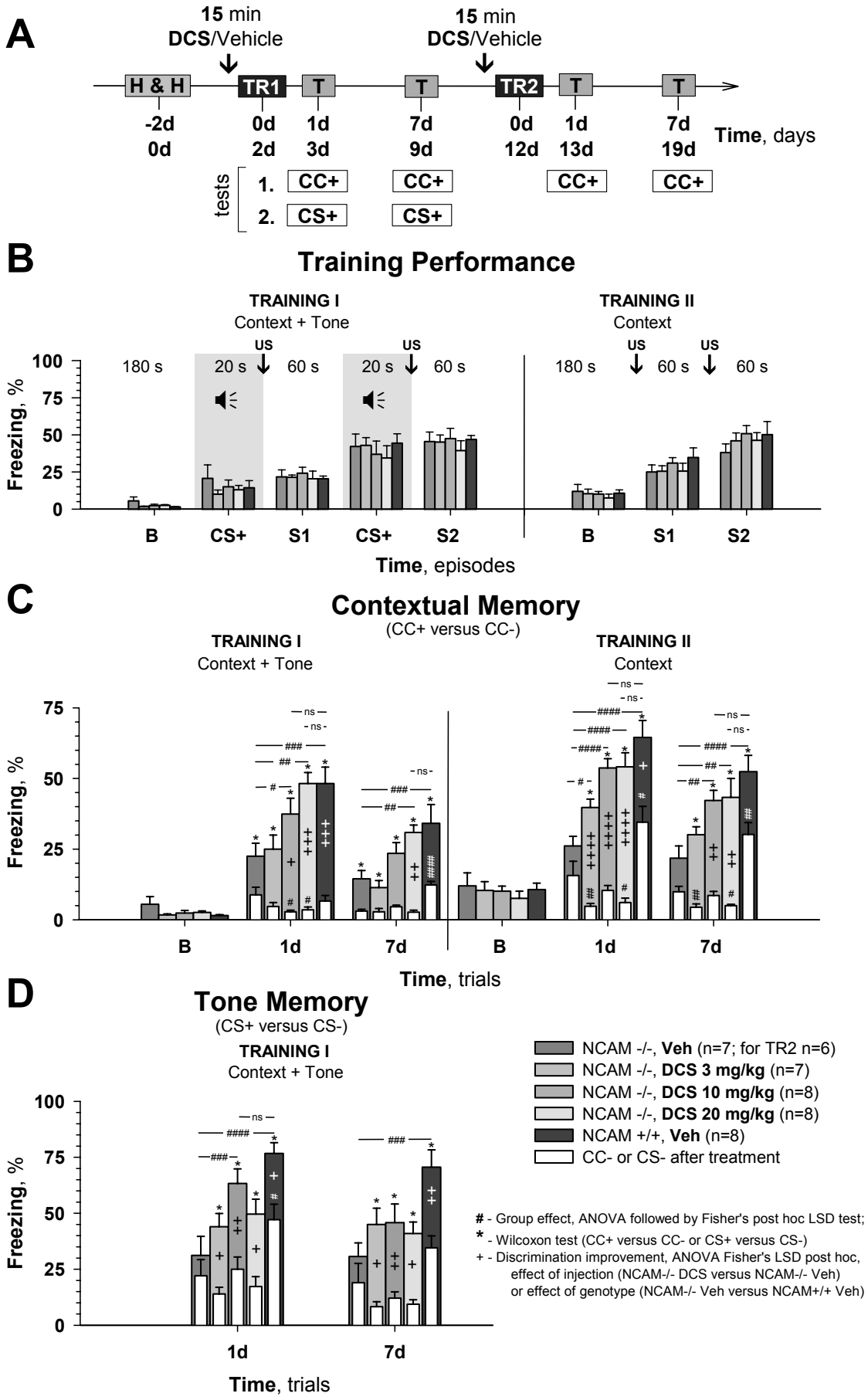
Comparison in contextual memory between the first and second trainings. After the second training there were many similarities in retention of contextual memory by all tested mice over the week of tests, however, there were and many differences, in comparison to the first training. For example, NCAM-/- mice injected with Veh after the second training showed no further improvement in the task, in opposite, if learning can be quantified as an increase in conditioned response compared to its pre-training value, then NCAM deficient mice absolutely did not acquire the task after second conditioning, since their levels of freezing on conditioned context (CC+) over the test days were similar to the pre-training value (*Learning:* $F_{2.12}$=2.09, p=0.16). However, for the first training, although NCAM knockouts exhibited impaired retention of contextual memory compared to wild-types, their learning was significant (*Learning:* $F_{2.12}$=23.32, p<0.001) for the 1st and 7th days compared to the baseline value (p<0.05, Wilcoxon test). Also, as it has been mentioned before, discrimination

of two contexts after the second training in NCAM deficient mice was significantly worse than after the first training for both test days.

Interestingly, more freezing was exhibited by wild-types in paired CC+ context before, and after second conditioning on both test days, as compared to corresponding values during the first training (p<0.05, Wilcoxon test). There was also greatly increased freezing of these mice in unpaired CC- context on both test days (p<0.05, Wilcoxon test). All these observations suggest that the second training may lead to an increase in some unspecific to learning components, like nonassociative generalization, in both genotypes. However, surprisingly, NCAM -/- mice of either DCS injected group after the second training had discrimination (as difference CC- from CC+) of two contexts even better than wild-types (*Group:* $F_{3.27}$=5.31, p=0.005). *Post hoc* Fisher's LSD analysis revealed that it was true for 10 and 20 mg/kg DCS groups (p<0.05 and p<0.005, respectively) on the 1st test day, and only for the 20 mg/kg of DCS group (p<0.01) on the 7th test day.

Thus, all the data mentioned above prove that DCS dose-dependently rescued formation of associative contextual learning in NCAM deficient mice measured in short- and long-term time scales (1st and 7th days, respectively) and improved discrimination of paired versus unpaired contexts.

Fig. 5-III Results of two repetitive fear conditioning and following retention tests after pre-training injection of DCS with increased concentration.
A – A time course of behavioural experiments; two consecutive trainings spaced apart about a week; CC+ contextual test, CS+ cued test, H & H handling and habituation. B – Training performance during two conditioning; one – auditory fear conditioning, another – contextual. C – Contextual memory of mice after two trainings. D – Tone memory after the first training.



← Fig. 5-III (the figure legend is on the previous page)

Dose-dependent rescue effect of D-cycloserine on cued memory in NCAM deficient mice

As shown in Fig. 5-IIID, NCAM-/- mice injected with Veh had also a deficit in retrieval of cued (tone) memory over the course of tests; repetitive two-way ANOVA found a highly significant difference between NCAM-/- and NCAM+/+ mice in freezing responses to CS+ (*Genotype:* $F_{1.13}$=20.87, p<0.001), which was trial-independent (*Day/Trail x Genotype:* $F_{1.13}$=1.37, p=0.26). However, injection of DCS before training significantly facilitated formation of cued memory and thus its consecutive retrieval at least on the 1st test day. Although repetitive two-way ANOVA detected only a tendency for a dose dependent effect of DCS on CS+ responses in NCAM-/- mice for the whole test (*Dose:* $F_{3.26}$=2.34, p=0.096), one-way ANOVA followed by post hoc Fisher's LSD revealed it for the 1st day (*Dose:* $F_{3.26}$=4.05, p=0.017). DCS significantly increased freezing during CS+ presentation for the dose of 10 mg/kg (p<0.005), fully recovering tone memory on this day compared to wild-type controls, but not for other DCS concentrations, as compared to the NCAM-/- Veh injected group (see Fig. 5-IIID).

Responses of the DCS injected NCAM-/- mice to unpaired CS- were dose-independent (*Dose:* $F_{3.26}$=0.91, p=0.44) over the course of tests (d1 and d7). Repetitive two-way ANOVA detected a tendency for NCAM-/- mice to have lower freezing time in CS- than NCAM+/+ Veh injected groups (*Genotype:* $F_{1.13}$=4.60, p=0.051) for the whole tests. It was trial-dependent (*Day/Trail x Genotype:* $F_{1.13}$=6.84, p=0.02). One-way ANOVA followed by *post hoc* Fisher's LSD test revealed that CS- response on the 1st day was significantly lower (p<0.05) in NCAM-/- *Veh* mice compared with control NCAM+/+ group.

Discrimination of CS+ versus CS- tones, measured as a difference in freezing times in response to CS+ and CS-, was highly genotype-dependent (*Genotype:* $F_{1.13}$=8.94, p=0.01) and trial-independent (*Day/Trail x Genotype:* $F_{1.13}$=1.2, p=0.29). *Post hoc* Fisher's LSD analysis found that it was significant for both 1st and 7th days (p<0.05, p<0.01, respectively). There was also a significant effect of DCS dose on the CS discrimination compared to NCAM-/- *Veh* injected group (*Dose:* $F_{3.26}$=3.74, p=0.023), which was consistent in time (*Day/Trail x Dose:* $F_{3.26}$=0.17, p=0.91). *Post hoc* Fisher's LSD revealed a significant effects of 3, 10 and 20 mg/kg of DCS for both test days (p<0.05; p<0.01; p<0.05, respectively) compared to NCAM-/- *Veh* injected mice. Discrimination of CSs measured as a difference by Wilcoxon test (indicated in

graphs by sign *, see Fig. 5-IIID) was detected (p<0.05) for all groups except for NCAM-/- mice injected with *Veh*.

Thus, we can conclude that pre-training injection of DCS dose-dependently facilitated formation and retrieval of cued memory in NCAM deficient mice and tone discrimination, which were completely rescued by 10 mg/kg DCS dose on the 1st test day. However, neither of the tested DCS doses could increase CS+ responses on 7th day, although discrimination was significantly normalized.

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DISCUSSION

Ablation of NCAM and cognitive deficits

As in previous experiments (see project I), NCAM-/- mice showed two-fold decrease in retention of contextual memory at the first test day following either first or second training. Additionally, we show here that contextual memory remains impaired in NCAM mutants also on the seventh day after training. In this study, for the first time we could show that deficits in fear learning and memory in NCAM knockout mice could not be overcome by getting familiar with the task or its simplification. There was no improvement in association of a context with US during the second training, even when the conditions of learning were significantly changed by removing the cued stimulus (tone) that may draw attention of mice away from the context. It is known that fear conditioning with context being in foreground facilitate retention of contextual information in comparison with situation when the context is in background.

Dose-dependent restoration of contextual memory in NCAM deficient mice by D-cycloserine

Since several published and ongoing studies indicate that there is a link between PSA-NCAM and NMDA-Rs, we used DCS as a partial agonist of the glycine site of NMDA-R to compensate a deficiency in NMDA-R activity in NCAM deficient mice. DCS has been proven, in a number of animal studies and clinical trials, to be a cognitive enhancer. Strikingly, DCS not only fully restored freezing responses to conditioned context in NCAM deficient mice in a dose-dependent manner, but even "made" these mutants to be better than wild-types in discrimination of conditioned versus neutral contexts, especially after the second fear conditioning. It can be argued that DCS simply decreased the level of generalized anxiety during tests in unpaired CC- contexts, since DCS is known for some experimental models as an anxiolytic drug (Anthony et al., 1993). However, this is unlikely due to several reasons. First, we injected DCS only once, rather than many times as in the studies where it showed anxiolytic properties (see for review Richardson et al., 2004). Second, since the half-life of DCS in mouse blood system is only 23 min (Conzelman and Jones, 1956), it is very unlikely that it could influence the level of anxiety 24 hours after its injection, at the first test day after training, and certainly not at the 7th

day after fear conditioning, since we did not inject DCS prior to any tests. Thus, the most plausible explanation would be that DCS could improve learning of conditioned context during acquisition of the task, so that animals could better recall and discriminate the conditioned context from a neutral one. Since the hippocampus, especially its CA1 area, plays a key role in contextual fear conditioning (Daumas et al., 2005; Inoue et al., 20005; Lee and Kesner, 2004), we think that systemically injected DCS could affect performance in this task, mostly acting at the hippocampus. However, we can not exclude that other areas of the brain, including the amygdala and/or prefrontal cortex, mediate action of DCS. To clarify this issue, additional acute intrahippocampal injections of DCS have to be performed.

Dose-dependent rescue of tone memory in NCAM deficient mice by D-cycloserine

In this study for the first time we showed that facilitation of NMDA-R signaling via systemic administration of DCS can rescue cued (tone) memory deficits in NCAM knockout mice in a dose-dependent manner. This improvement in recall of CS+ was apparent only for the first test day with the optimal concentration of DCS of 10 mg/kg, but surprisingly not for the seventh test day (with any tested concentrations of DCS). This, probably reflects the fact that NCAM is important not only for modulation of NMDA receptors but also involved in other processes. For instance, PSA-NCAM stimulates synaptogenesis via facilitation of FGF receptor signaling (Dityatev et al., 2004). It is unlikely that DCS would mimic this aspect of NCAM activity. Thus, other NMDA-R independent activity of NCAM may be critical for long-term (7 days) synaptic plasticity in the amygdala and/or other structures like auditory thalamus and auditory cortex, which are responsible for the tone processing.

Unfortunately, there is an apparent lack of information about effects of DCS on the amygdala-dependent learning, even in wild-type animals. There were just several studies mostly dealing with extinction models showing that, for example, DCS (3.25, 15 or 30 mg/kg, i.p, or 10 μg/amygdala side) facilitated conditioned fear extinction assessed with auditory fear-potentiated startle test (Walker et al., 2002) and its effect is abolished by HA-966, an antagonist of the glycine NMDA-Rs recognition site. Similar results were achieved for extinction of light-shock conditioning, which is also amygdala-dependent (Ledgerwood et al., 2003). For NCAM knockout mice, our experiments provide the first evidence that a deficit in the amygdala-dependent learning can be fully restored, at least for 1 day, via modulation of NMDA-R signaling.

Possible molecular mechanisms involved in impairment of LTP and learning and memory in NCAM knockout mice

Taking in consideration the behavioural data obtained in this project together with *in vitro* results we can speculate about possible molecular mechanisms involved in synaptic plasticity, learning and memory dysfunctions in NCAM deficient brain. First of all, it is noteworthy that a deficit in contextual fear conditioning in NCAM deficient mice nicely parallels impairment in LTP in CA3-CA1 synapses reported by our and other groups (see project I in this thesis, and Muller et al., 2000; Bukalo et al., 2004). Data obtained in project I of this thesis demonstrate that extracellular domain of PSA-NCAM may rescue LTP and contextual memory in NCAM deficient brains. Since no NCAM is expressed in these mice, the action of PSA-NCAM is not mediated by modulation of homophilic NCAM-NCAM interactions, but involves other molecules. It is likely that PSA-NCAM acts exclusively through PSA attached to NCAM backbone, since NCAM alone has effect neither on LTP nor learning. This idea is also in line with our observation that only PSA but not NCAM backbone appeared to inhibit NR2B-containing NMDA-Rs (Martin Hammond, Melitta Schachner, Alexander Dityatev, unpublished data).

This profound deficit in LTP in NCAM knockout mice can be completely restored by elevation of extracellular Ca^{2+}, lowering of extracellular Mg^{2+} and by application of DCS (Gaga Kochlamazashvilli et al., unpublished). All these three treatments result in facilitation of NMDA-R activity and/or higher Ca^{2+} influx to neurons during induction of LTP, thus supporting a view that a deficit in NMDA-R signaling underlies impaired LTP in NCAM deficient mice. There are several not mutually exclusive reasons for this. One is that NCAM is associated with spectrin and a lack of NCAM could lead to a deficit of spectrin and associated proteins, including NMDA-Rs, at synapses. This may lead to mistargeting of NMDA-Rs to extrasynaptic domain where they may trigger different signaling cascades than if placed synaptically (Liu et al., 2004; Clayton et al., 2002; Tang et al., 1999; Rodrigues et al., 2001; Kojima et al., 2005; Massey et al., 2004). Also, a lack of PSA carried by NCAM may be of critical importance since it has been found to inhibit NR2B-receptors activated by low concentrations of glutamate (Martin Hammond, Melitta Schachner and Alexander Dityatev, unpublished data). This would lead to preferential, if not exclusive, inhibition of NR2B-contaning NMDA-Rs by PSA-NCAM in extrasynaptic

domain, where the concentration of glutamate is low (see Fig. 6-III). The importance of this mechanism for induction of LTP is supported by our experiments demonstrating that a block of NR2B-containing NMDA-Rs by selective antagonist, Ro25-6981, recovers LTP in NCAM deficient mice (Gaga Kochlamazashvilli et al., unpublished). It would be important in the future to verify whether injection of this drug into the hippocampus would restore contextual fear conditioning in NCAM deficient mice.

Recent studies (Liu et al., 2004; Massey et al., 2004) suggest that synaptic NR2A-containing NMDA-Rs are the major contributors for induction of LTP at CA3-

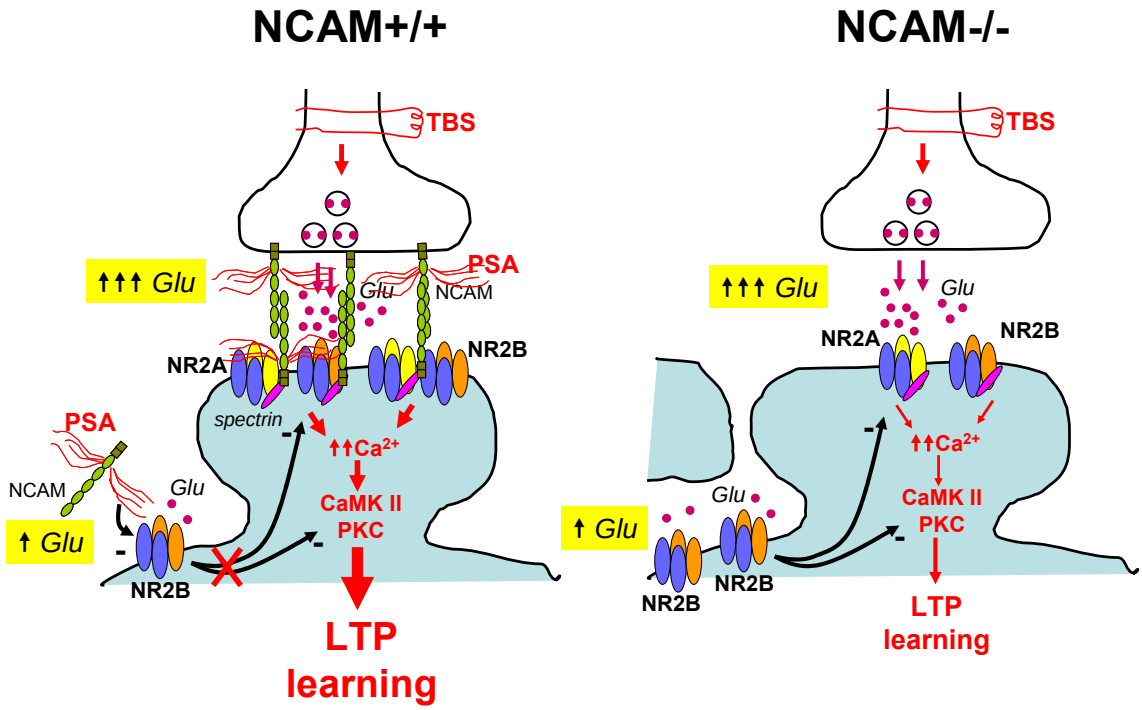

Fig. 6-III A diagram of possible molecular mechanisms laying behind deficits in synaptic plasticity, learning and memory in NCAM knockout mice. Since PSA is an inhibitor of extrasynaptic NR2B-containing NMDA-Rs, which are inhibitory for LTP, in NCAM deficient brain extrasynaptic NR2B NMDA-Rs are disinhibited due to absence of PSA. This hyperactivity of NR2B NMDA-Rs may result in reduced signaling via NR2A-containing NMDA-Rs, and impaired LTP and contextual memory.

CA1 synapses. In our experiments, LTP in NCAM deficient and wild-type mice was also strongly decreased by NVP, a drug preferably inhibiting NR2A-containing receptors. These results suggest that also in our conditions a significant part of the LTP is mediated via NR2A containing NMDA-Rs independently of NCAM genotype. This might appear puzzling in the light of the fact that DCS has slightly better affinity to NR2B containing receptors than to NR2A ones (O'Connor et al., 1996). If the

effects of DCS would be mostly on (extrasynaptic) NR2B- rather than on (synaptic) NR2A, one would expect a reduction of LTP by DCS, particularly in NCAM deficient brains, where signaling via extrasynaptic NR2B receptors is expected to be up-regulated. Thus, DCS has to target NR2A-containing receptors to have beneficial effects on LTP. It is likely that ambient concentrations of natural agonists of the glycine binding site of NMDA-Rs, D-serine and glycine, are sufficient to saturate NR2B-containing NMDA-Rs, thus preventing their further activation by exogenous DCS, which still could act on NR2A-containing NMDA-Rs, facilitating their down-stream signaling and leading to improvement of LTP in vitro experiments and contextual memory in the present study. This action appeared to be sufficient to overcome inhibitory activity of extrasynaptic NR2B-containing receptors.

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ABBREVIATIONS

A/C	associational/commissural;
aa	amino acids;
AA	Arachidonic Acid;
ACSF	artificial cerebrospinal fluid;
AG	Arachidonylglycerol;
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid;
AMPARs	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid Receptors;
ANOVA	Anylysis of Variances;
APV	2-D,L-aminophosphonovaleric acid;
Asp	Asparagin (amino acid);
ATP	Adenosine Triphosphate;
CaMK	Ca^{2+}/calmodulin kinase;
CAMKII	Calcium/Calmodulin-dependent Kinase II;
cAMP	Cyclic Adenosine-3-5-monophosphate;
CAMs	Cell Adhesion Molecules;
CB	Cannabinoid Receptors;
CC	conditioned context;
CHL1	close homologue of L1;
CNS	Central Nervous System;
CR	Conditioned or Conditional Response;
CREB	Cyclic-AMP Response-Element Binding protein;
CS	Conditioned or Conditional Stimulus;
CSPG	Chondroitin Sulfate Proteoglycan;
D1,2,3 etc	Domains;
DAG	Diacylglycerol;
DBD	DNA binding domain;
DCS	D-cycloserine;
DG	Dentate Gyrus;
DNA	deoxyribonucleic acid;
ECM	Extracellular Matrix;
Endo-N	Endoneuraminidase-N;
EPSCs	excitatory postsynaptic currents;
ER	estrogen receptor;
ERK	extracellular signal-regulated kinase;
ES	embryonic stem (ES) cells;
FAK	Focal Adhesion Kinase;
fEPSP	field excitatory post-synaptic potentials;
FGF-R or FGFR(s)	Fibroblast Growth Factor Receptor(s);
FN or (F3)	Fibronectin type three;
GABA	γ-aminobutyric acid;
GAP	Growth-associated protein;
GDNF	Glial Cell Line-Derived Neurotrophic Factor;
GFP	green fluorescence protein;
GluR	glutamate receptor;
GPI	Glycosylphosphatidylinositol;
GTP	Guanosine Triphosphate;
GTPases	guanosine triphosphatases;

HFS	high frequency stimulation;
HNK-1	3'sulfated glucuronic acid;
HSPG	Heparan Sulfate Proteoglycan;
Ig	Immunoglobulin domain;
Ig-CAMs	Immunoglobulin Superfamily of CAMs;
IP	inositol phosphates;
IP_3	Inositol 1,4,5-triphosphate;
LBD	ligand binding domain;
LTD	Long-Term Depression;
LTP	Long-Term Potentiation;
MAG	Myelin-Associated Glycoprotein;
MAP	Microtubuli-Associated Protein;
MAPK	Mitogen-Activated Protein Kinase;
MEK	MAP kinase kinases;
mGluR	metabotropic glutamatergic receptor;
MPP	medial perforant path;
mRNA	messenger ribonucleic acid;
MSD	Muscle Specific Domains;
MSK	Mitogen and Stress-Activated Kinase;
M_w	Molecular Wieght;
NCAM	Neural Cell Adhesion Molecule;
N-CAM	Neural Cell Adhesion Molecule;
Ng-CAM	neuron-glia cell adhesion molecule;
NMDA	N-methyl-D-aspartate;
NMDA-Rs	N-methyl-D-aspartate Receptors;
PDZ	postsynaptic density-95/Discs large/zona occludens-1;
PIP	phosphatidylinositol 4,5-bisphosphate;
PIP_2	Phosphatidylinositol 4,5-bisphosphate;
PKA	Protein Kinase A;
PKC	Protein Kinase C;
PLC	Phospholipase C;
PP	perforant path;
PPD	paired-pulse depression;
PPF	paired-pulse facilitation;
PrP	prion protein;
PSA	Polysialic Acid;
S/C	Schaffer collateral/commissural;
SEC	secreted exon;
SEM	standard error of mean;
SH2	Src homology domains;
STP	short-term potentiation;
SVZ	Subventricular Zone;
TAG	transient axonal glycoprotein;
Tamo	tamoxifen;
TBS	theta-burst stimulation;
UR	Unconditioned or Unconditional Response;
US	Unconditioned or Unconditional Stimulus;
VASE	Variable alternative spliced exon;
Veh	vehicle;

LIST OF PUBLICATIONS

ARTICLES:

1. Zhuravlev V, Bugaj V, Safonova T, Senkov O, Kodirov S. The chronoinotropic effects of new regulatory input to the heart of land pulmonates. Acta Biol Hung. 1999. 50:309-18.

2. Stoenica L., Senkov O., Gerardy-Schahn R., Weinhold B., Schachner M., Dityatev A. In vivo synaptic plasticity in the dentate gyrus of mice deficient in the neural cell adhesion molecule NCAM or its polysialic acid. European Journal of Neuroscience. 2006. *In press.*

3. Senkov O., Sun M., Weinhold B., Gerardy-Schahn R., Schachner M., and Dityatev A. Polysialylated NCAM is involved in induction of long-term potentiation and memory acquisition and consolidation in a fear conditioning paradigm. *Submitted.*

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ABSTRACTS:

1. Senkov O., Sun M., Dityatev A., Schachner M. The role of polysialylated NCAM in hippocampal long-term potentiation and acquisition and late consolidation of contextual memory, FENS, 2006, Austria

2. Behavioral and cardial reactions after removing of single identified neurons in central ganglia of giant enkov snail, Achatina fulica Ferrussac, V.Zhuravlev, V.Bugaj, T.Safonova, O.Senkov, V. Kochetova. VI East European Conference of the International Society for Invertebrate Neurobiology (ISIN), Simpler Nervous Systems, 2000, Russia.

3. Transcription factors CRE, C/EBP, AP-1, SRE and Helix memory formation. L.N.Grinkevich, P.D.Lisachev, O.V.Senkov. VI East European Conference of the international society for invertebrate neurobiology (ISIN), Simpler Nervous Systems, 2000, Russia

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ANIMAL WELFARE STATEMENT

In this work, I have used mice as experimental subjects. All surgical and behavioural procedures were approved by the Committee on Animal Health and Care of the local governmental body in Hamburg. I state here that I did treate all animals in most responsible and caring fashion, minimising all sources of discomfort for the animals, while always appreciating the ethical issues concerned with their work. A special care has been taken to minimize a number of animals in the experiments. At the same time, I want to stress that it is nevertheless very important to be critical towards the unnecessary use of animals in research, and to always appreciate that laboratory animals are living creatures that need to be treated with respect.

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30.03.2006

As an English native speaker hereby I confirm that the PhD thesis by Oleg Senkov titled as "Functional role of the polysialylated neural cell adhesion molecule in fear conditioning of mice (Mus musculus L., 1758)" is written in correct grammar and appropriate style.

Andrew Sharott, PhD