Research Report

Partial reduction in neural cell adhesion molecule (NCAM) in heterozygous mice induces depression-related behaviour without cognitive impairment

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ABSTRACT

The neural cell adhesion molecule (NCAM) plays an important role in brain plasticity. Using mice deficient in all isoforms of NCAM we have previously demonstrated that constitutive deficiency in the NCAM gene (NCAM−/−) resulted in cognitive impairment, anhedonic behaviour and a reduced ability to cope with stress. This was accompanied by reduced basal phosphorylation of the fibroblast growth factor receptor 1 (FGFR1) and reduced phosphorylation of calcium-calmodulin kinase II and IV and cAMP response element binding protein (CREB). The present study was aimed to investigate how partial deficiency in NCAM in mice (NCAM+/–) affected phenotype. We found that NCAM+/– mice showed a longer period of immobility in the tail suspension test, increased latency to feed in the novelty-suppressed feeding test and reduced preference for sucrose in sucrose preference test. Both NCAM+/– and NCAM−/– mice showed reduced extinction of contextual fear. In contrast to NCAM−/– mice, NCAM+/– mice did not demonstrate memory impairment in either object recognition or contextual fear conditioning tests. Levels of phosphorylated FGFR1 in the hippocampus and prefrontal/frontal cortex of NCAM+/– mice were partially reduced and no changes in the phosphorylation of CaMKII, CaMKIV or CREB in the hippocampus were found. We conclude that a constitutive partial reduction in NCAM proteins results in a behavioural phenotype related to depression without impairment in cognitive functions, also affecting the level of FGFR1 phosphorylation without major alterations in CaMKII and CaMKIV intracellular signalling. Partial reduction in FGFR1 phosphorylation might explain the observed behavioural phenotype in NCAM+/– mice.

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1. Introduction

One of the molecules involved in the regulation of brain plasticity is the neural cell adhesion molecule (NCAM). The neural cell adhesion molecule is a membrane-bound glycoprotein that belongs to the immunoglobulin superfamily of cell adhesion molecules. It is predominantly expressed on the surface of neuronal and glial cells at pre- and postsynaptic zones. The neural cell adhesion molecule has been implicated in cell–cell adhesion, neurite outgrowth, synaptic plasticity, neuronal development and neurogenesis (Persohn and Schachner, 1987; Rønn et al., 2000; Amoureux et al., 2001; Kiss and Muller, 2001; Schuster et al., 2001).
Neural cell adhesion molecule is present in three isoforms, which result from alternative splicing of a single gene. The three isoforms are called NCAM-180 and NCAM-140, which are transmembrane isoforms, and NCAM-120, which is a glycosylphosphatidylinositol-linked isoform. All three isoforms share the same extracellular domain comprising five Ig-like modules and two membrane-proximal fibronectin type-3 modules. The adhesive properties of NCAM can be regulated by the addition of long, linear homopolymers of alpha-2,8-linked sialic acid residues (polysialic acid, PSA), which attenuate NCAM-mediated cell interactions and thereby promote structural plasticity and affect cell functions (Bruses and Rutishauser, 2001; Rutishauser, 2008).

Altered levels of PSA and neural cell adhesion molecule have been associated with several neuropsychiatric disorders (reviewed in Brennaman and Maness, 2010). Accumulating evidence suggests that NCAM is involved in depression (Sequeira et al., 2007; Aonurm-Helm et al., 2008a; Tochigi et al., 2008). Adult mice that lack all three major isoforms of the NCAM (NCAM−/−) exhibit anhedonia, impaired cognitive functions and a reduced ability to cope with stress (Cremer et al., 1999; Bukalo et al., 2004; Aonurm-Helm et al., 2008a; Jürgenson et al., 2010).

It has been shown that NCAM and PSA-NCAM regulate plasticity by interacting with several interaction partners (Walmod et al., 2004). A major interaction partner of NCAM is the fibroblast growth factor receptor 1 (FGFR1, Doherty and Walsh, 1996; Cavallaro et al., 2001; Kiselyov et al., 2003; reviewed in Kiselyov et al., 2005; Francavilla et al., 2007).

Homophilic and heterophilic interactions of NCAM initiate signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) cascade and calcium/calmodulin dependent kinase II (CaMKII) and calcium/calmodulin dependent kinase IV (CaMKIV) pathways, and activates the transcription factor cAMP-response element binding protein (CREB) at Ser133 (Schmid et al., 1999; Kolkova et al., 2000; Jessen et al., 2001; Povlsen et al., 2003; Griffith, 2004; Dilevesen et al., 2008). Our recent studies showed that NCAM−/− mice demonstrated reduced ability to cope with stress as evidenced by an increased immobility time in the tail suspension test and anhedonia as evidenced by reduced preference for sucrose solution in the sucrose preference test (Aonurm-Helm et al., 2008a). These behaviours, but not cognitive impairment, were ameliorated by the antidepressants amitriptyline and citalopram and the NCAM mimetic peptide FGL (Aonurm-Helm et al., 2008a). Other studies have also demonstrated some morphological alterations in the brain of NCAM−/− mice, such as reduced olfactory bulbs size and reduced mossy fibre density in the hippocampus (Cremer et al., 1997; Stork et al., 1997). Furthermore, NCAM−/− mice showed reduced phosphorylation of the major NCAM interaction partner fibroblast growth factor receptor 1 (FGFR1) and impaired activity of several NCAM-mediated intracellular signalling pathways, demonstrated by the reduced levels of phosphorylated CREB and phosphorylated CaMKII and CaMKIV in the hippocampus (Aonurm-Helm et al., 2008b, 2010). However, the impaired intracellular pathways responsible for the cognitive dysfunction and those implicated in the formation of the depression-like phenotype remain unclear. It has been shown previously that mice with a partial reduction in NCAM expression (NCAM+/-) display increased anxiety and inter-male aggression with a post-aggression test increase in corticosterone plasma concentration, similarly to NCAM−/− mice (Stork et al., 1997, 1999). These data suggests that even a partial reduction in NCAM proteins may cause alterations in behavioural phenotype.

We therefore studied whether a partial reduction in NCAM was capable of inducing similar alterations in behaviour to those observed in NCAM−/− mice. We also studied the intracellular signalling pathways that were previously shown to be impaired in NCAM knockout mice.

For this purpose, we studied depression-related behaviours and memory function in heterozygous NCAM+−/ mice and measured the phosphorylation levels of FGFR1, CREB, CaMKII and CaMKIV in hippocampal tissues, all of which have been previously shown to be dysregulated in NCAM−/− mice.

2. Results

2.1. NCAM and PSA-NCAM protein levels

Western blots were performed to measure the amount of NCAM isoforms and PSA-NCAM in hippocampal and prefrontal/frontal cortical lysates from wild-type, NCAM+−/ and NCAM−/− mice. There was a 50% reduction in the immunoreactivity of all NCAM isoforms and PSA-NCAM in the hippocampus of NCAM+−/ mice compared with wild-type mice. The tissue from NCAM−/− mice did not demonstrate any immunoreactivity related to NCAM or PSA-NCAM (Fig. 1). Similar reductions in NCAM and PSA-NCAM levels were also observed in prefrontal/frontal cortex (Supplementary data, Fig. 1).

2.2. Tail suspension, novelty-suppressed feeding and sucrose preference tests in NCAM+−/− and NCAM−/− mice

To evaluate the ability to cope with stress and determine anhedonic behaviour in NCAM+−/− animals, we employed the tail suspension test (TST), novelty-suppressed feeding test and the sucrose preference test. The TST was first introduced as a test with predictive validity for detecting drugs with antidepressant-like activity (Porsolt et al., 1987; Bai et al., 2001; reviewed in Cryan and Mombereau, 2004 and Cryan et al., 2005). More recent studies, however, demonstrated relatively good face validity of the TST for describing depression-like phenotypes in genetic-, stress- or toxin-induced models of depression in mice (Dantzer et al., 2008; Ito et al., 2011; Monje et al., 2011; Popova and Tibeikina, 2010). As shown in Fig. 2A, the NCAM+−/− mice spent a significantly longer time immobile than their wild-type littermates. For comparison, we also tested NCAM−/− knockout mice and observed increased immobility time similar to our earlier publication (Aonurm-Helm et al., 2008a). A one-way ANOVA demonstrated a highly significant genotype effect: p<0.0001, F=10.57, d.f.=27 (n=8−10). Post-hoc analyses showed a significant increase in immobility time in both NCAM+−/ (p<0.01) and NCAM−/− (p<0.001) mice (Fig. 2A). To rule out the possibility that the longer period of immobility in the TST was due to an impairment in locomotion, we measured general locomotor activity. A one-way ANOVA revealed an effect of genotype:
p<0.05, F=4.63, d.f.=28 (n=9–10). Post-hoc analyses revealed that NCAM−/− mice were more active (p<0.01) whereas NCAM+− mice showed similar locomotor activity when compared with wild-type animals (Fig. 2B).

The novelty-suppressed feeding test (NSF) is thought to assess emotional reactivity toward a new environment and induces competition between motivational states (drive to eat vs. fear of venturing into the centre of the test arena). This

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

**Fig. 2** – Length of immobility in the tail suspension test (panel A), locomotor activity (panel B), latency to feed in novelty suppressed feeding test (panel C) and body weight (panel D) of wild-type, NCAM+− and NCAM−− mice. The data are expressed as mean±SEM (n=8–10). *p<0.05; **p<0.01; ***p<0.001 compared with wild-type mice (one-way ANOVA, followed by Bonferroni post-hoc test).
test is sensitive to antidepressant treatment and has excellent predictive validity for antidepressant efficacy (reviewed in Dulawa and Hen, 2005). Recently, this test was also used to assess depression-related behaviour (Santarelli et al., 2003; Uchida et al., 2011). As shown in Fig. 2C, the NCAM+/- and NCAM-/- mice demonstrated a significantly longer latency to feed than their wild-type littermates (one-way ANOVA, p<0.05, F=3.90, d.f.=19, n=6–7). Enhanced novelty suppression feeding behaviour in NCAM+/- and NCAM-/- mice appears to be unrelated to food consumption as the mutant mice had similar body weights to their wild-type littermates (Fig. 2D) and consumed similar amounts of food in their home cages (data not shown).

To test whether the animals exhibited a decreased ability to experience pleasure (anhedonia), wild-type and NCAM+/- mice were subjected to a sucrose preference test, which is commonly used to detect motivational deficits (Strekalova et al., 2004; Rygula et al., 2005). The sucrose preference test was performed over 19 days. Control mice demonstrated a clear preference for the sucrose solution because the sucrose solution comprised ~75–80% of the total liquid consumed. In contrast, the NCAM+/- mice showed decreased preference for sucrose, with ~50–65% of the total liquid consumption being the sucrose solution (Fig. 3). A two-way ANOVA with repeated measures showed a significant effect of genotype (F1.216 =16.33, p<0.01), but no significant effect of time (F1.216 =0.88, p<0.6) and no interaction (F1.216 =1.27, p<0.2). Total fluid consumption did not differ between the wild-type and NCAM+/- mice (Fig. 3).

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

2.3. Object recognition test

To evaluate learning and memory functions in NCAM+/- mice, object recognition test was performed. In the object recognition test both NCAM+/- mice and their wild-type littermates showed no significant preference for either of the two identical objects during the training phase (data not shown). During the test phase, both wild-type and NCAM+/- mice were presented with novel objects at 2 h (short-term memory, STM) and at 24 h (long-term memory, LTM) after the training phase. The NCAM+/- mice showed a similar STM and LTM preference for the novel object when compared with wild-type mice (Fig. 4).

2.4. Fear conditioning and extinction in NCAM+/- and NCAM-/- mice

To further explore cognitive functions and the ability to extinguish negative memories in NCAM+/- and NCAM-/- mice, the animals were subjected to tone and contextual fear conditioning and contextual fear extinction tests. In the tone and contextual fear conditioning test, the baseline freezing time prior to the test was similar between all genotypes (data not shown). 24 h after fear conditioning (day 1), the animals were placed in the same context without tone and foot shock and the freezing behaviour was measured. A one-way ANOVA revealed a significant effect of genotype: p<0.05, F=4.41, d.f.=23 (n=8) and post-hoc analyses showed that NCAM-/- mice showed significantly less freezing (p<0.05) compared with wild-type animals. The freezing time of NCAM+/- mice did not differ from that of wild-type mice, indicating that a partial deficiency in NCAM level does not affect contextual fear learning (Fig. 5A).

On the following day after testing animals for the retention of contextual fear memory, the extinctions sessions were initiated and they continued for 6 consecutive days. Experiments
showed that wild-type mice efficiently extinguished fear memory as demonstrated by the reduction in freezing time over the 6 days, whereas mice with partial or full deficiency in NCAM showed significantly reduced extinction of contextual fear as revealed by the continued increased level of freezing behaviour during extinction sessions (Fig. 5A). A two-way ANOVA with repeated measures showed a significant effect of genotype ($F_{1,105}=3.96, p<0.05$), a significant effect of time ($F_{1,105}=3.52, p<0.01$) and no interactions ($F_{1,105}=0.84, p<0.6$). Following extinction training (on the 8th day), we performed a tone-induced fear recall test. Freezing behaviour was measured after exposing the mice to the tone in a novel context. The freezing time in the novel context prior the recall test was similar between groups (Fig. 5B). In the tone-induced fear recall test, a one-way ANOVA revealed a significant effect of genotype: $p<0.05, F=3.99, d.f.=23 (n=8)$ and post-hoc analyses revealed that NCAM$^−/−$ mice showed significantly less freezing ($p<0.05$) compared with wild-type animals, whereas NCAM$^+/-$ mice froze for similar periods of time comparing with wild-type mice (Fig. 5B).

2.5. The brain weight of wild-type, NCAM$^+/-$ and NCAM$^−/−$ mice

Previous studies demonstrated some morphological changes, such as reduced brain weight, reduced size of the olfactory bulb, and a reduction in density of mossy fibres in the CA3 region in brains of NCAM$^−/−$ animals (Cremer et al., 1994, 1997). Our experiments also showed reduced brain weight in NCAM$^−/−$ mice but no changes in the brain weight of NCAM$^+/-$ mice. There were no differences in brain-to-body mass ratio between the experimental groups (Supplementary data, Table 1). No observable changes in the olfactory bulb of NCAM$^+/-$ mice were observed whereas olfactory bulbs in NCAM$^−/−$ mice were reduced in size (data not shown).

2.6. FGF receptor 1 phosphorylation and FGF-2 levels

The FGFR1 is the major interaction partner for NCAM and PSA-NCAM (Doherty and Walsh, 1996; Kiselyov et al., 2003; reviewed in Kiselyov et al., 2005). Therefore, it was of interest to study whether a partial deficiency in NCAM proteins could lead to an alteration in FGFR1 phosphorylation. We measured the levels of total FGFR1 and its phosphorylated (pFGFR1) form in the hippocampus and also in the prefrontal/frontal cortex of NCAM$^+/-$ mice. NCAM$^−/−$ mice were again used for comparison in these experiments.

Both the NCAM$^+/-$ and NCAM$^−/−$ mice demonstrated reduced levels of pFGFR1 in the hippocampal tissue (one-way ANOVA, $p<0.0001, F=22.96, d.f.=17$). Homozygous NCAM$^−/−$ mice showed a much greater reduction (65–70%) in the basal phosphorylation levels of FGFR1 than heterozygous NCAM$^+/-$ mice, which showed approximately 30% reduction from control levels (100%). No change in total FGFR1 protein level was found in either NCAM$^+/-$ or NCAM$^−/−$ mice compared with control animals (Fig. 6). A similar reduction in levels of pFGFR1 was found in the prefrontal/frontal cortical areas of these animals (Supplementary data, Fig. 2).

As basic fibroblast growth factor 2 (FGF-2) is an important activator of FGFR1 phosphorylation (Plotnikov et al., 1999; Frinchi et al., 2008), we also measured the levels of FGF2 in the hippocampus of NCAM$^+/-$ and NCAM$^−/−$ mice and their wild-type littermates. The results revealed a significant
increase in the levels of FGF-2 in both NCAM+/− and NCAM−/− mice compared with wild-type mice (Fig. 6).

2.7. The expression of phosphorylated CaMKII, CaMKIV and CREB

Our previous study (Aonurm-Helm et al., 2008b) has shown reduced levels of phosphorylated CREB and pCaMKII and pCaMKIV in the hippocampus of NCAM−/− mice, suggesting that this dysregulation might be responsible for the observed behavioural phenotype in NCAM−/− mice. Therefore, we measured the levels of phosphorylated CaMKII, CaMKIV and CREB proteins in NCAM+/− mice. No differences in the immunoreactivity of total and phosphorylated CaMKII, CaMKIV or CREB were found in the hippocampus of NCAM+/− mice compared with wild-type mice (Fig. 7).

3. Discussion

The results of our study show that NCAM+/− mice, with partial reduction in the levels of all isoforms of the NCAM protein and its polysialylated form, PSA-NCAM, demonstrate increased immobility time in the TST, reduced sucrose preference in the sucrose preference test, increased latency in the novelty-suppressed feeding test, and reduced extinction of contextual fear conditioning. Our previous study (Aonurm-Helm et al., 2008a, Jürgenson et al. 2010) and the data shown here demonstrate that similar behavioural changes occur in homozygous knockout (NCAM−/−) mice. On the basis of the observed phenotype of NCAM+/− mice, we propose that

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

Fig. 7 – The levels of phosphorylated and total CaMKII (panel A), CaMKIV (panel B) and CREB (panel C) in the hippocampi of wild-type and NCAM+/− mice. The data are expressed as a percentage of the wild-type±SEM (n=6).
these animals have enhanced emotional reactivity, reduced ability to cope with stress, exhibit anhedonia and retain aversive emotional memories. This behavioural phenotype might be, therefore, described as a depression-related phenotype. Although the behavioural tests employed in our study were developed and validated to predict efficacy of antidepressant drugs, some recent studies have explored the face validity of these tests for characterising depression-related phenotypes in several behavioural models of depression.

In the TST test, immobility behaviour represents a “behavioural despair”, which is based on the observation that after initial escape-oriented movements, animals develop an immobile posture when they are in an inescapable and stressful situation. Recent analysis has demonstrated that a number of interventions known to be involved in susceptibility to or induction of depression in humans induce a depression-like effect (increased immobility) in the TST (reviewed in Cryan et al., 2005). Similarly, novelty-suppressed feeding behaviour reflects the reaction of animals to stress, and the face validity of this test has been demonstrated in several behavioural models of depression (Santarelli et al., 2003; Uchida et al., 2011). It should be noted, however, that hyponeophagia-based models may be related to changes in appetite rather than anxiety levels. Several genetic manipulations and drugs may affect appetite and food preferences (reviewed in Dulawa and Hen, 2005). In our experiments, all genotypes had similar body weights and consumed the same amount of food in their home cages. Thus, we propose that the increased latency to consume food in the novel environment is related to levels of anxiety rather than changes in appetite. The sucrose preference test has also been used extensively to measure anhedonia as a core symptom of depression. It has been shown repeatedly, using several stress models, that chronic mild stress reduces preference for glucose consumption (Willner et al., 1987, 1992; Strekalova et al., 2004; Yu et al., 2011). It should be noted that the reduced preference for sucrose consumption may have resulted from a global impairment in the development of taste cells and receptors due to the deficiency in NCAM (Miura et al., 2005). We tested this possibility using bitter solutions containing HCl and demonstrated clear aversion in both wild-type- and NCAM+/− animals. However, the possibility that taste receptor functions were affected by the partial reduction in NCAM and PSA-NCAM cannot be fully excluded.

Another important finding in our study is that NCAM+/− mice were not impaired in the novel object recognition task and fear conditioning task. In contrast, NCAM−/− mice demonstrated impaired memory retention in both tasks, which confirms several previous studies (Stork et al., 2000; Senkov et al., 2006; Jürgenson et al., 2010). Previous studies have demonstrated that acquisition of the context during contextual fear conditioning reflects associative memory functions (Wellman et al., 2007; Tronson et al., 2008). Rats with depression-like behaviour caused by prenatal stress (Green et al., 2011) or rats predisposed to learned helplessness (Shumake et al., 2005) have reduced ability to extinguish contextual fear. In contrast, animals with an antidepressant-like phenotype due to deficiency of the ERK-1 kinase demonstrated enhanced extinction of contextual fear (Tronson et al., 2008). It appears that impaired fear extinction rather than the formation of fear memory is related to the depression-like state (Heldt et al., 2007; Wellman et al., 2007). In our experiments, both NCAM+/− and NCAM−/− demonstrated a reduced ability to extinguish contextual fear memory, and these data support our proposal that even partial reduction in NCAM proteins induces depression-related behaviour without an impairment in memory acquisition and recall, at least in the cognitive tests that were employed in our study. Our data are in accordance with previous studies that demonstrated greater inter-male aggression with a post-test increase in corticosterone plasma concentrations in NCAM+/− mice (Stork et al., 1997, 1999).

Numerous previous studies have demonstrated that impaired contextual fear extinction and increased immobility time in the TST and a reduced preference for sucrose in the sucrose preference test have also been associated with impaired hippocampal function (Paizanis et al., 2010; reviewed in Radulovic and Tronson, 2010; Shioda et al., 2010; Uchida et al., 2011). An abundance of data implicates the involvement of hippocampus in mood disorders (Brown et al., 1999; MacQueen et al., 2003).

Previous in vitro and in vivo studies have shown that the major interaction partner of NCAM is FGFR1 and that a deficiency in NCAM causes reduced FGFR receptor activation (Cavallaro et al., 2001; Kiselyov et al., 2003; reviewed in Kiselyov et al., 2005, Aonurm-Helm et al., 2010). We therefore measured levels of FGFR1 protein and its phosphorylated form in the hippocampus of NCAM+/− mice and compared results with NCAM−/− animals. In addition, we measured the levels of FG-2 protein in these animals. Our results demonstrated reduced levels of FGFR1 phosphorylation in both mutant genotypes without any changes in the total level of the FG receptor protein. It should be noted however, that the decrease in phosphorylated FGFR1 in NCAM+/− mice was less pronounced when compared with the NCAM−/− mice. In the NCAM+/− mice, the level of phosphorylated FGFR1 was 65–70% of control, whereas in NCAM−/− mice, the level was only 30–35% of control levels (100%). Similar changes in FGFR1 phosphorylation were also found in prefrontal/frontal cortex in these animals (Supplementary data, Fig. 2). In both mutant genotypes the reduction in phosphorylation of the FG receptor was accompanied by an increase in FG-2 protein in hippocampal tissues. Recently was shown, that hippocampal FG2 expression may represent a key mechanism involved in the genesis of anxiety disorders (Turner et al., 2011). To date, there is no evidence showing that NCAM directly affects the expression of FG-2. The observed increase in growth factor protein is likely a compensatory reaction due to the deficiency in FGF-mediated signalling. These data suggest important permissive roles of NCAM for FGF-mediated signalling because in NCAM deficient mice an increase in FG-2 protein was not capable of activating FGFR1. Our data also show that the behavioural phenotype due to NCAM deficiency may be dependent on the magnitude of FGFR1 phosphorylation. Indeed, the FGF signalling system has diverse biological functions during brain development and in adulthood (Powers et al., 2000). Several recent studies have proposed a link between the FGF system and depression (Evans et al., 2004; Gaughan et al., 2006).

In contrast to the NCAM−/− mice, the levels of phosphorylated CaMKII and CaMKIV were unchanged in NCAM+/− mice.
No difference in the phosphorylation level of the transcription factor CREB was observed between the NCAM+/- and wild-type animals either. It appears that a partial reduction in NCAM proteins does not affect the basal activity of CaMKII or CaMKIV signalling pathways.

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

Our data show that the observed behavioural phenotype in mice with a partial deficiency in NCAM is not associated with alterations in the basal phosphorylation levels of CaMKII and CaMKIV or CREB.

In conclusion, our data show that NCAM+/- mice, with a partial constitutive reduction in NCAM protein levels, exhibit a behavioural phenotype related to depression without impairment in cognitive function. This is accompanied by partial reduction in the levels of FGF receptor 1 phosphorylation, which may at least in part account for the observed behavioural phenotype in these animals.

4. Experimental procedures

4.1. Animals and drug treatment

All experiments were undertaken in accordance with the guidelines established in the Principles of Laboratory Animal Care (Directive 86/609EEC). All experiments were performed by individuals who held an appropriate licence. The NCAM-deficient mice were originally generated by Cremer et al. (1994). The NCAM+/-, NCAM+/- and wild-type mice used for this study were obtained by crossing C57BL6-Ncam tm1Cgn+/- heterozygous mice, which were purchased from Jackson Laboratories (Maine, USA). F5-generation NCAM+/- mice, NCAM+/- mice and their wild-type littermates, aged 2–4 months and with an average weight of 23.5 g, were used. All animals were housed under standard housing conditions as follows: the mice were group housed (five or six mice per cage) under a 12 h light–dark cycle. All mice had ad libitum access to food and water.

4.2. Tail suspension test (TST)

The TST is similar to the forced swim test and is based on the fact that mice suspended by the tail alternate between periods of struggle and immobility (reviewed in Cryan and Mombereau, 2004). The TST is commonly used as a model of depression, where mice subjected to an inescapable stressful situation develop an immobile posture after initial escape-oriented movements (Dantzer et al., 2008; Popova and Tibeikina, 2010; Ito et al., 2011; Monje et al., 2011). This test is regarded as useful for assessing the behavioural effects of antidepressant compounds and other pharmacological and genetic manipulations relevant to depression (reviewed in Cryan et al., 2009).

Mice (n=8–10 per group) were suspended by the tail from a wooden beam, using adhesive tape at 1 cm from the tip of the tail. We measured the total duration of immobility during the 6-min test period. Immobility was defined as a complete lack of movement other than respiration. The immobility times were timed, using a stop-watch, by a trained observer who was blinded to the genotypes of the animals.

4.3. General locomotor activity

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

4.4. Novelty-suppressed feeding test

The novelty-suppressed feeding test was performed as described previously (Uchida et al., 2011). Mice were weighed and food was removed from their cage. 24 h after the removal of food, mice were transferred to the testing room and placed in a clean holding cage where they were allowed to habituate for at least 30 min. The testing apparatus consisted of a square wooden box (50 × 50 × 50 cm). The floor was covered with 2 cm of wooden bedding. A small piece of mouse chow was placed in the centre of the arena. At the start of the experiment, each mouse was placed in the corner of the testing area, and the time to the first feeding event was recorded. Animals that did not feed within this 5-minute period were removed from all analyses. The latency to feed was timed, using a stop-watch, by a trained observer who was blinded to the genotypes of the animals.

4.5. Sucrose preference test

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

4.6. Taste aversion test

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

4.7. Object recognition test

The novel object recognition task is based on the innate tendency of rodents to differentially explore novel objects over familiar ones (Ennaceur and Delacour, 1988) and is widely used to assess memory functions in mice (Arqué et al., 2008; Coyle et al., 2009; Francis et al., 2012).

The object recognition task took place in a 50 cm × 50 cm × 50 cm (L × W × H) open field, made of brown wood, located in a testing room that was dimly (approximately 60 lux in the test arena). The objects chosen were porcelain cups of different shapes and sizes; these objects were sufficiently heavy that the mice did not move them. All objects had similar textures and colours but distinctive shapes. Each animal (n = 7 per group) was habituated where it was allowed to freely explore the open field for 5 min. No objects were placed in the box during the habituation session.

24 h after habituation, training took place by placing individual mice into the field for 5 min, in which two identical objects (A and A1) were positioned in two adjacent corners, 10 cm from the walls. The amount of time each mouse spent exploring both objects A and A1 was recorded. Short-term memory (STM) and long-term memory (LTM) tests were performed 2 h and 24 h after training, respectively. In both tests, the mice explored the open field for 5 min in the presence of one familiar (A) and one novel (B for STM or C for LTM) object and the time the mice spent exploring the objects was recorded. A preference ratio for each mouse was expressed as a ratio of the amount of time spent exploring the new object (B or C) (Tnew ×100)/(TA +Tnew), where TA and Tnew are the times spent exploring the familiar object A and the novel object, respectively.

Between trials, the objects were cleaned using a 5% ethanol solution. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. The time spent exploring each object was recorded manually with a stop-watch by an experimenter blinded to the genotypes of the animals.

4.8. Fear conditioning and extinction

The contextual fear memory test and contextual fear extinction test in NCAM+/−, NCAM−/− and wild-type mice were performed as described previously (Radulovic et al., 1998; Tronson et al., 2008). Fear conditioning, an experimental chamber of 22 cm × 22 cm × 35 cm (L × W × H) was used. The box was made from transparent plastic. The floor was made from stainless steel rods that were designed for mice and that were connected to a scrambled shock generator (TSE Systems, Germany). The chamber contained a speaker that emitted the audible tones.

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

4.9. Immunoprecipitation and western blotting

Adult (3 months old) wild-type, NCAM+/− and NCAM−/− mice (n = 6 per group) were weighted and euthanized by decapitation. The brains were removed from the skulls, weighted and placed on ice. The hippocampi and prefrontal/frontal cortex were quickly removed on ice in a +4 °C room and immediately placed into liquid nitrogen and stored at −80 °C until further processing. Tissues were lysed in 10 vol RIPA lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% glycerol, 1% NP-40, 2 mM EDTA) containing protease and phosphatase inhibitors, homogenised manually, incubated for 20 min on ice and then centrifuged (13,000 rpm for 20 min at 4 °C).

The following mono- or polyclonal primary antibodies were used: goat anti-pCaMKIIa (Thr286, 1:800; sc-12886; lot: 63007), rabbit anti-CaMKII (M-176, 1:800; sc-9035; lot: H1407), rabbit anti-pCaMKIV (Thr196, 1:800; sc-28443-R; lot: H1806), goat anti-CaMKIV (M-20, 1:800; sc-1546; lot: B1605), rabbit anti-pCREB-1 (1:1000; sc-101663; lot: D229), rabbit anti-CREB-1 (1:1000; sc-186; lot: 83374), mouse anti-FGF2 (1:1000; sc-186; lot: 136255; lot: H1309) and mouse anti-NCAM (1:1000; 556324; lot: 83374), goat anti-PSA-Ngam (1:10000; c0019; lot: 1107). The secondary antibodies used were: anti-goat IgG (HRP-conjugated, 1:400; cat no: PI-9500, Vector Laboratories, UK), anti-mouse IgG (HRP-conjugated), anti-mouse IgM (HRP-conjugated) and anti-rabbit IgG (HRP-conjugated, 1:400; cat no: 32530, 31440 and 32640, respectively, Pierce, US). All proteins of interest and actin were measured on the same membrane after stripping with buffer (10× stock; Millipore, CA, US). The
membranes were incubated with ECL detection reagent (ECL, Amersham, UK) for 5 min to visualise the proteins and were then exposed to an autoradiography X-ray film (Amersham hyperfilm ECL, UK). The blots were probed for the proteins of interest and the density of each band was then analysed and compared using the QuantityOne 710 System (BioRad).

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

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

4.10. Statistics

All values are presented as mean ±SEM. Statistical analyses were performed using Student’s t-tests and one-way ANOVAs or two-way ANOVAs followed by the Bonferroni post-hoc test, where appropriate. Any p-values less than 0.05 were considered significant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.brainres.2012.01.056.

REFERENCES


