Pharmacology, Biochemistry and Behavior 96 (2010) 461–468

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Behavioral profile of mice with impaired cognition in the elevated plus-maze due to a deficiency in neural cell adhesion molecule

M. Jürgenson, A. Aonurm-Helm, A. Zharkovsky *

Department of Pharmacology, Center of Excellence for Translational Medicine, University of Tartu, 19 Ravila Street, 51014 Tartu, Estonia

ARTICLE INFO

Article history:
Received 2 March 2010
Received in revised form 3 June 2010
Accepted 5 July 2010
Available online 13 July 2010

Keywords:
Anxiety
Learning
Neuronal cell adhesion molecule (NCAM)-knockout mice
Elevated plus-maze

ABSTRACT

The elevated plus-maze (EPM) test is one of the most used tests for screening levels of anxiety in rodents. In the present study, we studied how impaired cognition due to a deficiency in the neural cell adhesion molecule (NCAM) could affect the behavior of mice in the EPM task. NCAM-knockout mice demonstrated impaired learning in both object-recognition and fear-conditioning tasks. Analysis of the behavior of mice in the EPM task using a minute-by-minute method revealed a profound influence of genotype. Wild-type mice demonstrated quick learning of the aversive properties of the open arms during the first few minutes of a single EPM task, whereas NCAM−/− mice were unable to learn the aversive properties of the open arms of EPM. Wild-type mice also demonstrated habituation to the EPM task in a test/retest paradigm whereas NCAM-knockout mice failed to habituate during the second EPM presentation. Our data show that the anxiolytic-like behavior of NCAM-knockout mice is not just related to levels of innate anxiety but also to their inability to recognize potential danger associated with the open arms of the EPM task.

1. Introduction

The elevated plus-maze (EPM) test is one of the most used tests for screening anxioselective effects of drugs and anxious behavior in rodents (Pellow et al., 1985; Lister, 1987; Hogg, 1996). The ethological nature of the EPM was first suggested by Lister (1990). It was suggested that the test involves spontaneous exploration of the environment by rodents in the absence of explicit reward or consummatory behaviors and, therefore, the test might be considered as an ethological model (Lister, 1990; Rodgers and Dalvi, 1997). The unconditioned response characteristics seen in the EPM task are attributed to the spontaneous fear which the EPM elicits, given that during the regular 5 min session there is a clear preference to be in the enclosed arms rather than in the open arms. The total entries and total distance scores are considered as a useful index of general activity. There is also substantial evidence showing that drugs which increase open arm activity are anxiolytic compounds whereas drugs which reduce open arm activity are anxiogenic (Johnston and File, 1989; Carrasco et al., 2006; Vargas et al., 2006; Massé et al., 2007). A significant increase in the percentage of time spent on the open arms and the number of entries into the open arms were only observed when clinically effective anxiolytics had been administered to the rodents (Johnston and File, 1989; Calzavara et al., 2005; Vargas et al., 2006). Compounds that cause anxiety in humans, for example yohimbine, pentylenetetrazole, caffeine and amphetamine, significantly reduced the percentage of entries and time spent on the open arms by rodents (Lister, 1990). The use of this test for detecting such drug effects on rodents was validated behaviorally, physiologically and pharmacologically (Pellow et al., 1985).

However, it has been shown that there are some other factors which may have an impact on the performance of the animals in the EPM test. Detailed analysis of the behavior in the EPM demonstrated a gradual increase in avoidance of the open arms and a clear preference of animals for the enclosed arms during an EPM test (Bertoglio et al., 2006). On this basis, it was proposed that learning can occur during the EPM test session (Bertoglio and Carobrez, 2000; Calzavara et al., 2004; Calzavara, et al., 2005; Carobrez and Bertoglio, 2005; Bertoglio et al., 2006). Therefore, the possibility that any drug which could affect learning or memory capability in the animals may also affect behavior in the EPM test cannot be excluded.

If the animals could not remember that they were already in closed or open arms then, theoretically, they would enter the same arms again. If this type of response is learned through experience, then the EPM could not be classified as a test of unconditioned responses.

There are several data to demonstrate that brain plasticity plays a role in emotional behavior and memory formation. One of the molecules identified as being involved in the regulation of brain plasticity is the neural cell adhesion molecule (NCAM), a member of the immunoglobulin (Ig) superfamily, expressed at pre- and postsynaptic zones (Persohn and Schachner, 1987; Rønn et al., 2000; Schuster et al., 2001). Through homo- and heterophilic interactions, NCAM determines the binding forces between pre- and postsynaptic membranes. The

* Corresponding author. Department of Pharmacology, University of Tartu, 19 Ravila Street, 51014 Tartu, Estonia. Tel.: +372 7 374351; fax: +372 7 374352.
E-mail address: aleksander.zarkovski@ut.ee (A. Zharkovsky).

0091-3057/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2010.07.006

neural cell adhesion molecule undergoes post-translational modifications due to the addition of α-2,8-polysialic acid (PSA) chains to the Ig5 module (Rougon, 1993; Seki and Arai, 1993; Rutishauser, 2008). During development, PSA-NCAM is widely expressed during neuronal development and plays a crucial role in cell division, migration and differentiation (Kiss et al., 2001; Walmod et al., 2004).

Previous studies on mice with constitutive deficiencies in the *NCAM* gene demonstrated impaired learning and increased inter-male aggression, higher locomotor activity, increased anxiety and depression-like behavior (Cremer et al., 1994; Stork et al., 1999, 2000; Welzl and Stork, 2003; Aonurm-Helm et al., 2008).

Due to impaired cognitive functions, NCAM-knockout mice might represent a valuable model for testing the role of cognition in the performance of mice in the EPM test. In order to test whether impaired learning and memory can influence EPM test results, we performed the EPM test on NCAM-knockout mice and their wild-type littermates, and animal behavior was assessed using the minute-by-minute structure. Also, in order to assess the ability of NCAM-knockout mice to habituate to test situations, a test/retest protocol was employed in the EPM task. To determine which neuroanatomical substrates related to memory formation are altered due to NCAM deficiency, we decided to map the distribution of FosB/ΔFosB-positive cells in the brain regions which seemed to be involved in memory functions. The inducible transcription factor FosB and its truncated form ΔFosB (35–37 kDa) have been found to have prolonged induction characteristics and might be the markers of chronic activation of neuronal populations (Chen et al., 1997; Nestler et al., 2001).

2. Materials and methods

2.1. Animals

All experiments were undertaken in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86 / 609 / EEC). All experiments were carried out by individuals who hold an appropriate license. The NCAM-knockout (NCAM−/−) mice and control mice used for this study were obtained by crossing C57BL/6-Ncam tm1Cgn+/− heterozygotic mice purchased from Jackson Laboratories (Maine, USA). F2-generation NCAM−/− mice and their wild-type (NCAM+/+) littermates at the ages of 4–6 months and with an average weight of 22.0 g were used. All animals were housed under standard housing conditions: the mice were group housed (five mice per cage) under a 12 h light–dark cycle. All mice had free access to food and water. The experiments with the open-field test, object-recognition test, and fear-conditioning test were performed on the same groups of animals. The EPM test and hot-plate test were assessed on separate, naive and previously unhandled groups of animals.

2.2. Open-field test

Open-field activity was determined between 09.00 and 11.00. The open-field apparatus consisted of a four-sided 52 cm × 52 cm × 50 cm (L × W × H) dark colored wooden box which was covered inside with foil to increase the reflectivity of the walls. The floor of the box was divided into 16 squares (Swiergiel and Dunn, 2006; Hefner and Holmes, 2007). Four squares were defined as the central area and the twelve squares along the walls as the periphery. Each mouse (n = 12 in each group) was gently placed into the very center of the box and left to freely explore for 5 min and its activity was scored as a line crossing when a mouse removed all four paws from one square and entered another. Line crossings in the central four squares and in the peripheral twelve squares of the open field were counted separately. During the test the number of passed squares, time spent on the central squares and the number of rearings were measured. All behaviors were recorded manually. After each animal the test cage was cleaned with a 5% ethanol solution and water to remove any olfactory cues.

2.3. Object-recognition test

The novel object recognition task is based on the innate tendency of mice to differentially explore novel objects over familiar ones (Arqué et al., 2008).

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 dimly lit by a constant illumination of about 60 lux in the test arena. The objects chosen were porcelain cups of different shapes and sizes; these objects were heavy enough to prevent the mice from moving them. All objects presented similar textures and colors but distinctive shapes. All animals (n = 12 in each group) were given a habituation session where they freely explored the open field for 5 min. No objects were placed in the box during the habituation session.

Twenty-four hours after habituation, training was conducted by placing individual mice into the field for 5 min, into which two identical objects (objects A and A1) were positioned in two adjacent corners, 10 cm from the walls. The amount of time spent exploring both objects A and A1 was recorded. Short-term memory (STM) and long-term memory (LTM) tests were performed 1.5 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 and C for LTM) object and time 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) $(T_{new} \times 100)/(T_A + T_{new})$, where T_A and T_{new} are the times spent exploring familiar object A and the novel object, respectively.

Between trials, the objects were cleaned with 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.

2.4. Hot-plate test

The sensitivity of animals to painful stimuli was tested in the hot-plate test. The hot plate was heated to 55 °C ± 0.5 °C. Each mouse was placed into a transparent beaker made of Plexiglas with a height of 20 cm and a diameter of 14.5 cm to avoid the animals escaping from the plate on the hot-plate apparatus. The latency to licking of the paws or jumping was recorded as response latency. A 30 s cut-off time was used to prevent tissue damage to the paws. After each animal the plate and the baker were cleaned with 5% ethanol solution.

2.5. Contextual and tone fear-conditioning test

Learning and memory in NCAM-knockout mice were also examined using contextual and cued fear-conditioning paradigms. For fear conditioning, an experimental chamber 22 cm × 16 cm × 16 cm (L × W × H) was used. Three sides of the box were made of non-transparent plastic and the fourth side was made of transparent plastic. The floor was made of stainless steel rods designed for mice and connected to a scrambled shock generator (TSE Systems, Germany). The chamber contained a speaker through which audible tones were delivered.

The chamber was housed inside a larger noise-attenuating box and a ventilation fan provided background noise. After each trial the chamber was cleaned with 5% ethanol solution. On the first day, each mouse (n = 12 in each group) was allowed to explore the conditioning chamber during a 3 min contextual pre-exposure session. After this, three conditioned stimulus (CS)/unconditioned stimulus (US) pairings were given with 1 min intervals and freezing during the CS was also recorded. The CS was an 85 db, 2800 Hz, 20 s tone and the unconditioned stimulus was a scrambled foot shock at 0.75 mA presented during the last 3 s of the CS. Freezing was defined as the absence of any movement other than that due to respiration. Each mouse was returned to the shock chamber 24 h later and freezing

responses were recorded for 3 min (contextual testing). Two hours later each mouse was tested for freezing to the conditioned tone in a modified chamber. The modified chamber included a white Plexiglas floor and a triangular insert which altered the lighting. Freezing behavior was recorded for 3 min before and during three CS presentations (cued conditioning). The time of freezing was recorded in seconds. Six hours after exposure of animals to CS (tone) presentations, animals were submitted for FosB/ΔFosB immunohistochemistry.

2.6. The elevated plus-maze test

The EPM was made of metal and black plastic and the apparatus consisted of two opposite open arms (45 cm × 10 cm) without side walls and two enclosed arms (45 cm × 10 cm × 30 cm) with sides and end walls, extending from a central square (10 cm × 10 cm). The maze was elevated to a height of 60 cm above the floor and placed in a dimly lit room (8 lux as measured at the center of the maze). At the onset of the test, the animals (n = 10 in each group) were placed in the center of the EPM facing towards an open arm and the experimenter recorded the number of open and closed arm entries, as well as the time spent on either type of the arms, during a 5 min test period . An entry was counted when animal was on an arm with all four paws. The maze was cleaned with 5% ethanol solution and water after each trial. All experiments were also recorded on video and later analyzed by each minute. Besides minute-by-minute scoring, a test/retest protocol was put into practice. Twenty-four hours after the first trial (Trial 1) a second trial was performed (Trial 2). All tests were performed at the same time of day, between 11.00 and 14.00, and with the same experimenter. The percentage of entries onto open arms from total arm entries and the percentage of time spent on the open arms were taken as measures of anxiety. In addition, the total number of entries was scored as a measure of locomotor activity.

2.7. FosB/ΔFosB immunohistochemistry

To determine whether conditioned fear-related neuronal activity is induced in NCAM-knockout mice, animals of both genotypes (n = 5), which had been exposed to CS in the tone-induced fear-conditioning task, were taken for FosB/ΔFosB immunohistochemistry. In addition, naive wild-type (n = 5) and NCAM-knockout mice (n = 5) were taken for immunohistochemical determination of FosB/ΔFosB and served as controls. Mice were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and transcardially perfused with 0.9% saline and then with 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.1 M, pH 7.4). After a post-fixation of the brain in paraformaldehyde/PBS solution for 24 h, 40 μm thick sections were cut on a vibromicrotome (Leica VT1000S, Germany), placed in PBS and kept at 4 °C until further processing.

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

2.7.1. Quantification of FosB/ΔFosB-positive cells

The number of FosB/ΔFosB-positive nuclei was counted in the following brain areas according to the Mouse Brain Atlas (Paxinos and Franklin, 2001): prefrontal cortex (PFC) and frontal cortex (FC), dentate gyrus of hippocampus (DG), basolateral nucleus of amygdala (BLA), basomedial nucleus of amygdala (BMA) and piriform cortex (Pir). For each structure, four random sections per animal were taken and positive nuclei were manually counted according to the optical fractionation method (West, 1993) where the number of counting frames in the delineated region was randomly applied by the CAST program (Olympus, Denmark). Counting was performed using an Olympus BX-51 microscope. The immunoreactivity was expressed as the number of positive nuclei per 0.1 mm^2 of brain region. The experimenter was blind to the experimental grouping at all stages of the assessments.

2.8. Statistics

All values are presented as mean ± SEM. Statistical analysis was performed using either the Student's t-test or two-way ANOVA followed by Bonferroni post-hoc test, where appropriate. Any p-value less than 0.05 was considered significant.

3. Results

3.1. Open-field test

Behavior of the wild-type and NCAM-knockout mice was assessed in the open-field test. There were no statistically significant differences in locomotor activity between groups. The NCAM-knockout animals displayed similar numbers of line crossings when compared to the wild-type mice (Fig. 1). However, there were significant differences between groups in the time spent in the central areas of the field ($p<0.05$) and in rearing activities ($p<0.05$) (Fig. 1). Neuronal cell adhesion molecule (NCAM)-knockout mice spent less time in the center of the open field and showed significantly higher rearing activities as compared with wild-type littermates ($p<0.05$) (Fig. 1).

3.2. Object-recognition test

In the object-recognition test, NCAM−/− mice spent significantly ($p<0.01$) more time exploring both objects during the training phase (Table 1). No significant preference was observed for either of the two identical objects during the training phase in NCAM−/− and their wild-type littermates (data not shown). During STM and LTM phases wild-type animals spent significantly more time exploring the new objects, as was reflected by their preference ratios (Fig. 2). No preferences for novel object recognition were observed for NCAM-knockout mice in both STM and LTM tests (Fig. 2).

3.3. Fear-conditioning tests

The baseline freezing times prior to fear-conditioning were similar in NCAM−/− mice and their wild-type littermates (3.46 ± 0.33 and 4.90 ± 0.9 s, respectively). Compared to wild-type mice NCAM−/− mice demonstrated significantly less freezing if they were exposed to the same context 24 h later ($p<0.05$) (Fig. 3). In the tone-cued test, baseline (pre-tone) freezing was slightly but significantly ($p<0.05$) lower in NCAM−/− mice as compared to wild-type mice. When the animals were exposed to CS (tone) in a new context, marked differences in the freezing times were observed: freezing time in NCAM−/− mice was considerably lower ($p<0.001$) as compared with wild-type mice (Fig. 3). To ensure that the performance of NCAM −/− animals in the fear-conditioning task was not affected by differences in pain sensitivity, wild-type and NCAM-knockout mice were exposed to the hot-plate test. The experiment showed that there was no significant difference in pain sensitivity between wild-type and NCAM-knockout mice. The latency to lick the paws in wild-type mice was 12.20 ± 0.7 s and in NCAM−/− mice it was 12.64 ± 0.8 s (NS, n = 12).

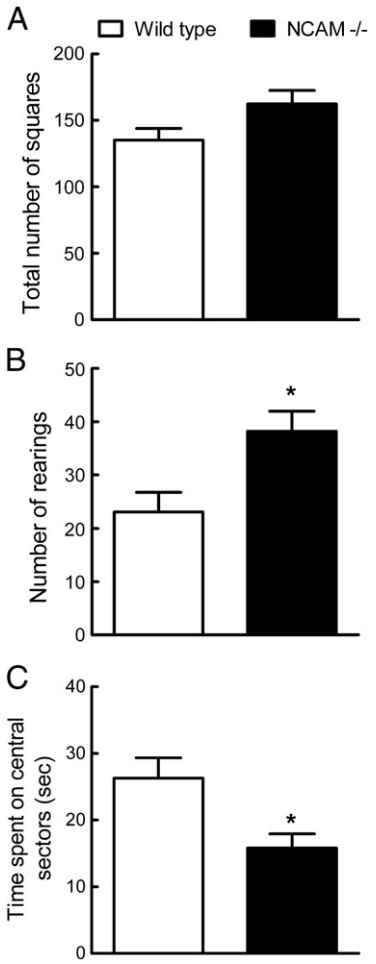

Fig. 1. Open-field test performance of NCAM-knockout mice and their wild-type littermates. Total number of passed squares (A), rearing activity (B) and time spent on the central sectors of the field (C) are shown. Data expressed as mean ± SEM (n = 12) *p<0.05 (Student's t-test) as compared to wild-type mice.

3.4. Elevated plus-maze (EPM) test

Next, we tested NCAM−/− mice for levels of anxiety using the elevated plus-maze test. In this test, NCAM−/− mice demonstrated an increased percentage of entries onto open arms and an increased percentage of time spent on the open arms of the elevated plus-maze test during the 5 min test period as compared with wild-type animals (Fig. 4). They also had a higher number of total entries onto the arms of the EPM. This would suggest that NCAM−/− mice have lower level of innate anxiety than their wild-type littermates. Previous studies have suggested that a rapid learning of the aversive properties of the open arms in the EPM occurs during a 5 min test period and that this might have an impact on the performance of the animals in the test

Table 1
Total time (s) of NCAM-knockout and wild-type mice spent exploring both objects in the object recognition test.

Session	Group	Time exploring objects (s)
Training	Wild-type	17.7 ± 1.3
	NCAM−/−	28.2 ± 2.3***
Short-term memory	Wild-type	8.2 ± 0.8
	NCAM−/−	18.7 ± 2.4***
Long-term memory	Wild-type	8.6 ± 1.0
	NCAM−/−	14.4 ± 2.5*

The data are expressed as mean ± SEM. *p<0.05; ***p<0.0001 (Student's t-test), n = 12.

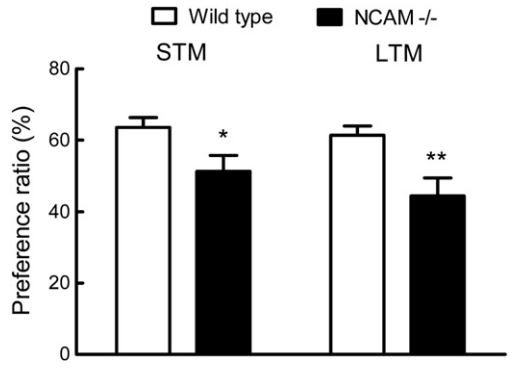

Fig. 2. Exploratory preference (%) of NCAM-knockout mice and their wild-type littermates in the novel object-recognition test of short-term memory (STM) and long-term memory (LTM) tasks. Data expressed as mean ± SEM (n = 12) *p<0.05, **p<0.01 (Student's t-test) as compared to wild-type mice.

(Carobrez and Bertoglio, 2005). To test such a possibility, we compared the scores of the percentage of entries onto the open arms of the plus-maze by a minute-by-minute basis and found that in wild-type mice, the percentage of entries onto the open arms was high during the first minute of the test but then dropped dramatically throughout the following 4 min of the 5 min test (Fig. 5). Thus, in wild-type mice the test situation led to a gradual increase in avoidance behavior and the decision to stay inside the safer part of the maze, the enclosed arms, and suggests that some learning might have occurred during the testing session. In contrast, NCAM−/− mice demonstrated a stable and high percentage of entries onto the open arms throughout the 5 min test period (Fig. 5). No significant effect of

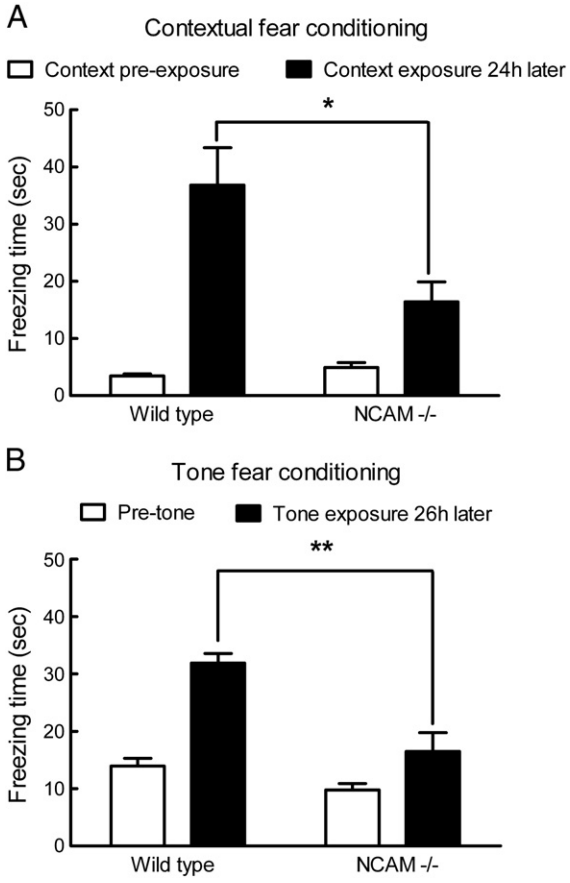

Fig. 3. Contextual (A) and tone fear conditioning (B) of NCAM-knockout mice and their wild-type littermates. Data expressed as mean ± SEM (n = 12) of freezing time (s) *p<0.05, **p<0.01 (Student's t-test) as compared to wild-type mice.

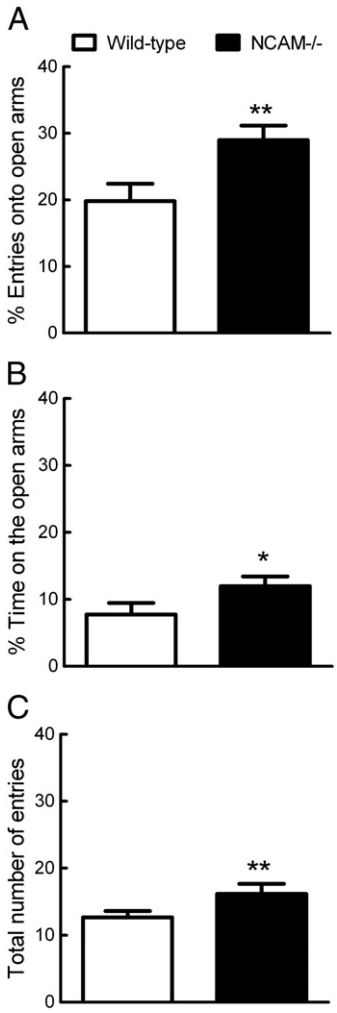

Fig. 4. Behavior of NCAM-knockout mice and their wild-type littermates on the elevated plus-maze test. Percentage of entries onto open arms (A), percentage of time spent on the open arms (B) and total number of entries for detection of locomotion (C) were scored. Data expressed as mean ± SEM (n = 10) *p<0.05; **p<0.01; (Student's t-test) as compared to wild-type mice.

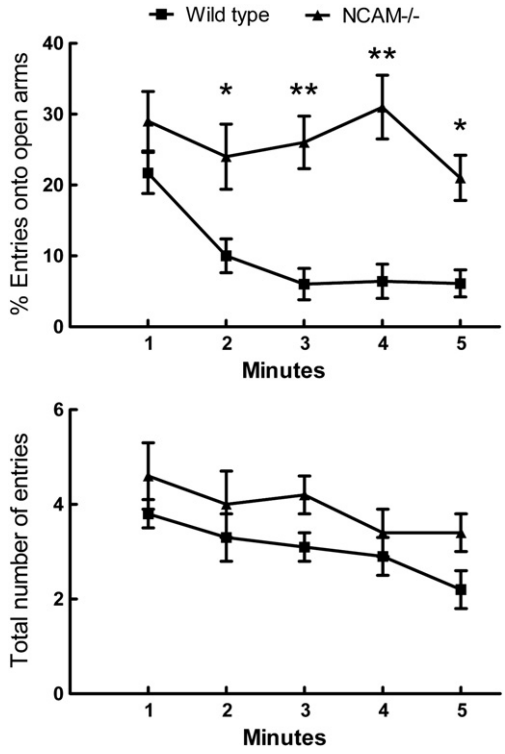

Fig. 5. Behavior of NCAM-knockout mice and their wild-type littermates on the elevated plus-maze test using minute-by-minute scoring. The figure shows the percentage of entries onto the open arms (left) and the number of total entries (right). Data expressed as mean ± SEM (n = 10) *p<0.05; **p<0.01; (Student's t-test) as compared to wild-type mice.

genotype on the total number of entries at each time point during the 5 min session was found. Previous studies have demonstrated that re-exposure of rats to EPM results in a dramatic reduction of the percentage of entries onto open arms, which represents an avoidance learning response to open arms (Bertoglio and Carobrez, 2000). Therefore, in our experiments, we re-exposed NCAM−/− mice and their wild-type littermates to the EPM. The results of this study are shown in Fig. 6. Two-way ANOVA of the percentage of entries showed a significant effect of genotype ($F1.36 = 280.7$, $p<0.001$) and a significant effect of trial ($F1.36 = 10.0$, $p<0.01$) but no significant interaction between genotype and trial ($F1.36 = 3.8$, $p = 0.059$). Post-hoc analysis revealed that re-exposure to the EPM reduced the percentage of entries only in wild-type mice and not in NCAM−/− mice (Fig. 6). Analysis of the percentage of time spent on the open arms also demonstrated a significant effect of genotype ($F1.36 = 280.7$, $p<0.001$) and a significant effect of trial ($F1.36 = 9.9$, $p<0.05$), but no significant interaction between genotype and trial ($F1.36 = 3.8$, $p = 0.06$). Re-exposure of animals to the EPM did not affect the total number of entries onto both open and closed arms as compared with the first trial (Fig. 6). However, there was a significant effect of genotype ($F1.36 = 146.4$, $p<0.001$) suggesting that NCAM−/− mice had higher locomotor activity as compared with their wild-type littermates.

3.5. FosB/ΔFosB expression in wild-type and NCAM−/− mice

Next, we evaluated the distribution of FosB/ΔFosB-positive cells in the different brain regions of control mice and mice which had been exposed to tone fear-conditioning. Mice with a constitutional deficiency of NCAM demonstrated a significantly higher number of cells expressing FosB/ΔFosB in PFC, FC and BLA. Two way-ANOVA revealed a significant effect of genotype (PFC: $p<0.01$, $F1,16 = 57.03$; FC: $p<0.01$, $F1,16 = 61.45$; BLA: $p<0.05$, $F1,16 = 7.336$), a significant effect of tone fear conditioning (PFC: $p<0.01$, $F1,16 = 124.1$; FC: $p<0.01$, $F1,16 = 110.0$; BLA: $p<0.05$, $F1,16 = 407.3$) and a significant interaction between genotype and tone fear conditioning (PFC: $p<0.01$, $F1,16 = 121.4$; FC: $p<0.01$, $F1,16 = 107.6$; BLA: $p<0.05$, $F1,16 = 473.3$). The Bonferroni post-hoc test revealed an effect of tone fear conditioning on the number of FosB/ΔFosB positive nuclei only in control mice and not in NCAM−/− mice (Fig. 7, Table 2). There were no differences in the number of FosB/ΔFosB-positive cells between wild-type and NCAM−/− mice in BMA, DG and Pir (Fig. 7, Table 2). Re-exposure of wild-type mice, which had been conditioned for fear to the CS, induced an increase in the number of FosB/ΔFosB-positive cells in PFC ($p<0.001$), FC ($p<0.01$) and BLA ($p<0.001$). Re-exposure of NCAM−/− mice to the conditioned stimulus did not affect FosB/ΔFosB cell counts in any structure under study (Fig. 7, Table 2).

4. Discussion

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

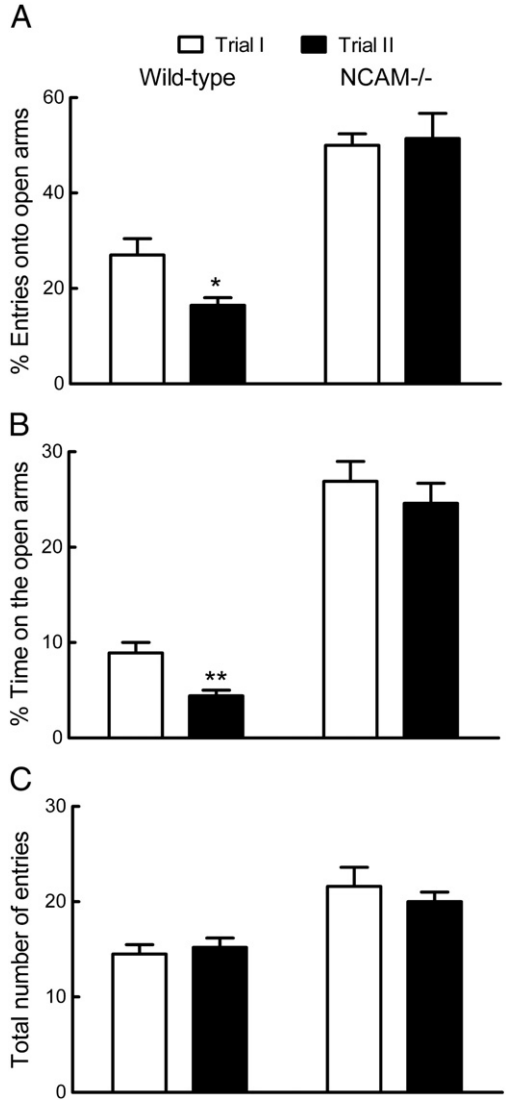

Fig. 6. Behavior on the elevated plus-maze in test/re-test scoring tasks of the NCAM-knockout mice and their wild-type littermates. Percentage of entries onto open arms (A), percentage of time on the open arms (B) and total number of entries for locomotion (C) in Trial I and Trial II are shown. Data expressed as mean ± SEM (n = 10) *p<0.05; **p<0.01; (two-way ANOVA followed by Bonferroni post-hoc test) comparing performances of Trial I and Trial II.

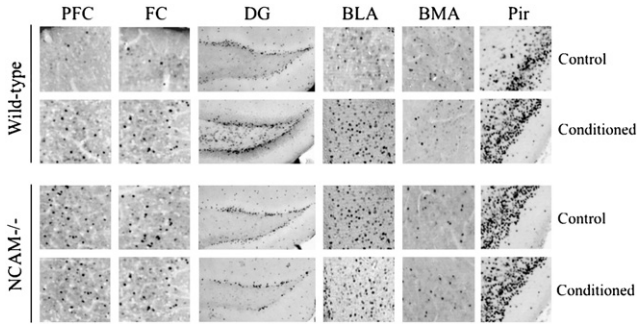

Fig. 7. Representative microphotograph demonstrating FosB/ΔFosB-positive cells in wild-type and NCAM-knockout mice 6 h after the presentation of CS in the tone-fear conditioning task in the following brain regions: prefrontal cortex (PFC), frontal cortex (FC), dentate gyrus of hippocampus (DG), basolateral nucleus of amygdala (BLA), basomedial nucleus of amygdala (BMA) and piriform cortex (Pir). During training mice received CS (tone) with US (shock) and 24 h later they were tested for freezing behavior. Six hours after re-exposure to CS the brain tissue was harvested for immunohistochemical detection of FosB/ΔFosB. Animals unexposed to fear conditioning were used as naive controls for both genotypes (magnification ×100 for PFC, FC, BLA BMA and Pir; magnification ×40 for DG).

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

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

During training phase mice received CS (tone) with unconditioned stimulus (US) (shock) and 24 h later were re-exposed to the CS and the duration (s) of freezing behaviour was measured. Six hours after re-exposure to CS the brain tissue was harvested for immunohistochemical detection of FosB/ΔFosB. Animals unexposed to tone fear conditioning were used as naive controls for both genotypes. The numbers of FosB/ΔFosB-positive nuclei were counted in the following brain areas: prefrontal cortex (PFC) and frontal cortex (FC), dentate gyrus of hippocampus (DG), basolateral nucleus of amygdala (BLA), basomedial nucleus of amygdala (BMA) and piriform cortex (Pir). The data are expressed as mean ± SEM of the number of positive cells per 0.1 mm^2. Each group consisted of 5 mice. ***p<0.001 as compared with corresponding control; #p<0.05, ###p<0.001 as compared to wild-type mice from control group (Two-way ANOVA, Bonferroni post-hoc test).

results corroborate earlier studies in which the NCAM−/− mice showed impaired learning (Cremer et al., 1994; Bukalo et al., 2004).

Previous studies also demonstrated that deficiency in the *NCAM* gene results in increased inter-male aggression (Stork et al., 1997), depression-like behavior (Aonurm-Helm et al., 2008) and anxiety-like behavior (Stork et al., 1999). In the experiments of Stork et al. (1999), the anxiety-like behavior was evidenced by an increased preference for the dark compartment in the light/dark avoidance test (Stork et al., 1999). When the NCAM-knockout mice were exposed to the EPM they demonstrated an increased preference for open arms, which is suggestive of anxiolytic-like behavior (Stork, et al., 1999, 2000). Thus, it seemed that the performance of the NCAM-knockout mice in the tests for anxiety was dependent on the test employed. Our study also demonstrated some discrepancies when we measured the anxiety-like behavior in the open field and EPM. The open-field test may produce moderate anxiety by the mice being placed in an open space without the possibility of escape (Prut and Belzung, 2003). Indeed, in our experiments NCAM−/− mice showed a reduced number of entries into the central area, which can be interpreted as an indicator of increased anxiety. They also demonstrated increased exploratory activity in the open field, as was evidenced by an increased number of rearings and an increased number of total sectors crossed. On the other hand, when the NCAM-knockout mice were exposed to the EPM task they demonstrated an increased percentage of entries onto and an increased percentage of time spent on the open arms of the EPM, which would indicate anxiolytic-like behavior. Since we used relative measures expressed as a percentage of entries and time, an increased exploratory behavior, which indeed was observed in the NCAM-knockout mice, should have little if any impact on these relative measures. Previous studies using precise spatiotemporal analysis employing minute-by-minute analysis of the rodent's behavior in the EPM task demonstrated a continuous increase in open arm avoidance during a 5 min session. These data demonstrated that during exposure to the EPM task, animals quickly learn aversive properties of the open arms, implying that learning can occur during the EPM session (Carobrez and Bertoglio, 2005). Therefore, we performed a minute-by-minute analysis of the behavior of NCAM-knockout and wild-type littermates in the EPM task. Indeed, the analysis showed that the wild-type animals demonstrated a high percentage of entries onto the open arms only during the first minute of the EPM task. During the last 4 min the percentage of entries onto the open arms declined considerably, suggesting that learning of the aversive properties of the open arms occurred. In contrast, the NCAM-knockout animals did not demonstrate

any decline in the percentage of entries during the 5 min session. This shows that the NCAM-knockout mice were unable to learn or to recognize the aversive properties of the open arms and that the observed anxiolytic-like behavior was largely due to this dysfunctional cognition. This proposal was further confirmed when the test/re-test protocol of the EPM task was employed. In the second EPM trial, wild-type animals demonstrated clear avoidance of the open arms. This indicates that mice acquired information related to the exploration of potentially dangerous areas of the maze during the first trial, and that they consolidated it and retrieved it during the second session. These data are consistent with those previously found by others (File, 1990; Lister, 1990; Treit et al., 1993; Bertoglio and Carobrez, 2000). By contrast, when the NCAM-knockout mice were exposed to the test/re-test paradigm of the plus-maze, no evidence for habituation was observed.

Abundant evidence suggests that the expression of families of immediate early genes such as fos, fra, jun, krox and zif are critical in changing the expression of genes which are important to memory formation (Dragunow et al., 1989; Hall et al., 2001; Moller et al., 1994, Tischmayer and Grimm, 1999). In contrast to c-fos, which is transiently induced upon stimulation, FosB and its truncated form ΔFosB, generated by alternative splicing of the FosB transcript, have prolonged induction characteristics and may be markers of chronic neuronal activation (Chen et al., 1997). Our experiments showed that re-exposure of wild-type mice to CS (tone) induced expression of FosB/ΔFosB in discrete neuronal populations within the PFC, FC and BLA, and confirmed that FosB/ΔFosB is also induced by memory retrieval in regions which are related to the formation of fear memories (Hall et al., 2001). In contrast, fear conditioning in the NCAM−/− mice did not induce FosB/ΔFosB expression in the BLA, PFC or FC, as was seen in wild-type mice. Furthermore, NCAM−/− mice under basal conditions demonstrated significantly higher numbers of FosB/ΔFosB-positive cells in the BLA, PFC and FC as compared with wild-type littermates. Assuming that FosB/ΔFosB might serve as relatively stable indicators of neuronal activity (Chen et al., 1997), our data might suggest that in NCAM-knockout mice, structures involved in fear memory formation are constantly being activated and their activation interferes with their ability to form new fear-related memories. Previous studies have demonstrated an increased Fos protein expression in PFC and BLA following the second but not first exposure to the EPM test in rats (Albrechet-Souza et al., 2008). Therefore, it seems that alterations in the neuronal activity in the PFC and BLA due to NCAM deficiency play a role in both cognition and performance of animals following their second exposure to the EPM.

In conclusion, the data obtained in the present study show that the anxiolytic-like behavior of NCAM-knockout mice in the EPM is largely related to their inability to recognize and learn the danger associated with the open arms. Thus, in animals with impaired cognition the EPM cannot be considered as a valuable test for assessing innate anxiety, but rather it reflects the impaired cognitive functions of the animals.

Acknowledgements

The study was supported by the EU 6FWP grant LSHM-CT-2005-512012 (Promemoria), EU 7FWP grant HEALTH-F2-2008-223077 (NEUROPRO), Estonian Science Foundation Grant 7955, the European Regional Development Fund and the Archimedes Foundation.

References

Aonurm-Helm A, Jurgenson M, Zharkovsky T, Sonn K, Berezin V, Bock E, et al. Depression-like behavior in neural cell adhesion molecule (NCAM)-deficient mice and its reversal by an NCAM-derived peptide, FGL. Eur J Neurosci 2008;28:1618–28.

Albrechet-Souza L, Borelli KG, Brandão ML. Activity of the medial prefrontal cortex and amygdala underlies one-trial tolerance of rats in the elevated plus-maze. J Neurosci Meth 2008;169:109–18.

Arqué G, Fotaki V, Fernández D, Martínez de Lagrán M, Arbonés ML, Dierssen M. Impaired spatial learning strategies and novel object recognition in mice haploinsufficient for the dual specificity tyrosine-regulated kinase-1A (Dyrk1A). PLoS One 2008;7:e2575.

Bertoglio LJ, Joca LSR, Guimarães FS. Further evidence that anxiety and memory are regionally dissociated within the hippocampus. Behav Brain Res 2006;175:183–8.

Bertoglio LJ, Carobrez AP. Previous maze experience required to increase open arm avoidance in rats submitted to the elevated plus-maze model of anxiety. Behav Brain Res 2000;108:197–203.

Bukalo O, Fentrop N, Lee AYW, Salmen B, Law JWS, Wotjak CT, et al. Conditional ablation of the neural cell adhesion molecule reduces precision of spatial learning, long-term potentiation and deptession in the CA1 subfield of mouse hippocampus. J Neurosci 2004;24:1565–77.

Calzavara MB, Lopez GB, Abílio VC, Silva RH, Frussa-Filho R. Role of anxiety levels in memory performance of spontaneously hypertensive rats. Behav Pharmacol 2004;15:545–53.

Calzavara MB, Patti CL, Lopez GB, Abílio VC, Silva RH, Frussa-Filho R. Role of learning of open arm avoidance in the phenomenon of one-trial tolerance to the anxiolytic effect of chlordiazepoxide in mice. Life Sci 2005;76:2235–46.

Carobrez AP, Bertoglio LJ. Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neurosci & Biobehav Rev 2005;29:1193–205.

Carrasco M, Vicens P, Redolat R. Effects of co-administration of bupropion and nicotinic agonists on the elevated plus-maze test in mice. Prog Neuropsychopharmacol Biol Psychiatry 2006;30:455–62.

Chen J, Kelz MB, Hope BT, Nakabeppu Y, Nestler EJ. Chronic Fos-related antigens: stable variants of delta FosB induced in brain by chronic treatments. J Neurosci 1997;17:4933–41.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, et al. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 1994;367:455–9.

Dragunow M, Abraham WC, Goulding M, Mason SE, Robertson HA, Faull RL. Long-term potentiation and the induction of c-fos mRNA and proteins in the dentate gyrus of unanesthetized rats. Neurosci Lett 1989;101:274–80.

File SE. One trial tolerance to the anxiolytic effect of chlorodiazepoxide in the plus-maze. Psychopharmacology 1990;100:281–2.

Hall J, Thomas KL, Everitt B. Fear memory retrieval induces CREB phosphorylation and fos expression within the amygdala. Eur J Neurosci 2001;13:1453–8.

Hefner K, Holmes A. Ontogeny of fear-, anxiety- and depression-related behavior across adolescence in C57BL/6 J mice. Behav Brain Res 2007;176:210–5.

Hogg S. A review of the validity of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 1996;54:21–30.

Johnston AL, File SE. Sodium phenobarbitone reverses the anxiogenic effects of compounds acting at three different central sites. Neuropharmacology 1989;28:83–8.

Kiss JZ, Troncoso E, Djebbara Z, Vutskits L, Muller D. The role of neural cell adhesion molecules in plasticity and repair. Brain Res Rev 2001;36:175–84.

Lister R. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 1987;92:180–5.

Lister R. Ethologically-based animal models of anxiety disorders. Pharmacol Ther 1990;46:321–40.

Massé F, Dhonnchadha BÁN, Hascoët M, Bourin M. Anxiolytic-like effect of 5-HT2 ligands and benzodiazepines co-administration: comparison of two animal models of anxiety (the four-plate test and the elevated plus maze). Behav Brain Res 2007;177:214–26.

Moller C, Bing O, Heilig M. c-Fos expression in the amygdala: in vivo antisense modulation and role in anxiety. Cell Mol Neurobiol 1994;14:415–23.

Nestler EJ, Barrot M, Self SW. ΔFosB: a molecular switch for addiction. Proc Nat Acad Sci USA 2001;98:11042–6.

Paxinos G, Franklin KBJ. Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press; 2001.

Pellow S, Chopin P, File SE, Briley M. Validation of open-closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Meth 1985;14:149–67.

Persohn E, Schachner M. Immunoelectron-microscopic localization of the neural cell adhesion molecules L1 and N-CAM during postnatal development of the mouse cerebellum. I Cell Biol 1987;105:569–76.

Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003;463(1–3):3-33.

Rodgers RJ, Dalvi A. Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev 1997;21:801–10.

Rougon G. Structure, metabolism and cell biology of polysialic acids. Eur J Cell Biol 1993;61:197–207.

Roullet P, Mileusnic R, Rose SPR, Sara SJ. Neural cell adhesion molecules play a role in rat memory formation in appetitive as well as aversive tasks. Neuroreport 1997;8:1907–11.

Rønn LCB, Berezin V, Bock E. The neural cell adhesion molecule in synaptic plasticity and ageing. Int J Develop Neurosci 2000;18:193–9.

Rutishauser U. Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. Nat Rev Neurosci 2008;9:26–35.

Schuster T, Krug M, Stalder M, Hackel N, Gerardy-Schahn R, Schachner M. Immunoelectron microscopic localization of the neural recognition molecules L1, NCAM, and its isoform NCAM180, the NCAM-associated polysialic acid, beta1 integrin and the extracellular matrix molecule tenascin-R in synapses of the adult rat hippocampus. J Neurobiol 2001;49:142–58.

Seki T, Arai Y. Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci Res 1993;17:265–90.

Stork O, Welzl H, Cremer H, Schachner M. Increased intermale aggression in mice deficient for the neural cell adhesion molecule (NCAM). Eur J Neurosci 1997;9: 1117–25.
Stork O, Welzl H, Wolfer D, Schuster T, Mantei N, Stork S, et al. Recovery of emotional behavior in neural cell adhesion molecule (NCAM) null mutant mice through transgenic expression of NCAM 180. Eur J Neurosci 2000;12:3291–306.
Stork O, Welzl H, Wotjak CT, Hoyer D, Delling M, Cremer H, et al. Anxiety and increased 5-HT1A receptor response in NCAM null mutant mice. J Neurobiol 1999;40:343–55.
Swiergiel AH, Dunn AJ. Feeding, exploratory, anxiety- and depression-related behaviors are not altered in interleukin-6-deficient male mice. Behav Brain Res 2006;171:94-108.
Tischmayer M, Grimm R. Activation of immediate early genes and memory formation. Cel Mol Life Sci 1999;55:564–74.
Treit D, Menard J, Royan C. Anxiogenic stimuli in the elevated plus-maze. Pharmacol biochem Behav 1993;44:463–9.
Vargas KM, Da Cunha C, Andreatini R. Amphetamine and pentylenetetrazole given post-trial 1 enhance one-trial tolerance to the anxiolytic effect of diazepam in the elevated plus-maze in mice. Prog Neuropsychopharmacol Biol Psychiatry 2006;30: 1394–402.
Walmod PS, Kolkova K, Berezin V, Bock E. Zippers make signals: NCAM-mediated molecular interactions and signal transduction. Neurochem Res 2004;29:2015–35.
Welzl H, Stork O. Cell adhesion molecules: key players in memory consolidation? News Physiol Sci 2003;18:147–50.
West MJ. New stereological methods for counting neurons. Neurobiol Aging 1993;14: 275–85.