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A synthetic peptide ligand of NCAM affects exploratory behavior and memory in rodents

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Abstract

The neural cell adhesion molecule (NCAM) is an important modulator of neuronal development and plasticity associated with learning and memory. Previously, a synthetic peptide ligand of NCAM, called C3, has been identified and shown to modulate neuronal plasticity in vitro and memory in a step-through passive avoidance task in rats in vivo. In this study, we extended these findings and found that intraventricular injection of C3 prior to training impaired learning or memory processes in rats and mice in an approach avoidance task and decreased exploratory behavior in rats. The effect of C3 was additionally evaluated in the Morris water maze; memory impairment was observed in the second training trial 24 h after the injection of C3 only, indicating an effect on short-term memory. The C3-mediated memory impairment observed in the approach avoidance and water maze tests is suggested to be the result of C3-inhibiting NCAM functions in the brain. This study demonstrates that it is possible to modulate learning/memory processes in rodents in vivo with small synthetic NCAM-binding peptides that induce developmental plasticity in vitro.
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Keywords: NCAM; Approach avoidance; Water-maze; Exploration; Learning; Memory; Behavior

1. Introduction

The neural cell adhesion molecule (NCAM) is a membrane-bound glycoprotein involved in neuronal development and learning/memory. It mediates cell adhesion and intracellular signalling by promoting homophilic and heterophilic binding between cells, as well as to components of the extracellular matrix (Doherty and Walsh, 1994; Fields and Itoh, 1996). NCAM knock-out mice exhibit deficient learning in the Morris water maze, and intracranial injections of NCAM antibodies induce amnesia in rats (Alexinsky et al., 1997; Arami et al., 1996; Doyle et al., 1992; Roullet et al., 1997) and chicks (Mileusnic et al., 1995; Scholey et al., 1993). It is believed that NCAM is a modulator of synaptic plasticity promoting stability and structural remodelling of synaptic connections, events regulated by expression, down-regulation and posttranslational modifications of NCAM. Previously, we have demonstrated that it was possible to modulate NCAM-dependent learning using a synthetic NCAM-binding peptide, C3 (Foley et al., 2000). In vitro, C3 has been shown to modulate neural plasticity by inducing neurite outgrowth and inhibiting cell adhesion (Rønn et al., 1999). Intraventricular injection of this peptide-impaired memory formation in a step-through passive avoidance task in rats when injected within specific time constraints pre- and posttraining (Foley et al., 2000). In the present study, we further investigated the effect of intraventricular injections of this NCAM-binding peptide on memory and behavior in rats and mice using approach avoidance, water maze, motility, open field and rotarod tests.

2. Methods

2.1. Animals

Three-month-old male Wistar rats (250–350 g) from Møllegård, Denmark, were used in these experiments. After surgery, they were housed individually with free access to food and water. The animals were kept in a temperature- and humidity-controlled "Scantainer" (Scanbur, Denmark).

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Female NMRI mice (20–25 g) from Møllegård, Denmark, were also used. They were housed in groups of 10 and allowed free access to food and water. The animals were kept in a temperature- and humidity-controlled "Scantainer."

All experiments were carried out in accordance with the guidelines for care and use of laboratory animals as outlined by the Danish Committee for Experiments on Animals.

2.2. NCAM-binding peptide, C3

We used the NCAM-binding peptide, C3, in a dendrimeric version consisting of four monomers (ASKKPKRNIKA) bound to a backbone of three lysines. This peptide ligand of the NCAM-Ig1 module has previously been identified by screening of a library composed of synthetic peptides bound to resin beads (Rønn et al., 1999). The doses used in this study were based on effective concentrations on neurite outgrowth in vitro (Rønn et al., 1999).

2.3. Intracerebroventricular injections

2.3.1. Surgery

A guide cannula was implanted into the right lateral ventricle at stereotaxic coordinates 1 mm (rats)/0 mm (mice) posterior and 1.4 mm (rats)/0.8 mm (mice) lateral to Bregma (skull in flat position) under deep halothane (rats)/Hypnorm–Dormicum (mice) anesthesia. Analgesics (Carprofen, Rimadyl; Pfizer, Denmark) were given before the surgery. The guide cannula was held in place using dental cement (Dentalon Plus; Agnthos, Sweden). The animals were allowed a minimum of 5 days (rats)/3 days (mice) to recover before testing. For all tests in rats, a 5-μl saline solution of C3 was injected intracerebroventricularly (icv) at a depth of 5 mm below the skull, via the guide cannula at an infusion rate of 2 μl/min using a micropump connected to a polyethylene tube (CMA/Microdialysis, Sweden). For the approach avoidance test in mice, a 1-μl saline solution consisting of 0.1, 0.5 or 2 μg C3 was injected intracerebroventricularly at a depth of 3 mm below the skull, via the guide cannula at an infusion rate of 1 μl/min using a micropump connected to a polyethylene tube (CMA/Microdialysis, Sweden). The animals were allowed to move freely during the injection.

2.4. Test procedures

2.4.1. Rat rotarod

The rotarod consisted of a wooden rod (49 cm in length, 11 cm in diameter) divided into four separate compartments (11.5 cm in width) by plastic discs (41 cm in diameter). Rats were placed on the rotating rod (4 rpm) and latency to fall from the rod within 120 s was taken as a measure of motor deficits. Data were analysed using the nonparametric Kruskal–Wallis test. The rats in this study were the same as those in the water maze experiment, and they were tested on the rotarod on Day 2 of water maze training after the fourth trial (about 24 h after the injection of 0.7 or 2 μg C3).

2.4.2. Rat exploratory motility

Exploratory motility was measured in novel cages (30 × 20 cm), which automatically detected activity when photo beams were interrupted. The boxes consisted of a row of eight opposing emitters and sensors placed 5 cm apart. The activity boxes were connected to a computer for automatic recording of activity counts. A rat was placed in the cage for 30 min and allowed to explore the environment. The animals in this study were those used in the open-field experiments, and motility was measured after the third exposure to the open field (about 24 h after injection of 0.1, 0.7 or 2 μg C3). Data were analysed using parametric one-way analysis of variance (ANOVA).

2.4.3. Rat open field

Spontaneous exploratory behavior and habituation to a novel environment were evaluated in a circular open field (87 cm in diameter, 20 cm in height) made of wood. The floor was subdivided into 12 compartments, each compartment demarcated by lines. A rat was placed in the open field close to the wall and allowed to explore the environment for 3 min before being returned to its home cage. The number of line crossings was taken as a measure of spontaneous activity. Rats were reexposed to the open field for 3 min 3 and 24 h after the initial exposure, and habituation to the open field was determined by the reduction in line crossings compared to the initial exposure. In the current study, we have deemed these 3- and 24-h time points as roughly reflecting short-term and long-term memory, respectively (Cerbone and Sadile, 1994; Vianna et al., 2000). As well as line crossings, rearings were also counted and used as an additional measure of exploratory behavior. C3 was injected intracerebroventricularly once (0.1, 0.7 and 2 μg), 15 min prior to the first exposure to the open field. Data were analysed using parametric two-way ANOVA with repeated measures (time as repeated factor) followed by multiple comparisons.

2.4.4. Rat Morris water maze

The Morris water maze (Morris, 1984) consisted of a circular pool (1.6 m in diameter, 51 cm deep, water temperature of 21 °C and depth of 21 cm) made of black-painted stainless steel. A platform (11 × 11 cm) made of steel grid was submerged approximately 2 cm below the water surface in a fixed position in the northeast (NE) quadrant. Rats were placed in the water and trained to locate the platform on five consecutive days with four successive trials per day. Each trial began from a different start position (north, south, east, west) with the order changed every day), and rats used extramazial cues (paintings on the wall, lamps, location of the experimenter, etc.) for navigation to the platform. On each trial, animals were placed in the water maze with the head facing the wall of the maze and were allowed a maximum of 60 s to locate the hidden platform. Rats failing to locate the platform within 60 s were placed on the platform

by the experimenter and each was allowed to stay there for 15 s before the next trial was initiated. The mean latency of Trials 1–4 was used as a measure of learning over days. Latency on Day 2 was taken as a measure of short-term memory. A probe trial on Day 7 constituted of placing the rat in the water maze with the platform removed and assessing time spent in the NE-quadrant for a maximum of 60 s. This test determined if the rats had a long-term memory for the location of the platform. The swimming of the animals was tracked by a video camera above the pool connected to the Videotrack computer sytem (VIDEOTRACK, version NT; Viewpoint, France). The latency, swimming distance and speed to find the platform were measured automatically. In this study, C3 (0.7 and 2 μg) was injected only once, 10 min before the first training session on Day 1. Data were analysed by parametric two-way ANOVA with repeated measures (time as the repeated factor) followed by pairwise multiple comparisons.

2.4.5. Approach avoidance

Animals were water deprived for 48 h prior to training. On the training day, animals were placed in the test box (rats: 18 × 21 cm, mice: 15 × 15 cm) prior to training to allow habituation to the test environment for a maximum time of 6 min (rats)/10 min (mice). During habituation, animals had free access to a waterspout and were removed from the test box after licking the waterspout for approximately 60 s (rats)/5 s (mice). In the training session, animals were placed in the test box and received a shock (rats: 1.6 mA, mice: 0.3 mA) to the tongue through the waterspout after completing 20 licks for rats and after approximately 5 s of contact with the waterspout for mice. Animals received maximally only five shocks. The animals were allowed free access to water for 1 h (rats)/10 min (mice) in their cages at a random time after the training session and for mice also on the following day. Retention was assessed 24 h or 48 h after the training session in rats and mice, respectively, determining the latency to the 20th lick (rats)/5 s of contact with the waterspout (mice) when animals were reexposed to the boxes. Animals were injected intracerebroventricularly with the peptide, C3, 20 min (rats)/10 min (mice) prior to training. Data from the rat experiment were analysed using parametric *t* test and data from the mouse experiment were analysed using parametric one-way ANOVA followed by multiple comparisons. This procedure has been modified from a water-lick conflict paradigm previously described (Petersen and Lassen, 1981).

3. Results

3.1. Rotarod

In order to evaluate the effect of C3 on sensorimotor function, rats were placed on a rotarod 24 h after injection of C3 (0.7 μg or 2 μg). No statistically significant effects were observed at 0.7 μg or 2 μg (data not shown).

3.2. Motility

Exploratory motility was measured for 30 min in novel boxes 24 h after injection of C3 (see Fig. 1). The results showed no significant change in activity compared to vehicle after C3 treatment (0.1, 0.7 and 2 μg), but a tendency to decrease motility was observed at 0.7 μg.

3.3. Open field

When reexposed to the open field 24 h after the first exposure, rats treated with 0.7 and 2 μg C3 made significantly ($P<.05$) fewer line crosses than vehicle-treated animals (Fig. 2a). There was no significant effect on rearings relative to vehicle treatment (Fig. 2b). At the 24-h time point, animals treated with 0.1, 0.7 and 2 μg C3 showed a significant reduction in line crosses and/or rearings relative to the 0-h time point. Likewise, at the 3-h time point, animals treated with 0.1 μg C3 and vehicle showed a significant reduction in rearings relative to the 0-h time point. On the second exposure 3 h after the treatment, the only effect of C3 was observed on rearings, as 0.1 μg C3 reduced the number of rearings compared to the first exposure ($P<.01$). On first exposure, C3 (0.1, 0.7 or 2 μg) did not have an effect on spontaneous exploratory behavior; neither crossings (Fig. 2a) nor rearings (Fig. 2b) were affected by treatment.

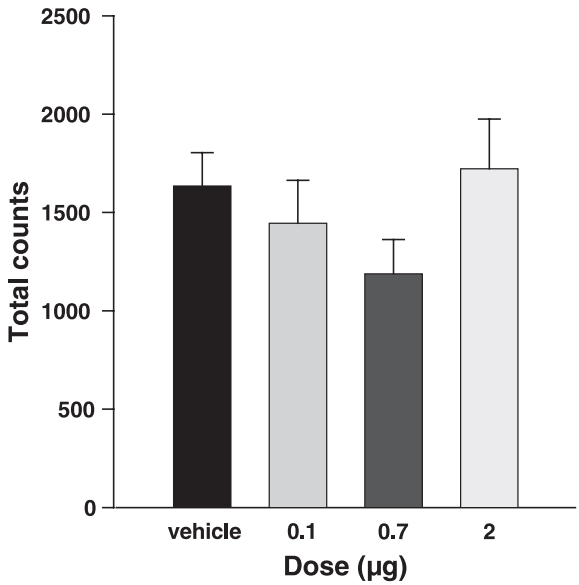

Fig. 1. Effect of C3 on exploratory activity in unfamiliar cages in rats when C3 was injected intracerebroventricularly 24 h prior to testing. Data are depicted as mean + S.E.M. of counts of activity in 30 min ($n=6$).

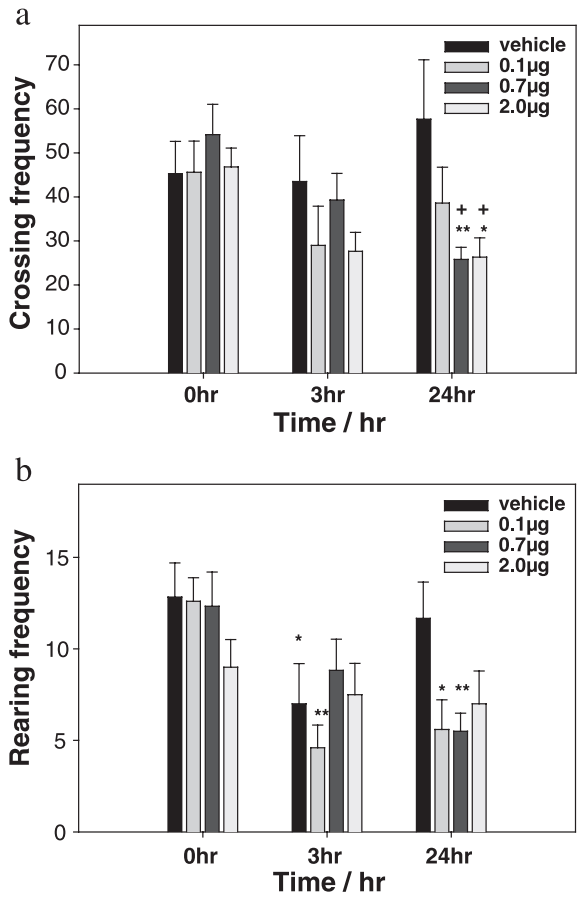

Fig. 2. (a) Effect of C3 on crossings after repeated exposure to an open field. C3 was injected before the first exposure (0 h). Data are depicted as mean + S.E.M. of crossings in 5 min ($n = 6$). Levels of statistical significance are * $P < .05$, * * $P < .01$. (*) Compared to 0 h; (+) compared to vehicle. (b) Effect of C3 on rearings after repeated exposure to an open field. C3 was injected before the first exposure (0 h). Data are depicted as mean + S.E.M. of crossings in 5 min ($n = 6$). Levels of statistical significance are * $P < .05$, * * $P < .01$, when compared to 0 h.

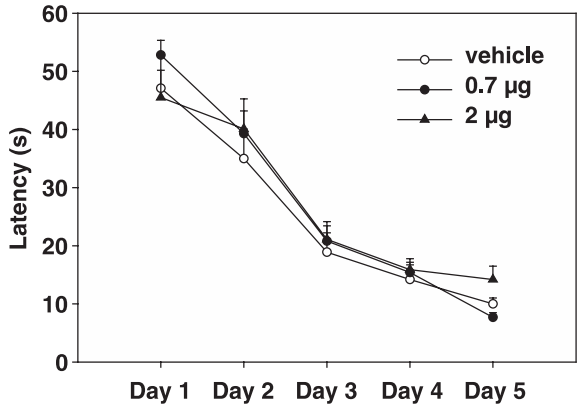

Fig. 3. Effect of C3 on acquisition in a water maze. Data are depicted as mean + S.E.M. of four training trials for latency. C3 was injected prior to the first training session ($n = 8$).

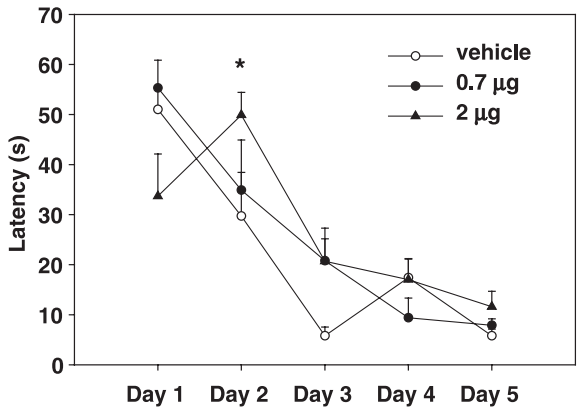

Fig. 4. Effect of C3 on second training trial in a water maze. Data are depicted as mean + S.E.M. for latency. C3 was injected prior to the first training session ($n = 8$). Levels of statistical significance are * $P < .05$.

3.4. Water-maze

The C3 peptide was injected only on the first day of training prior to the first training session, and the results are shown in Figs. 3 and 4 and Tables 1 and 2. No difference in the latency to reach the platform (Fig. 3, Table 1), in distance swum (Table 2) or in speed of swimming (data not shown), was seen between the C3-injected animals and the controls when the means of all four trials on each day were investigated. Furthermore, no effect was observed in the probe trial on Day 7 (data not shown). In order to investigate the effect of C3 on short-term memory, the second trial on each day was considered. When evaluating the second trial on Day 2 (24 h after the injection), the latency to reach the platform and the distance travelled were both significantly increased after injection of 2 µg C3 compared to the control group ($P < .05$) (Fig. 4, Tables 1 and 2), without any effect on speed of swimming (data not shown). At the 0.7-µg dose, a tendency to an increase in latency was also observed over the first 3 days conferred to

Table 1
Water-maze latency

Trial	Dose	Day 1	Day 2	Day 3	Day 4	Day 5
Trial 1	Vehicle	51 ± 6	50 ± 9	35 ± 9	19 ± 7	7 ± 3
	0.7 µg	60 ± 0	58 ± 3	28 ± 7	33 ± 7	9 ± 2
	2 µg	52 ± 5	49 ± 8	28 ± 7	24 ± 7	20 ± 3
Trial 2	Vehicle	51 ± 5	30 ± 9	6 ± 2	17 ± 4	6 ± 1
	0.7 µg	55 ± 6	35 ± 10	21 ± 7	9 ± 4	8 ± 1
	2 µg	33 ± 8	50 ± 5 *	21 ± 4	17 ± 4	12 ± 3
Trial 3	Vehicle	44 ± 7	34 ± 9	17 ± 3	13 ± 5	12 ± 4
	0.7 µg	47 ± 7	36 ± 8	12 ± 3	10 ± 3	6 ± 2
	2 µg	48 ± 8	24 ± 9	16 ± 2	14 ± 4	10 ± 4
Trial 4	Vehicle	42 ± 9	27 ± 6	18 ± 7	11 ± 3	14 ± 5
	0.7 µg	49 ± 8	29 ± 6	22 ± 9	10 ± 2	9 ± 1
	2 µg	48 ± 7	37 ± 9	20 ± 6	9 ± 2	15 ± 7

Latency is shown as mean ± S.E.M. (s) ($n = 8$).
* $P < .05$.

Table 2
Water-maze distance

Trial	Dose	Day 1	Day 2	Day 3	Day 4	Day 5
Trial 1	Vehicle	1297 ± 216	1425 ± 230	1045 ± 289	574 ± 234	164 ± 45
	0.7 μg	1569 ± 118	1755 ± 82	815 ± 235	795 ± 179	177 ± 47
	2 μg	1241 ± 128	1628 ± 255	553 ± 170	582 ± 231	519 ± 93
Trial 2	Vehicle	1253 ± 101	901 ± 291	149 ± 55	522 ± 127	149 ± 26
	0.7 μg	1358 ± 141	932 ± 276	552 ± 150	267 ± 133	188 ± 32
	2 μg	1128 ± 297	1583 ± 137 *	601 ± 149	384 ± 127	316 ± 93
Trial 3	Vehicle	980 ± 186	859 ± 227	467 ± 112	375 ± 138	377 ± 141
	0.7 μg	1065 ± 188	966 ± 219	356 ± 100	230 ± 84	153 ± 53
	2 μg	1318 ± 267	677 ± 238	447 ± 72	361 ± 129	263 ± 100
Trial 4	Vehicle	918 ± 208	731 ± 139	511 ± 194	338 ± 121	442 ± 147
	0.7 μg	1063 ± 186	703 ± 153	623 ± 268	242 ± 66	233 ± 28
	2 μg	1284 ± 237	929 ± 208	552 ± 176	201 ± 26	422 ± 197

Distance is shown as mean ± S.E.M. (cm) ($n = 8$).
* $P < .05$.

the control group. No differences were observed on Trials 3 or 4 (Tables 1 and 2).

3.5. Approach avoidance test

The effect of C3 on memory retention in rats and mice was assessed in an approach avoidance test. Results are depicted in Figs. 5 and 6 as the latency to drink water from a waterspout through which the animals received shocks during training (see Methods). In rats, injection of 4 μg C3 prior to training had no significant effect on retention latency (data not shown). However, a twofold increase in dose to 8 μg C3 showed that the peptide induced a significant decrease in latency to drink water 24 h later as compared to the controls ($P < .05$). No differences in latency were observed during training indicating that C3 did not induce any acute effects on acquisition (data not shown). In addition, no motor disturbances were observed at the 8-μg dose of C3 at the time of testing.

In mice, three doses of C3 were tested in the approach avoidance test (0.1, 0.5 and 2 μg). Fig. 6 demonstrates that injection of 2 μg C3 pretraining reduced approach avoidance latency ($P < .05$) and thus impaired memory retention 48 h posttraining. In contrast, injection of neither 0.1 nor 0.5 μg C3 affected retention of the task.

4. Discussion

The effect of the NCAM-Ig1-binding peptide, C3, on learning and memory was assessed in the rat using an open field, the Morris water maze and an approach avoidance task in both rats and mice. Additional measures of exploratory motility and sensorimotor function at relevant time points provided further characterisation of the effect of C3 in the learning and memory tests.

In the open-field test, no acute effect of C3 (0.1–2 μg) was observed, whereas exploratory behavior decreased 3 and 24 h after the initial exposure to C3. These results indicate that C3 treatment enhanced habituation to a novel environment after repeated exposures or alternatively, that it reduced exploratory behavior/activity at the tested time points. Additional studies were designed to investigate whether the effect of C3 really was the result of enhanced

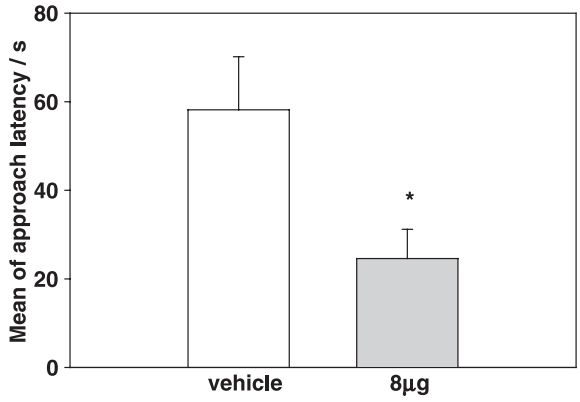

Fig. 5. Effect of C3 on retention in an approach avoidance task in rats and tested 24 h after training. C3 was injected intracerebroventricularly prior to training. Data are depicted as mean + S.E.M. ($n = 10$ and 7, respectively). Statistical significance is indicated as * $P < .05$.

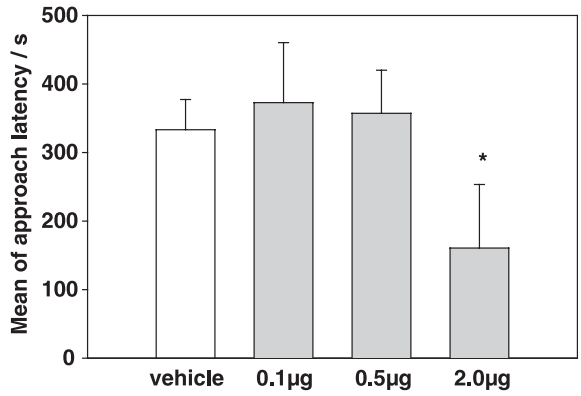

Fig. 6. Effect of C3 on retention in an approach avoidance task in mice and tested 48 h after training. C3 was injected intracerebroventricularly prior to training. Data are depicted as mean + S.E.M. ($n = 7–18$). Statistical significance is indicated as * $P < .05$.

memory or a consequence of C3-induced effects on exploratory behavior and/or motor disturbances. At the same dose range as used in the open-field test, C3 had no significant effect on exploratory motility when tested in novel motility cages shortly after the third test in the open field. However, a tendency to decrease exploratory motility was observed after 0.7 μg C3 administration. The decrease in exploratory behavior observed in the open field was probably not a general consequence of sensorimotor impairments as C3-treated rats showed no strong alterations in swim speed in the water maze or in rotarod performance 24 h after injection. Clearly, C3 enhanced habituation in the open-field test although minimal habituation was seen in the control group suggesting caution in interpreting the C3 data without corroborating evidence from other tests (see below). In addition, the reduction in exploratory motility at 24 h after C3 administration may indicate a general decrease in exploratory behavior, thus affecting open-field behavior.

Acquisition and retention of learning in the water maze by rats appeared unaffected by a single injection of C3 prior to first-day training when the mean of all four daily trials was considered for all measures. However, an increased latency to find the platform was observed on the second day of training (24 h after the injection) when the second trial alone was evaluated. This may indicate that C3 affects short-term memory 24 h after treatment since on the second training trial, rats recall memory from the previous training trial as well as from the preceding day. However, no memory deficits were found on the third or fourth trials suggesting caution in interpreting the data. Interestingly, continuous infusion of NCAM antibodies has previously been demonstrated to impair acquisition and retention of learning in a water maze, and this effect was not apparent until the day after the infusion started in accordance with our results (Arami et al., 1996).

In the approach avoidance task, administration of C3 prior to training decreased approach latency in rats when tested 24 h after training and in mice when tested 48 h after training indicating that memory retention was impaired. Retention at 48 h could not be determined in this task for rats as the rats approached the waterspout too fast due to increased motivation at this time point. We decided to extent the approach avoidance study to mice in addition to rats in order to investigate whether the C3-mediated memory impairment was expressed across species as we saw the most pronounced memory impairment in this test. We demonstrate a C3-mediated memory impairment in both rats and mice; however, the effective dose of 8 μg in rats and 2 μg in mice show that mice are less sensitive (in relation to the body weight) to the memory impairment induced by C3 than rats. In addition, learning in the approach avoidance test and in the water maze shows different sensitivity to C3. The effective dose of C3 is much higher in the rat approach avoidance test (8 μg) than in the water maze (2 μg). However, as these tests represent different forms of learning with different stressor intensities, conditioned, nonspatial (approach avoidance) and unconditioned, spatial (water maze) learning, C3 may affect these forms of learning differently. Nevertheless, both the approach avoidance data and the water maze data support our previous studies in a step-through passive avoidance task in rats showing that intraventricular injections of C3 impaired long-term memory when injected prior to training or 6 h after training (Foley et al., 2000), the effect being apparent 24–48 h after training. The impairments observed in this and previous studies implicate a time-restricted role for NCAM in memory formation.

Several possibilities exist for how C3 may influence learning and memory. C3 may interfere with a learning-related intracellular signalling cascade as C3 has been shown to induce signal transduction pathways similar to NCAM-mediated signalling involved in synaptic plasticity (Fields and Itoh, 1996). Another hypothesis is that C3 binds to NCAM-Ig1 and thereby inhibits homophilic NCAM binding, resulting in reduced cell adhesion as has been demonstrated in vitro (Rønn et al., 1999). This may result in reduced stability in new synaptic contacts established during learning (Wenzel et al., 1980). In vitro, C3 acts as a plasticity promoter in a simple system nonpermissive of homophilic NCAM binding in which it probably mimics NCAM function. By contrast, in more complex systems permissive of homophilic NCAM binding, C3 may act as an inhibitor of NCAM functions (Rønn et al., 1999). Thus, in a complex system such as the brain, C3 is presumed to inhibit NCAM function acting as a partial agonist in the sense that it may inhibit NCAM–NCAM interactions but in itself promotes plasticity, however, not to the same extent as an NCAM–NCAM interaction. This may explain the memory impairments observed in our studies despite the positive influence on developmental plasticity in vitro. Thirdly, it has been demonstrated that NCAM is internalised 3–4 h posttraining in the step-through inhibitory avoidance task and that C3 may block this internalisation (Foley et al., 2000). This is consistent with studies in astrocytes in culture indicating that NCAM is internalised and also with studies in *Aplysia* in vitro demonstrating a learning-related internalisation of an NCAM analogue, ApCAM (Bailey et al., 1992; Mayford et al., 1992). Thus, a C3-mediated block of NCAM internalisation may decrease the structural remodelling occurring at the synapse as a consequence of learning and memory consolidation and this may result in the demonstrated amnesia (Foley et al., 2000).

In summary, the present study shows that intracerebroventricular administration of an NCAM-binding peptide prior to training in rats and mice impairs learning or memory processes in the brain, probably acting as a partial agonist resulting in inhibition of NCAM functions in the brain. Thus, it is possible to modulate learning/memory processes in rodents in vivo with small synthetic NCAM-binding peptides that induce developmental plasticity in vitro.

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