Sequence-Specific Impairment of Memory Formation by NCAM Antisense Oligonucleotides

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Abstract

The functional role of NCAM gene expression in memory formation was studied in the one-trial passive avoidance task in day-old chicks by pretraining injections of one of three different 18-mer end-protected oligonucleotides corresponding to positions 190-, 207-, and 332- of the NCAM Ig1 domain. Twenty-four-hour-old chicks were trained by pecking at a bitter-tasting bead and tested for avoidance 30 min, 3, 8, or 24 hr later. Memory retention was significantly reduced only in the group of animals injected with the NCAM antisense corresponding to position 207- (AS-ODN-207), and only if given twice, both immediately after hatching and 12 hr before training. This antisense was without effect on the general behavior of the chicks, training or acquisition, and did not produce observable neurotoxic damage. Under such conditions amnesia was evident by 3 hr after training and lasted until at least 24 hr after training. The two other tested oligonucleotides were without behavioral effect. To control for nonsequence-specific effects of AS-ODN-207, brains from injected and trained animals were processed for Western blotting and probed using anti-NCAM, anti-L1, and anti-actin antibodies. NCAM antisense corresponding to position 207- significantly reduced the level of NCAM, whereas the level of L1 and actin remained unchanged. These results confirm our earlier conclusion that NCAM is necessary for longer term memory retention.

Introduction

Memory retention for a one-trial passive avoidance task in day-old chicks involves a well-characterized molecular and cellular cascade, in particular, in the forebrain regions, the intermediate medial hyperstriatum ventrale (IMHV), and the lobus parolfactorius (for reviews, see Rose 1996; Rose and Stewart 1999). This cascade begins with a series of synaptic transients and proceeds through gene activation, ultimately (within 24 hr) resulting in the remodeling of pre- and postsynaptic structures that modulate synaptic connectivity and electrophysiological response (Rose and Mason 1988; Anokhin et al. 1991; Rose 1991, 1993; Doubell and Stewart 1993; Gigg et al. 1994). After the training, there are two anisomycin-sensitive waves of protein synthesis, the first, involving immediate early genes, occurring within 1 hr of the training event and the second, beginning 4 hr after training and persisting for the next hour (Freeman et al. 1995). After this wave, there is a period of up to 8 hr after training of post-translational modification and insertion of newly synthesized proteins in synaptic membranes. It is this second wave that involves the late genes necessary for the stabilization of long-term memory, and that include coding for synaptic membrane cell adhesion molecules (Schroley et al. 1993, 1995; Mileusnic et al. 1995).

The suggestion that glycoproteins play a part in the synaptic plasticity underpinning memory formation is not novel (Routtenberg et al. 1974; Pohle 1979; Popov et al. 1981). Antibodies raised against the glycoprotein or protein epitopes of glycoconjugates and recombinantly expressed fusion proteins or peptides have been used previously to

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identify specific glycoproteins involved in memory formation (Lapuke et al. 1987; Stanton et al. 1987; Pirot and Schmidt 1988; Schlosshauer 1989; Staubli et al. 1990; Jork et al. 1991, Luthi et al. 1994; Scholey et al. 1995). Because the extracellular domain of the glycoprotein that is responsible for cell–cell recognition and hence, synaptic connectivity, injection of antibodies or peptides that bind to this domain during a period of synaptic reorganization and plasticity might impair synaptic modulation after training. This possibility has been confirmed by the use of an antibody and peptides to the L1 cell adhesion molecule. Injections at either 30 min before training or 5.5 hr after training, but not at intervening times, result in amnesia for the passive avoidance task (Scholey et al. 1995). In contrast, injection of a polyclonal anti-neuronal cell adhesion molecule (NCAM) antibody, recognizing the three major isoforms of the NCAM, results in amnesia only if injected during the second time window (i.e., 5.5 hr after training) (Scholey et al. 1993; Mileusnic et al. 1995).

NCAM was the first molecule mediating cell adhesion to be identified on the basis of functional criteria (for review, see Edelman 1988; Thiery et al. 1997). NCAM is a complex of three immunologically related membrane proteins of 180, 140, and 120 kD, each having identical amino-terminal domains and differing primarily in their region of membrane association. These isoforms are primary translation products that arise through alternative splicing of a single gene. Each isoform contains five immunoglobulin (Ig) domains and two fibronectin type III sequences. NCAM is expressed on neuronal and glial cells and on a variety of peripheral tissues including skeletal, cardiac, and smooth muscle cells (for review, see Goridis and Brunet 1992). Within the central nervous system, NCAM 180 and 140 appear to be characteristic of neurons, whereas NCAM 140 and 120 are expressed predominantly by glial cells (Pollenberg et al. 1987; Rutishauser and Jessel 1988). Unique to NCAM are the post-translational modifications associated with the glycan structures (Doherty et al. 1990; Doyle et al. 1992a,b; for review, see Rougon 1993). Studies on cell adhesion molecules (Doherty and Walsh 1992) suggest that an individual NCAM can function both to promote synaptic plasticity and maintain the structure of the synapse. Mice lacking all protein isoforms of the NCAM gene (NCAM−/−), although phenotypically showing surprisingly mild deficits, appear to have problems with spatial learning and exploratory behavior and almost total loss of polysialylation (Cremer et al. 1994). More recently, another study revealed deficits in long-term potentiation in the NCAM−/− mice (Müller et al. 1996).

To clarify the question of the functional importance of NCAM gene expression we have used antisense methods to deplete the protein during and after training chicks on the passive avoidance task, using three different synthetic oligonucleotides (ODNs) corresponding to the Ig1 domain of NCAM. Our results suggest that (1) to induce significant depletion of NCAM that might in turn affect memory formation, antisense oligonucleotides should be injected twice at appropriate pretraining times; (2) not all sequences targeting the NCAM mRNA are effective in attenuating memory retention; and (3) when high doses of oligonucleotides are used, although efficient in producing significant reduction of NCAM gene expression and induction of amnesia, sequence nonspecific effects influencing behavior might occur.

**Materials and Methods**

**OLIGODEOXYNUCLEOTIDES**

Three different end-protected antisense (AS) ODNs corresponding to nucleotides position: 190-, 207- and 332- were used [Exon 1; source: chicken, cDNA to mRNA, clones pEC(254, 265)]. Controls had the same base composition as the AS oligonucleotides but in a random sequence, which is not complementary to any other mRNA sequence in the species (scrambled controls, SC). Oligonucleotides 190- and 332- were synthesized in King’s College (Department of Molecular Medicine, University of London, UK; oligonucleotide 207- and anti-L1 antibody were gifts from Professor M. Schachner (Centre for Molecular Neurobiology, Hamburg, Germany). Monoclonal anti-polysialic acid antibody was a gift from Dr. G. Rougon. Anti-actin antibody was purchased from Sigma.

**ANIMALS, INJECTION PROCEDURES, AND TRAINING**

One-day-old Ross Chunky chicks (*Gallus domesticus*) of either sex were hatched in our own brooders. Bilateral intracranial injections of 2 µl per hemisphere (0.30 µg/µl of either saline, AS, or SC-ODNs) were made using a Hamilton syringe fitted with plastic sleeve as a stop, and a Plexiglas head-holder designed to direct the injection into the IMHV. This injection volume is safe and reliable...
to use, granted the relatively large extracellular volume of the young chick brain. Chicks can be injected without the use of anesthesia and suffer no apparent behavioral or physiological distress. They appear to be completely normal within 20 sec of being returned to their pens after injections. Chicks were injected twice, first 18–20 hr before training (6–8 hr after hatching) and then 11–12 hr before training.

On the morning after hatching, chicks were placed in pairs in aluminium pens (20 × 25 × 20 cm) with scattered chick crumbs. The pens were maintained at 28°–30°C and illuminated by an overhead red light. Chicks were allowed to settle for 1 hr before being pretrained by two 10-sec presentations of a small (2.5 mm) white bead. Birds were scored as either peck or nonpeck. Ten minutes after the last pretraining trial, each bird was trained by a single presentation of a 4-mm bright chrome bead coated with methylanthranilate. On this training trial, each bird’s behavior was scored as peck, peck and shake (the disgust response to methylanthranilate) or nonpeck. Birds that failed to peck twice during pretraining or failed to peck at training were not used for further analysis. At 30 min, 3, 8, or 24 hr after training, each chick was tested by being offered a dry chrome bead for 30 sec, and its response (peck or avoid) was noted. Each bird was trained and tested only once. Learning and retention are expressed by avoidance of the bead dry during the retention test. Retention was calculated as percent avoidance score. Forgetting, or amnesia, is indicated by trained birds pecking the dry bead on test. Scoring during both training and testing was done by an experimenter blind as to the previous treatment. Retention scores between groups were compared by G-test using a χ² table (Sokal and Rohlf 1981). To detect any gross morphological abnormalities that might be induced by multiple injections of end-protected ODNs, 8 hr after training and after testing, chicks were decapitated and their brains examined by staining sections with toluidine blue.

All animal experiments were done in accordance to the Home Office Code of Practice approved by the local Animal Ethical Committee.

**SDS-PAGE AND WESTERN BLOTTING**

After testing, brains were removed, checked for correct placement of injections, and processed for Western blotting. Regions of forebrains enriched in IMHV were pooled from two to three animals for each experimental condition, homogenized in Tris-buffered saline [TBS, 1:10 (wt/vol)] and centrifuged at 3000g for 10 min. Supernatants were collected and spun again at 100,000g for 60 min. Twenty to 25 µg of soluble fraction (for actin detection) or pellet (membrane fraction) (for NCAM and L1 detection) was separated by SDS-PAGE under reducing conditions on 5%–15% pre-made Bio-Rad polyacrylamide gels (Laemmli 1970). Proteins were transferred to nitrocellulose according to the method of Burnette (1981) in buffer containing 10% methanol, followed by Western blotting (Towbin et al. 1979). Blots were checked routinely by post-transfer staining in amido black. After overnight incubation with anti-NCAM, anti-L1, and anti-actin antibodies (diluted 1:500) in TBS (pH 7.2), containing 5% defatted milk powder, immunoreactive bands were detected using horseradish peroxidase-coupled secondary antibodies (Sigma) and dianinobenzidine.

**Results**

Chicks were injected twice with one of the three different NCAM AS-ODNs or their SCs, or saline as described above. Memory retention was significantly reduced only in the group of animals injected with the AS-ODNs corresponding to position of 207–223 of the Ig1 domain, whereas the two other tested oligonucleotides injected according to the same protocol showed no behavioral effect and therefore, were not tested for effect on NCAM levels.

The apparent slight reduction in memory retention after injection of SC-ODNs, when compared with saline injected controls (Fig. 1), was not statistically significant. It is important to note that there was no effect of any of the AS-ODNs or their SC counterparts on behavior during pretraining or training; the proportions of chicks that failed either at pretraining or training to peck at the beads, or show the disgust response, were the same for all groups (<12% in any group).

To determine the time of onset of amnesia in the animals injected twice with the ODN-207, the time of test relative to training was varied. As shown in Figure 2, amnesia was not apparent in chicks tested 30 min after training but was evident in animals tested 3 hr after training, and was still apparent at 24 hr after training. The double injections were essential. If the entire dose of ODNs was applied in one injection, 12 hr before training, chicks were very nervous and could not be trained,
most probably attributable to accumulation of degradation products of ODNs (Dragunow et al. 1994). By 24 hr there were some deficits in motor coordination. If only a single injection at the reduced dose was given, there were no behavioral deficits, but also no effect on retention.

To determine the specificity of the effect of the antisense both on expression of its target gene and on the behavior, two types of control were run. First, to determine whether injections resulted in either mechanical or neurotoxic lesions, which could account for the amnestic effect, tissue sections from the double-injected animals were taken for toluidine blue staining. Figure 3 is a photomicrograph taken near the injection site. Histological examination revealed only minor local damage that did not extend far from the site of injection and did not differ from saline controls.

To determine the level of NCAM gene suppression and specificity of the antisense effect, brain proteins were probed with antibodies to NCAM, L1, and actin antibody, and subjected to Western blotting. Chicks were injected singly or doubly with AS-ODNs-207 and SC or saline controls. Twelve hours the second injection they were trained, 8 hr later tested and killed, the brains removed, and soluble proteins and crude membrane fraction isolated. As shown in Figure 4, the doubly injected AS-ODN chicks showed a considerable reduction of NCAM protein, but the AS had no effect on either L1, a structurally similar member of the CAM family, or on actin, a functionally and structurally unrelated protein.

Discussion

CAMs expressed in neural tissues have been implicated in the control of axonal growth, fasciculation, and pathfinding (for review, see Rougon 1993). During the past 10 years there has been a remarkable convergence of evidence pointing to a key role for the cell adhesion molecules in the modulation of neuronal circuitry by way of the restructuring of synaptic connectivity, believed to be required for memory formation (for reviews, see Rose 1996; Rose and Stewart 1999).

NCAM is probably the best-characterized and most abundant neuronal CAM and appears to play...
NCAM mediates cell–cell and synaptic bouton–dendrite interaction through Ca²⁺-independent, homophilic-binding mechanisms and enhances L1-dependent cell–cell adhesion (Kadmon et al. 1990), as well as cytoskeletal–membrane interactions (Pollenberg et al. 1987). NCAM is extensively glycosylated and represents a major carrier of PSA in the nervous system (Rutishauser and Landmesser 1991). The amount of PSA on the NCAM is low during embryogenesis but increases significantly during the postnatal period of intense synaptogenesis (Hoffman and Edelman 1983). In the central nervous system NCAM appears to be involved in synaptic recognition, selection, and stabilization during development (Rutishauser and Jessel 1988). This, along with evidence from in vitro-binding studies (Rutishauser et al. 1985; Doherty et al. 1990), has led to the hypothesis that selection and stabilization of synaptic contacts might be inversely proportional to the extent of NCAM polysialylation.

Of particular importance are the results suggesting a role of NCAM in activity-dependent synaptic plasticity, which indicate that antibodies to NCAM down-regulate a variety of plasticity-associated responses in neurons such as long-term potentiation (Luthi et al. 1994) and several different forms of memory retention (Doyle et al. 1992a; Scholey et al. 1993; Mileusnic et al. 1995; Roullet et al. 1997). The results reported here show that, just as blockade of glycoprotein synthesis in general, or injection of antibodies to NCAM at an appropriate time after training are amnestic, therefore, down-regulation of NCAM gene expression by injection of an appropriate antisense ODN does indeed interfere with retention of memory for the passive avoidance task.

The AS-ODNs used were designed to correspond to the Ig1 domain Exon 1 and intended to inhibit expression of all three isoforms of NCAM. As shown in Figure 2, injection of one of these ODNs had marked effects on memory retention 3 hr after training, which persisted for at least 24 hr after training. The fact that a double injection of the ODN was required, at 18–20 and 11–12 hr pre-training for amnesia to result, and that under these circumstances it sets in by 3 hr after training, suggests that the amnestic effect results from two different mechanisms: depletion of already existing NCAM in the hours before training because of the rapid turnover rate of NCAM (Bloch 1992; Olsen et al. 1993) and inhibition of the NCAM gene expression that must precede the “second wave” of protein synthesis necessary for long-term memory formation (Scholey et al. 1993; Freeman et al. 1995). Moreover, down-regulation of NCAM gene expression was sequence specific, as not all sequences, although positioned very close to each other in Exon 1, were effective in inducing amnesia (Fig. 1).

We used end-protected phosphorothioate oligonucleotides to increase the nuclease resistance and half-life of ODNs (Campbell et al. 1990), although the benefits of increasing half-life must be balanced against the fact that they have been shown to increase cytotoxicity (Krieg 1993). Phosphorothioate protection may also result in neurotoxic effects through the inactivation of some proteins essential for basic cellular functions. Because Dragunow et al. (1994) showed that c-fos antisense reduced constitutive egr-1 expression, it was necessary to establish the level of toxic damage and also determine the specificity of any NCAM depletion. Histological examination of the brain tissue (Fig. 3) showed that the observed lesions were no greater than in controls, and therefore, could only have been created by the mechanical damage resulting from injection, which does not affect either learning or retention. Spreading the time of administration of the ODN by using two injections, each of low concentration of end-protected ODNs, on an 8- to 12-hr interval schedule greatly reduced the damage seen either after a single injection of high concentration of ODNs (data not shown) or multiple injections of fully phosphorothioated ODNs (Chaissong et al. 1997).
The question of the biochemical specificity of the NCAM antisense was addressed by examining its effects on the expression of NCAM itself, on its close congener L1, and on actin. L1 (NgCAM), like NCAM and actin, belongs to the superfAMILY of immunoglobulins. It shares many functional and structural similarities with NCAM. The extracellular domains of both CAMs consist of immunoglobulin-like domains followed by fibronectin type III-like domains. However, anti-L1 antibody, unlike anti-NCAM, if injected either 30 min before or 5.5 hr after training induces amnesia in the chicks tested 24 hr after training, whereas anti-NCAM is effective only at the second time point (Scholey et al. 1995). As shown in Figure 4, the AS-ODN reduced NCAM levels in whole brain crude membrane fraction, nevertheless leaving levels of expression of L1 and actin unaffected. Although this is indicative of a degree of specificity, the results must still be viewed with caution, as a variety of unexpected sequence nonspecific effects of ODNs have been observed (for review, see Branch 1998), including preliminary suggestions that NCAM-AS-ODN may affect levels of expression of other proteins, such as synaptosomal-associated protein-25 (SNAP-25) (Roberts et al. 1998). Available commercial SNAP antibodies do not recognize chick antigen in Western blots, therefore, direct quantitation was not possible.

This caveat apart, and taken in conjunction with our other evidence concerning the role of NCAM, our results demonstrate that only one ODN sequence, corresponding to the position 207 of the Ig1-like domain, produced depletion of NCAM protein, and that it did so without affecting levels of either L1 or actin. The same ODN induced a specific impairment in memory formation with an onset time between 30 min and 3 hr after training. Bearing in mind that our laboratory has shown that an injection of anisomycin 4–5 hr after training results in amnesia apparent at 24 hr, the implication is that for the transition from short- to long-term memory to occur, NCAM must be synthesized de novo during the second wave of protein synthesis. It may also be relevant that we have shown recently that a related molecule, the amyloid precursor protein \( \beta \)APP, which is a cell-cell and cell-matrix adhesion molecule, is also necessary if memory consolidation is to occur, as pretraining injections of either AS-ODNs or antibodies to APP, although without effect on training or acquisition, result in amnesia for the passive avoidance task with an onset time no later than 30 min after training (R. Mileusnic, C. Lancashire, and S.P.R. Rose, in prep.). This is before the onset time of amnesia after injection of the AS-ODNs to NCAM described here, suggesting that APP is required at an early phase, rather than in the transition to long-term memory, which we have postulated for NCAM and L1 (Scholey et al. 1995; Rose 1996).

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