Apolipoprotein E Antibodies Affect the Retention of Passive Avoidance Memory in the Chick

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SUMMARY

Isoforms of apolipoprotein E (ApoE) have been implicated as risk factors in Alzheimer's disease. We have, therefore, examined the possible role of ApoE in memory formation, using a one-trial passive avoidance task in day-old chicks. Birds were trained on the task and then at various times pre or post-training were injected intracerebrally with anti-ApoE. Immunofluorescence staining demonstrated the presence of the antibody bound to the neuropil, close to the injection site and adjacent to the ventricle, with a residence time in the brain of up to 30 min. Chicks that were injected 30 min pre-training or just post-training with 5 pg/hemisphere of the antibody learned the task, but were amnesic when tested at 30 min or at subsequent times up to 24 hr post-training. When tested at 24 hr, birds injected 5.5 hr post-training showed unimpaired retention. Birds injected with 5 ng/hemisphere of anti-ApoA-I (which has a brain distribution similar to that of anti-ApoE) at 30 min pretraining showed no amnesia, indicating the specificity of the effect to the ApoE. Possible mechanisms for this effect are discussed.

KEYWORDS

amnesia, learning, Alzheimer's disease, ApoA-I

INTRODUCTION

Apolipoprotein E (ApoE) is a low-molecular-weight, 299 amino-acid protein, with a molecular size variously described as ranging from 34–37kDa (Beffert et al., 1998; Puttfarken et al., 1997; Stone et al., 1997). ApoE is involved in cholesterol and phospholipid transport and metabolism, mediating lipid uptake by cells by acting as a ligand for the neuronal and astrocytic receptors for low density lipoprotein (LDL) and very low density lipoprotein (VLDL), among others. The LDL and VLDL receptors bind and internalize ApoE-containing lipoproteins, and the complex is then degraded (for review see Beffert et al., 1998). ApoE mRNA is richly present in the brain; ApoE is synthesized notably by astrocytes (Poirier et al., 1991). In mammals, ApoE is one of eight exchangeable and soluble lipoproteins (including ApoA I, II, IV and ApoC-I, II, III; Babin et al., 1997). Until recently, the ApoE form was believed to be absent from other non-mammalian vertebrate species; Babin et al., (1997) have shown, however, that the ApoE gene is not confined to mammals but is also present in other vertebrates, being highly expressed, for instance, along with ApoA-I in zebra fish during development.

ApoE exists in a number of isoforms, ApoE2, ApoE3, and ApoE4. Although their exact function is unclear, either in normal or in pathological metabolism, apolipoproteins have recently been implicated as potential risk factors in Alzheimer's disease (AD), with the APOE4 allele increasing and the APOE2 and APOE3 alleles decreasing the risk of early onset of the disease (Strittmatter & Roses, 1996). Just why this association should be the case is not known, although ApoE has been described as interacting with the Alzheimer's-associated amyloid...
precursor protein (APP) by way of a common receptor, the LDL-receptor-related protein (Kounnas et al., 1995). ApoE also interacts, presumably following internalization, with tau and other cytoskeletal proteins (Fleming et al., 1996). Such interactions might imply that ApoE could play a role during aging by helping to stabilize dendritic Arborizations or synaptic junctions. ApoE has been shown to be neurotrophic, increasing neurite length in culture, as well as ameliorating β-amyloid-induced toxicity (Puttfarcken, 1997).

As one of the early characteristics of progressive AD is memory dysfunction, notably an inability to transfer new information into longer-term memory, exploring the possible role of ApoE in memory formation is relevant. Some groups have claimed that Apo-E-deficient mice show cognitive impairments (Masliah et al., 1997), although such impairment may depend on genetic background. Other groups (Anderson et al., 1998) did not find major behavioral consequences of the deletion. Following Hebb (1949), long-term memory is generally believed to be stored in the brain in the form of altered patterns of neuronal connectivity. Modifying these patterns requires the remodeling of synapses by changing their number, dimensions, or morphology (Rose, 1995). The biochemical cascade required for such remodeling has been studied in a variety of vertebrate species and training tasks. Convergent evidence from several laboratories has identified a sequence of events which, beginning with synaptic transients, second messenger release, and the activation of transcription factors and immediate early genes, culminates in the synthesis and insertion into pre- and post-synaptic membranes of a variety of glycoprotein cell-adhesion molecules (Rose, 1996).

A model system in which this cascade has been mapped in detail is the one-trial, passive avoidance learning task in day-old chicks. In this task, birds are offered a small bright bead, at which they will peck spontaneously within a few seconds. If the bead is coated in a bitter tasting substance they will peck once, evince a disgust response, and avoid similar but dry beads subsequently (Andrew, 1991). The merit of the one-trial task is that it is quick, reproducible, and sharply timed, and further, that the soft, unossified skull of the chick enables agents that putatively might either enhance or inhibit memory formation to be directly injected into the brain within a few seconds, without anesthesia or overt behavioral distress.

We have identified [for references see Rose (1995, 1996)] a sequence of biochemical changes occurring in a specific region of the chick forebrain, the intermediate medial hyperstriatum ventrale (IMHV), in the minutes to hours following pecking at the bitter bead. The changes include, within minutes of training, the release of glutamate, upregulation of NMDA glutamate receptors, opening of presynaptic calcium channels, and altered phosphorylation of the presynaptic protein B50 (GAP 43). One hr after training, expression of the protein product of the immediate early gene c-fos is enhanced, which is then followed 4–8 hr post-training by enhanced protein synthesis, post-translational glycosylation, and transport to the synaptic membranes of several species of cell adhesion molecules, including the neural cell adhesion molecule NCAM and L1 (NgCAM). Blockade of any step in this sequence results in the onset of amnesia for the task, as a result of which chicks peck at rather than avoid the bitter bead on test. Thus, antagonists of the NMDA receptor, of presynaptic calcium channels, or of inhibitors of the nitric oxide synthase enzyme, injected either just before or just after training, produce an early onset of amnesia and are presumed to prevent activation of later steps in the cascade.

Antibodies to specific synaptic membrane proteins, such as NCAM and L1, which can bind to the external domains of these molecules, also produce a time-dependent amnesia for the task. Anti-NCAM produces amnesia only if injected during a late phase in the cascade, 5.5 hr post-training, whereas anti-L1 is amnestic at two time windows, whether injected 30 min before training or 5.5 hr post-training (Scholey et al., 1993, 1995). We suggested that this late phase is a period that is associated with the transition from short-term to long-term memory (Rose, 1995).

Granted the extracellular location of the apolipoproteins and their interaction with membrane-bound receptors, a logical approach to inquiring whether
they have a specific role to play in the cascade would be, therefore, to examine the effect of antibodies to such molecules on learning and memory for the passive avoidance task. In this paper we report that antibodies to ApoE, but not to ApoA-I, when injected around the time of training, are indeed amnestic.

**EXPERIMENTAL**

**Animals and training**

Commercially obtained Ross Chunky eggs were incubated and hatched in our own brooders and held until 24±6 hr old. The chicks were then placed in pairs in small aluminum pens, pretrained, and trained essentially as described by Lossner & Rose (1983). Briefly, following an equilibration period of 1 hr, chicks were pretrained by three 10-sec presentations of a small (2-mm diameter) white bead, at approximately 5 min intervals. Chicks failing to peck at the bead at least twice in three presentations (<5%) were not used subsequently, but remained in their pens for the duration of the experiment. Following the last pretraining trial, the chicks were injected, where appropriate, as described below, before being trained by a 10 sec presentation of a 4-mm-diameter chrome bead that had been dipped in the bitter-tasting methylanthranilate. At various times after training, the chicks were tested by offering them a small (2-mm-diameter) white bead, followed 10 min later by a dry 4-mm-diameter chrome bead, each for 20–30 sec. The chicks were considered to remember the task if they pecked at the white bead but avoided the chrome bead at test, and to have forgotten the task if they pecked at both beads. Only chicks pecking at the bitter bead on training and on the white bead at test were included in the final results. Among the chicks meeting the criterion (>80%), recall was calculated as a percent avoidance score (that is, the percentage of chicks in each group that avoided the chrome bead). Each chick was trained and tested only once, and the differences between the groups were tested for statistical significance using the $\chi^2$ test. The significance levels were set at 5%.

**Antibodies and injections**

Antibodies to ApoE and ApoA-I were purchased from Boehringer Mannheim (East Sussex, UK) and from Calbiochem-Novabiochem (Nottingham, UK) respectively. Anti-ApoE is a murine monoclonal antibody (mAb) prepared against human apolipoprotein E; anti-ApoA-I is a polyclonal antibody raised in rabbits; both antibodies belong to the IgG class. Each antibody preparation was diluted appropriately with saline for injection. Bilateral injections of antibody or saline control (5μl) were made using a 10-μl Hamilton syringe fitted with a plastic sleeve to allow a penetration of 3.5 mm. The correct placement of injections into the IMHV was ensured by using a custom-built headholder (Davis et al., 1979), and the injections were routinely visually monitored post-mortem. The injections are rapid (<30 sec per bird) and cause no observable distress to the chicks. Normally, one chick of a pair in each pen was injected with the antibody and the other with saline. The animals were injected and tested by an experimenter who was blind regarding which treatment each chick had received.

**Western blotting technique**

Synaptic plasma membrane (SPM) proteins were prepared as described by Murakami et al. (1986), separated by SDS-PAGE under reducing conditions using a 12% polyacrylamide gel (Laemmli, 1970), and then transferred to nitrocellulose (Burnette, 1981). The blots were routinely checked by post-transfer staining with Ponceau S. The nitrocellulose was incubated in blocking buffer [Tris-buffered saline (TBS) pH 7.5 containing 5% skimmed milk powder and 0.05% Tween 20]. The antibodies were diluted in blocking buffer (ApoE 30μg/ml, ApoA-I 20μg/ml), and the blot was incubated overnight at 4°C. After three 10 min washes, peroxidase-conjugated anti-mouse IgG (for ApoE) and peroxidase-conjugated anti-rabbit IgG (for ApoA-I) were added at a dilution of 1:500 in blocking buffer, and the blot was incubated overnight at 4°C. The immuno-reactive bands were detected as described previously (Scholey et al., 1993). To check the specificity of the anti-ApoE,
Fig. 1: (a) Immunoreactivity of ApoE and Apo-A-I antibodies with SPM proteins. SDS-PAGE and Western immunoblotting were performed as described in the Experimental section. Each track contained 50 µg of SPM protein from day-old chicken forebrain. Lane 1: Rainbow standard; Lane 2: anti-ApoE (30 µg/ml); Lane 3: anti-Apo-A-I (20 µg/ml). (b) SDS-PAGE and Western blot of authentic human Apo-E. Lane 1: Rainbow standard; Lane 2: SDS-PAGE of 3 µg of authentic human ApoE; Lane 3: Western blot of ApoE with monoclonal anti-human Apo-E antibodies (2.5 µg/ml).
3 μg of human ApoE (Human Plasma, VLDL Calbiochem-Novabiochem) was electrophoresed on 12% SDS-PAGE, and a Western blot was performed as above, using anti-ApoE at 2.5 μg/ml.

RESULTS

Characterization of antibodies

To determine the pattern of SPM proteins isolated from chick brain that were recognized by mAb anti-human ApoE and polyclonal anti-ApoA-I, the gels were overloaded with 50 μg of SPM. Figure 1a shows the Western blots obtained when chick SPMs were probed with antibodies to ApoE and ApoA-I. Anti-ApoE gave a relatively weak signal, recognizing bands running at 41, 50, 72, 90, and 110 kD. Anti-ApoA-I gave a much stronger signal, recognizing two major bands with relative molecular masses of 60 and 140 kD, as well as a number of minor bands, two (at 71 and 90 kD) corresponding to those for ApoE. Figure 1b shows the SDS gel pattern obtained from 3 μg of human ApoE, and the corresponding Western blot when probed with the mAb to human ApoE. Although the ApoE showed only one major band on the gel, at around 34 kD, the corresponding blot showed multiple bands; the major band was very diffuse, as is characteristic of glycosylated proteins, and overlapped with the 41 kD band found in chick SPMs; approximate correspondences with the 50 and 71 kD bands were also seen.

Recognition and residence time of antibody in the chick forebrain

To determine the pattern of antibody binding in the chick IMHV, and the residence time therein, chicks were injected into the IMHV bilaterally with a solution containing 5 μg of each antibody per hemisphere, and then killed either 10 min, 30 min, or 60 min after the injection. The brains were frozen and prepared for immunocytochemistry. Figure 2 is a montage showing the distribution of immunofluorescence 10 min after injection of the antibodies. The photographs show a concentration of immunofluorescence around the injection site in the IMHV (Fig. 2, a,b), in what appears to be ‘trails’ through the neuropil (Fig. 2, c,d) and around the ventricle (Fig. 2, e,f). Note also (Fig. 2, g) the very circumscribed region of damage around the...
Fig. 2: Immunocytochemistry of anti-ApoE and anti-Apo-A-I localization at 10 min post-injection. One-day-old chicks were injected bilaterally into the IMHV with anti-ApoE (a,c,e) and anti-ApoA-I (b,d,f). Ten min later, the chicks were killed, brain tissue frozen, and immunocytochemistry carried out on 15 μm coronal sections as described in the Experimental section. Texas Red conjugated anti-mouse IgG was used to locate the injected anti-ApoE and Fluorescein-conjugated anti-rabbit IgG to locate the anti-ApoA. Sections (a-f) were examined on a Leica confocal microscope (x100). Toluidine blue counterstained sections (g) were examined on a Zeiss Axiophot (x25). Figures show coronal sections through the forebrain in the region of A 8.8 (Kuenzel & Masson, 1988). Sections a-b: injection site; c-d: Neuropil; e-f: Ventricles; g: Toluidine blue counterstaining, arrow indicates the injection site.
Fig. 3: Immunocytochemistry of anti-ApoE and anti-Apo-A-I localization 30 min post-injection. One-day old chicks were injected bilaterally into the IMHV with anti-ApoE (a) and anti-Apo-A-I (b). Thirty min later chicks were killed, brain tissue frozen and immunocytochemistry carried out on 15 μm coronal sections as described in the Experimental section. Texas Red conjugated anti-mouse IgG was used to locate the injected anti-ApoE and Fluorescein-conjugated anti-rabbit IgG to locate the anti-ApoA. Sections (a-b) were examined on a Leica confocal microscope (x100). Toluidine blue counterstained sections (c) were examined on a Zeiss Axiophot (x25). Sections a-b show the remaining fluorescence in ventricles; c: Toluidine blue counterstaining, the arrow indicates the injection site. V- ventricle, IMHV- intermediate medial hyperstriatum ventrale.

injection site. The two antibodies were remarkably similar in the distribution of immunofluorescence, suggesting that both recognized antigens located next to plasma membranes or in extracellular material in the IMHV. The residence time (or at least activity) was relatively brief; however, as Fig. 3 shows, by 30 min after the injection, the fluorescence had migrated to the ventricles and was barely visible. At 1 hr post-training (data not shown), no fluorescence could be detected. Thus, the time window for the putative biological activity was short.

The effects of antibodies on learning and memory for the passive avoidance task

Initial studies (data not shown) had indicated that the chicks could tolerate injection loads of up to 10μg/hemisphere of anti ApoE without suffering overt physiological or behavioral stress. As Fig. 4
Fig. 4: The effects of anti-Apo-E and anti-ApoA-I on training retention. Chicks were trained and tested as described in the Experimental section. At 30 min pretraining or just post-training, chicks were injected bilaterally with saline or with anti-ApoE (1 or 5 μg/hemisphere), or anti-ApoA-I (5 μg/hemisphere), and then were tested for retention 24 hr post-training. The number of chicks in each group is shown in columns. Injections of 5 μg/hemisphere anti-ApoE, either 30 min pretraining or just post-training, resulted in amnesia when analyzed by χ² (30 min pretraining, χ² = 5.65, p<0.025; just post-training, χ² = 4.67, p<0.05).

Fig. 5: Time of onset of amnesia for a passive avoidance task in chicks injected with anti-ApoE. Chicks were pretrained, trained, and injected with either saline or anti-ApoE (5 μg/hemisphere) 30 min pretraining, as described in the Experimental section. Different groups of chicks were tested for retention at various times post-training. (each chick was trained and tested only once). The number of chicks in each group is shown in columns. 30 min post-training, χ² = 2.68, p < 0.1; 1 hr post-training, χ² = 3.35, p < 0.05; 6 hr post-training, χ² = 4.70, p < 0.05; 24 hr post-training, χ² = 8.12, p < 0.005).
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shows, chicks injected with 5μg, but not 1μg, anti-ApoE per hemisphere 30 minutes before training showed significant amnesia when tested 24 hr later. The amnesia was also apparent if the antibody was injected just post-training.

As Fig. 4 also shows, no amnesia occurred if, instead of anti-ApoE, the same concentration (5μg/hemisphere) of anti-ApoA-I was injected. Amnesia did not occur in chicks injected with anti-ApoE 5.5 hr post-training (retention with saline controls: 70%, n=14; retention in antibody-injected chicks: 70%, n=14, ns). The amnesia was of rapid onset; chicks injected with 5μg/hemisphere of anti-ApoE 30 min before training were amnesic when subsequently tested at 1, 6, or 24 hr. Although the degree of amnesia at 30 min did not quite reach a level of significance, the overall trend itself is clear (Fig. 5): The amnesia was of rapid onset and irreversible. The antibody did not, however, affect the behavior, visual capacity, or motor coordination of the birds during the training trials, as indicated by the finding that of the chicks injected with saline pretraining, 53/54 trained successfully; of those injected with anti-ApoE, 55/59 trained successfully.

DISCUSSION

Because ApoE isoforms have been implicated as risk factors for Alzheimer’s disease, and the early stages of that disease are characterized by loss of the ability to transfer items from short-term to long-term memory, it has become of importance to assess the putative role of ApoE in that transfer process. One way of approaching this question is by examining the effect of gene deletions of ApoE, or of the specific isoform (ApoE 4), known to be a major risk factor for the disease (Strittmatter & Roses, 1996).

Whereas one group (Masliah et al., 1997) reported cognitive impairment in mice with ApoE deletions, others have found relative slight effects (Anderson et al., 1998). Such differences may be related to genetic background or to other factors affecting the interpretation of all such deletion experiments, notably the confounding effect of developmental and neuronal plasticity (Gerlai, 1996).

A more direct approach would be to attempt to interfere with the functioning of the protein during the actual processes of training and recall. One such method is provided by the use of antisense, an approach that we have employed previously in identifying a role for the protein product of the immediate early gene c-fos (Mileusnic et al., 1996) and for APP (Mileusnic et al., 1998). An unequivocal sequence for chick ApoE, however, has not yet been reported. An alternative is offered by the use of antibodies, which can be injected directly into the relevant brain region and can bind to either secreted proteins or the external domains of membrane-bound proteins. We have used such methods to identify time windows, during which antibodies to the cell adhesion molecules NCAM and L1 will cause amnesia for a passive avoidance task in the chick (Scholey et al., 1993, 1995; Tiunova et al., in press).

In rodents, similar methods have shown comparable results (Doyle et al., 1992; Fox et al., 1995; Alexinsky et al., 1997; Roullet et al., 1997). In particular, antibodies to L1 cause amnesia for the passive avoidance task if injected into the IMHV either 30 min pretraining or 5.5 hr post-training, whereas antibodies to NCAM are amnestic only if injected during the second time window. We have argued that this finding indicates a role for the cell adhesion molecules in the transition from short-term to long-term memory.

ApoE, a low molecular weight protein that is secreted and binds to membrane receptors, meets the criteria for proteins whose function might be disrupted by the use of antibodies in this way; hence our decision to explore the effect of anti-ApoE in our chick learning model. Until relatively recently, although ApoA-I is known to be present in chicks, and indeed its sequence and expression have been studied in detail (Rajavashisth et al., 1987; Ferrari et al., 1987), ApoE was thought to be present only in mammals. As non-mammalian vertebrates have now been shown to express ApoE, however, especially during development (Babin et al., 1997), the way seemed open to explore the role of ApoE in the passive avoidance paradigm.

Western blotting showed that when authentic human VLDL ApoE was probed with the mAb anti-ApoE, a number of bands, in addition to the expected one at 34–37 kD, were recognized. The
bands were not only very broad, making the precise molecular mass identification uncertain, but also showed the smearing that is characteristic of glycosylated proteins. Comparison with the blot obtained using chick SPMs showed a number of possible correspondences, including those found in the chick at 41, 50, and 71 kD (Fig. 1, a,b). The chick SPM signal was weak, however, perhaps because much soluble ApoE had been removed during the preparation of the SPMs. By contrast, and not unexpectedly, antibody to ApoA-I gave a much stronger signal on Western blotting, with major bands seen at 60 and 140 kDa. The implication of the presence of multiple bands, even for the authentic human ApoE, is not known.

When injected into the IMHV, however, both antibodies showed strong immunofluorescence, with no obvious differences in distribution and with extensive binding to the neuropil and around the ventricles in the minutes after injection (Fig. 2). The residence time of the antibodies was relatively brief; within 30 min post-injection most fluorescence was concentrated around the ventricles (Fig. 3), and had entirely disappeared by 60 min. This result contrasts with the longer residence times found, for instance, for antibodies to NCAM (Scholey et al., 1993). The ventricular concentration implies that much of the signal is lost by diffusion; some might be lost, however, because of internalization and inactivation of the antibody.

The apparent identity of the intracerebral distribution of the immunofluorescence of both antibodies points to a related functional distribution of their respective antigens, lending strength to the significance of the clear separation of effects on memory retention between the two. Anti-ApoA-I gave a stronger signal on Western blots of chick SPMs than did anti-ApoE, and had a similar distribution pattern upon injection into the IMHV. Anti-ApoE, however, and not anti-ApoA-I, was amnestic when injected into the IMHV either 30 min pre- or just post-training on the passive avoidance task. This result may be related to that of Masliah et al., (1997) who found that infusion of recombinant ApoE could alleviate some of the cognitive deficits that they observed resulting from the deletion of the gene for the protein. We can conclude that ApoE, while without effect on learning the avoidance task, is required for the task to be effectively remembered, even for a period as brief as 30 min.

The experiments described here point to a significant role for ApoE in the biochemical cascade that is associated with memory formation. That pre- and early post-training injections, but not those 5.5 hr after training, resulted in amnesia suggests that this function is required at an early phase, rather than in the transition to long-term memory that we have postulated for NCAM and L1 (Rose, 1995, Scholey et al., 1995). As the possibility that the transport of LDL or VLDL is directly required for memory formation is not considered likely, at least not at this early time point, the implication would be that the effects of anti-ApoE are mediated via a blockade of some other function of the protein.

If, as we believe, memory formation requires the modulation of synaptic connectivity, the finding that ApoE affects neurite extension (Puttfarken et al., 1997) may be relevant. The most direct mechanism, however, may be via the interactions between ApoE and β-amyloid (Kounnas et al., 1995; Beffert et al., 1997). We have found that antibodies to APP, injected around the time of training, or antisense oligomers injected 12 hr previously, are profoundly amnestic in the chick passive avoidance model (Mileusnic et al., 1998). The time course of onset of this amnesia parallels that found with the anti-ApoE. One implication is that the anti-APP amnesia occurs as a result of blocking the adhesion functions of the external domains of APP, which, like those of NCAM and L1, are necessary for synaptic remodeling. If ApoE and APP interact via a membrane-bound lipoprotein receptor at the synapse, the amnestic effect of antibodies to ApoE could be explained. We are currently exploring these putative interactions and their effect on memory formation and retention.

ACKNOWLEDGEMENTS

We thank Dr Charles Large, of GlaxoWellcome, and members of the Brain and Behaviour Research Group for helpful discussions, Verina Waights for advice on the confocal microscopy, and
Steve Walters and Dawn Sadler for care of the chicks. This work was supported by an MRC-Foresight LINK grant to SPRR.

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