Sense and antisense transcripts of the apolipoprotein E gene in normal and ApoE knockout mice, their expression after spinal cord injury and corresponding human transcripts

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The apolipoprotein E (ApoE) gene has been linked to maladies such as hypercholesterolemia, CNS injury and disease. In this study, we present evidence that, in addition to the known transcript (ApoE S1) that translates into ApoE, there are three additional transcripts in mice. Two of these transcripts, ApoE S2 and ApoE S3, which are predicted to be transmembrane proteins, are transcribed from the sense strand. ApoE AS1 is transcribed from the antisense strand and is complementary to exon 4 of ApoE S1. The open reading frame of ApoE AS1 is conserved between human and mouse. The antisense transcript falls within the region of the human epsilon 4 allele that has been linked to the familial onset form of Alzheimer’s disease. We also demonstrate the expression of ApoE S3 and ApoE AS1 in ApoE knockout mice, and ApoE S1 and ApoE S2 do not get transcribed. We had previously identified ApoE S1 as being upregulated in mice after spinal cord injury. In this study, we show that in spinal cord-injured C57BL/6 mice, both ApoE S1 and ApoE S3 transcripts are 10-fold upregulated and the antisense ApoE AS1 is 100-fold upregulated compared with normal levels. Such data suggest that these alternate transcripts are involved in the molecular pathogenesis of CNS disease and perhaps in ApoE expression in general, as we show that ApoE S2 and AS1 are also transcribed in human.

INTRODUCTION

The molecule apolipoprotein E (ApoE), originally found in the liver where it associates with plasma lipoproteins, is a 34 kDa glycosylated protein, which was first shown to be a plasma protein involved in systemic lipid metabolism and cholesterol transport [1]. This molecule has two structural domains, the N-terminus (residues 1–191) which can bind a low-density lipoprotein (LDL) receptor and can interact with heparin sulfate proteoglycans on the cell surface and the C terminus (residues 222–299) which has a high affinity for lipids and is responsible for lipoprotein binding (2–4). These subunits are connected by a flexible and unstructured region encompassing amino acids 191–216 (3).

ApoE is also synthesized and secreted by most tissues including the CNS, spleen, kidney, adrenal glands, large intestine and lung (5,6) and is associated with cardiovascular and CNS disease through its use of different alleles of the same gene (7–10). In the CNS, the ε4 ApoE allelic form was identified and shown to be associated with Alzheimer’s disease (9).

ApoE is thought to be important in the normal functioning of neural tissue and in response to injury, although the exact mechanisms are not clear. It has been shown to be synthesized and secreted by both astrocytes (6,11) and monocytes (12,13). The finding that ApoE is in neurons in the brain (14) is explained by the presence of ApoE receptors and reflect the uptake of the molecule (15). Using a spinal cord injury model (16), we previously showed that both ApoE mRNA and protein levels are upregulated after spinal cord injury in the mouse (17).

The effects of knocking out the ApoE gene have been studied in mice (18). Creation of the knockout involved the insertion of the neomycin resistance gene by homologous recombination in place of exon 3 and part of intron 3 of the ApoE gene. Mice that were heterozygous for the knockout gene were reported to be healthy at 3 months of age and...
produce homozygous knockouts at the expected frequency. These homozygous knockout progeny appear healthy despite the absence of ApoE in their plasma.

In the present study, we show evidence for two additional mouse sense transcripts generated from the ApoE gene, which could be transmembrane proteins, and an antisense transcript which has an open reading frame in exon 4 that is conserved between mouse and human. The antisense protein falls within the region of the epsilon 4 allele that has been linked to the familial onset form of Alzheimer’s disease (9,19). We also demonstrate the expression of one sense and antisense ApoE transcripts in ApoE knockout mice. Furthermore, we show that these transcripts are upregulated in B6 mice after spinal cord injury and follow the upregulation of ApoE itself. Finally, we find that for one of the additional sense transcripts and the antisense transcript, corresponding RNAs exist in human.

RESULTS

Database support for new ApoE gene products in the mouse

We previously showed that both ApoE mRNA and protein levels are upregulated after spinal cord injury in the mouse (17). In examining the cell types involved, in situ hybridization was carried out using both the ApoE antisense and sense probes (exon 4) on tissue sections. Both probes produced a signal suggesting the presence of an antisense transcript in the absence of ApoE in their plasma.

The Gene2EST BLAST Server (39) was used to align ESTs against mouse, human and rat genomic sequences corresponding to ApoE gene (no. D00466.1) and the mappings were visualized with Artemis (40). This search as well as our own RT–PCR and sequencing efforts uncovered several new transcripts relative to the original known transcript. Our current model of the mouse ApoE gene, including both sense and antisense transcripts, as well as primer and RNA probe locations used in subsequent experiments, is represented in Figure 1. All positional information is made in reference to the genomic sequence of ApoE (D00466), which was corrected for two deletions (discussed subsequently).

ApoE S2 (EST accession nos A1049252, BF236692, A1313990, AA537923) lacks exon 1 and has a translation start site (ATG) 99 bases upstream of ApoE S1 exon 2. Therefore, the amino acid sequence is identical to ApoE S1. Starting transcription in such a manner upstream of exon 2 transforms the secretory signal peptide into a transmembrane helix. Thus, ApoE S2 could encode a 352 amino acid protein with a molecular weight of 40.3 kDa and a transmembrane helix amino acids 43–65 (SOSUI) (23). A BlastP similarity search of ApoE S2 against NCBI’s non-redundant protein sequence database (nr) did not return any hits.

A third transcript, ApoE S3 (EST accession nos AA067884, A1614580), codes for a transmembrane protein as well. Using the published sequence of the mouse gene (D00466.1), we predicted its translational start (ATG) 117 bases before ApoE S1 exon 4 using the same reading frame as ApoE S1 within exon 4. However, our own sequencing could not account for two bases in the published sequence (D00466.1), a C at position 2882 [TTG (C) CTC] and a C at 2898 [TGG (C) CCC]. This was confirmed on the genomic and mRNA level. The corrected sequence of ApoE S3 still predicts an open reading frame; however, the resulting amino acid sequence is entirely different from ApoE S1 even in the shared part of exon 4 because ApoE S3 is using a different reading frame. ApoE S3 is, if translated, 156 amino acids long with a molecular weight of 15.9 kDa and a predicted transmembrane helix (SOSUI) (23) amino acids 16–38.

The antisense transcript ApoE AS1 is complimentary to ApoE S3 (Fig. 1A). Its translational start site is near the stop codon of ApoE S1, with a frame that is open throughout exon 4 and a polyA signal at 2744. It encodes a 219 amino acid long soluble protein with a molecular weight of 26.6 kDa (23). A conserved domain database search did not return any hits (24). An NCBI blastp protein search uncovered low-level similarity with other proteins in the last 100 amino acids, with class I helical cytokine receptor member 2 (Tetradon nigroviridis) (accession no. AAR25665.1) having the highest score (40).

We used the Neural Network Promoter Prediction software (25) to predict the promoters and transcriptional start sites for ApoE S2, ApoE S3 and ApoE AS1. No appropriate targets were found. The program, however, did predict the correct promoter and transcriptional start for ApoE S1.

Expression of new ApoE-derived transcripts in the mouse

Expression of the predicted forms of ApoE was initially tested by RT–PCR. Reverse transcription reactions of RNA from a variety of tissues were primed with oligo dT and amplified with transcript-specific pairs of PCR primers (Fig. 2A). Although this approach could not differentiate between sense and antisense transcripts, it does illustrate a tissue-specific expression pattern for different ApoE transcripts. ApoE S1 is present in all of the tissues tested. ApoE S2 is expressed almost exclusively in the liver, with only marginal levels of expression in the cerebellum, parotis, kidney, lung and thymus. ApoE S3 (or the antisense ApoE AS1) is found in the liver and thymus with lower levels in the lung, parotis, heart and small intestine. Marginal levels appear in skeletal muscle and in the cerebellum (with brainstem). The rest of the organs, the brain, ear, spleen, adrenal gland, bone marrow and fat, were negative.

To distinguish between ApoE S3/AS1, RT–PCR was performed on liver RNA using both RT and subsequent PCR primers that specifically amplify ApoE S3 or ApoE AS1 (Fig. 2B and C). Both ApoE S3 (Fig. 2B) and ApoE AS1 (Fig. 2C) are present in liver.

The presence of ApoE S1, ApoE S2, ApoE S3 and ApoE AS1 was also confirmed by northern analysis (Fig. 4A–D). Probes were designed to hybridize only to their specific target. In addition to binding the sense probe no. 3 (Fig. 1), ApoE AS1 also hybridized to a sense probe no. 4 in C57BL/6 liver (data not shown), confirming that the antisense transcript at least extends between its start and stop codon.
Expression of the new ApoE-derived transcripts in APOE knockout mice

The ApoE knockout mouse has been an important tool in determining the role of ApoE in a variety of systems. Therefore, we wanted to investigate the effect of knocking out this gene on the expression of ApoE transcripts. In the knockout mouse that we used in this study, the ApoE gene is disrupted upstream of exon 4 (Fig. 1).

Again, we employed oligo dT-primed RT–PCR with transcript-specific PCR primers to investigate whether any of the ApoE transcripts could be detected in the ApoE knockout mouse. Only ApoE S3/AS1 could be amplified by this method (Fig. 3A). Furthermore, the expression profile differed from the wild-type where ApoE S3/AS1 could not be amplified in normal brain and spleen (Fig. 2).

The use of specific RT and PCR primers revealed that both ApoE S3 and ApoE AS1 are expressed in liver of the knockout (Fig. 3B). Northern blot analysis also demonstrates the presence of ApoE AS1 in the knockout mouse. Although the knockout seems to affect the transcription level of ApoE S3 such that it is almost below the detection limit of this technique (Fig. 4C), ApoE AS1 mRNA (Fig. 4D) is present in both the knockout and wild-type mice.

Functional expression of new ApoE transcripts

As a follow-up to our previous studies showing ApoE expression following spinal cord injury in C57BL/6 mice (17), we examined the expression of various transcripts of the ApoE gene after spinal cord injury. ApoE S2 could not be demonstrated in brain but only at marginal levels in the cerebellum (Fig. 2A). Using RT–PCR, ApoE S2 also could not be detected in uninjured or injured spinal cord

Figure 1. (A) Representation of the ApoE gene in the mouse (accession no. D00466.1 with two corrections made, deletion of a C at position 2882 [TG (C) CTC] and a C at 2898 [TGG (C) CCC]. The known transcript is named ApoE S1. Start and stop sites of translation are indicated by black lines in the bars, predicted transmembrane regions are shown as a solid black rectangle. ApoE S1 and S2 use the same frame, ApoE S3 uses a different frame. ApoE AS1 translates a protein in the antisense direction of the gene. Primers are shown with their 5′ location. Also shown is the location of the neo insert of the ApoE knockout mouse (C57BL/6J-Apoptm1Unc, Jackson) (18). (B) Representation of the human APOE gene (accession no. M10065). ApoE S2 corresponds to the mouse transcript; however, the protein translated is identical to ApoE S1. The antisense transcript is highly conserved between human and mouse. There are two alternative stop codons shown for the AS1 molecule, as the one at 3819 will be open in one of the allelic variations (Table 1).

Figure 2. (A) RT–PCR showing expression levels of the different transcripts on a variety of tissues. Primers used are: for ApoE S1, 52F–40R; ApoE S2, 40F–40R; ApoE S3/AS1, 44F–40R; GAPDH, 47F–47R. Controls with samples that omitted the enzyme in the RT reaction were included for each primer pair and were negative (data not shown). (B) and (C) Directional-specific RT–PCR demonstrating the presence of sense (ApoE S3) and antisense transcripts (ApoE AS1) in liver. (B) ApoE S3: RT primer used 45R, PCR with 69F–70R. Lane 1, +RT; lane 2, −RT. (C) ApoE AS1: RT primer used 44F, PCR with 69F–45R in lanes 1 and 4, 49F–46R in lanes 2 and 5, 49F–40R in lanes 3 and 6. Lanes 1–3 +RT, lanes 4–6 −RT. All products were gel purified and sequence verified.
Transcripts of the human gene

Finally, to investigate whether corresponding transcripts to the mouse ApoE S2, S3 and AS1 transcripts are also present in human, an RT–PCR analysis was carried out on human liver RNA. The resulting model of the human gene and its transcription products are shown in Figure 1B. Human ApoE S2 could be amplified by RT primed with oligo 1R and subsequent PCR primed with either 6F–5R (lane 1, Fig. 7A) or 7F–5R (lane 2, Fig. 7A). The human ApoE S2 sequence is also backed up by an EST (accession no. CA771535). In contrast to mouse, in human, the protein translated from ApoE S2 is the same as ApoE S1.

A human sequence corresponding to ApoE S3 could not be amplified. RT using 1R and subsequent PCR with primer combinations 3F–2R, 8F–2R and 4F–2R did not yield products that are transcribed from the ApoE gene.

The presence of human ApoE AS1 was demonstrated by RT primed with 3F followed by PCR with primer 8F–1R (lane 1, Fig. 7B). Two products were amplified by this reaction, and after sequencing, the lower band was confirmed as originating from ApoE AS1 message. The upper band is not a product of the ApoE gene. If translated, the human and mouse proteins are highly conserved.

DISCUSSION

In this study, we have shown that multiple RNAs, in both the sense and antisense orientation, are produced by the mouse and human ApoE gene, thereby adding this gene to a growing list of genes that are known to be transcribed from both strands. This list is conservatively estimated to number about 1600 or ~8% of the genes contained in the human genome (26). In a recent paper, Chen et al. (27) show that 22% of clusters transcribe sense–antisense pairs, the highest number so far supported by EST evidence. Matching accession numbers with their data, it was found that ApoE was not one of the genes. However, the number of genes that transcribe from both strands identified so far show that antisense transcription has been greatly underestimated in its contribution to genomic complexity.

One set of ApoE transcripts that we identified code for putative transmembrane proteins, one of which is frame shifted, the other is an antisense RNA. Both sense and antisense messages have sequences recognized as translation start sites and therefore could exist as protein. Examples demonstrating the expression of antisense message have been shown for a variety of genes (reviewed in 28 and 29) including urocortin (30), cardiac troponin (31) and basic fibroblast growth factor (bFGF) (32). The scenario we describe here for ApoE most closely resembles that of bFGF expression. The bFGF gene locus is known to be transcribed into multiple mRNA products, including at least one antisense transcript. This antisense transcript may be involved in regulating gene expression by hybridizing the sense transcript while still in the nucleus and perhaps affecting developmental patterns and tissue differentiation (32). The bFGF transcript isolated from Xenopus could also, however, be translated into a 25 kDa protein of as yet unknown function (33). At this point, it is unclear whether any of our newly identified ApoE
transcripts, including the antisense transcript, are in fact translated except for ApoE S2 protein that is found in the serum. Beyond being translated into a protein, all the transcripts could participate in regulating each other’s levels through a process like RNAi.

Marking yet another similarity with bFGF, the antisense transcript is present in both human and mouse (33). For the ApoE antisense transcript, we can say that the reading frame is also open in humans and, consequently, there is the possibility that an antisense message is actually translated into a protein. The protein predicted from sequence M10065 has 198 amino acids and has a signal peptide predicted by the SignalP-3.0 HMM program (34) with a probability of 0.973. In contrast, in the mouse AS1 protein, the same program predicts a signal peptide with a probability of only 0.265. Even deleting the first 21 amino acids in the mouse protein that the human protein lacks increases the probability only to 0.38. Therefore, even though the human sequence probably enters the secretory pathway, the mouse sequence seems to lack a signal peptide. As programs like SignalP-3.0 HMM are focusing on prediction of classical signal peptides, one cannot exclude the presence of other signal motifs in the mouse protein.

The ApoE antisense protein is encoded by exon 4, the exon that accounts for the three major allelic variants in humans. These allelic variations of the sense protein are defined by the substitution of an arginine (a positively charged polar amino acid) for a cysteine (a non-polar amino acid) at positions 112 and 158 of the protein (35,36). In the putative antisense protein, an arginine is substituted for a histidine, both positively charged polar amino acids. Therefore, these two variants are probably affecting the function of the antisense protein less than that of the sense protein and are therefore more conservative in the antisense direction.

However, as studies of allelic variations and their implications in disease have always been conducted in reference to the sense protein, other more prominent variations (Table 1) might lead to functional and more relevant changes. One dramatic change is the variation at positions 3817–3819 (sequence position in reference to M10065). The substitution of TAG to GCT on the antisense strand opens the reading frame, so translation can continue for another 113 amino acids. The E4 allele as published by Paik et al. (35) and Emi et al. (36) contains a stop codon at positions 3817–3819. However, at this point, it is not clear if the allelic variation that opens the reading frame is linked with any of the major allelic forms like the E4, E3 or E2 genotype (for some of the observed haplotypes, see Table 1 in 37; for other relative frequencies of ApoE sequence variants within the E4, E3 and E2 genotypes, see Table 3 in 38). Therefore, an involvement of the putative antisense protein in diseases correlated with the allelic variants cannot be excluded. For example, in Alzheimer’s disease, the specific molecular pathway by which ApoE modifies the expression of this disease remains elusive (19). Furthermore, other sequence variations in the different alleles (34) that are conservative in the sense direction might lead to amino acid substitutions or even stop signals in the antisense direction.

Even though our sequence work thus far suggests that human ApoE S2 is translated to the same protein as ApoE S1, allelic variation might cause an open reading frame similar to the one seen in C57BL/6 mice, leading to the presence of a transmembrane version of ApoE.

Knockout organisms are created to investigate any phenotypic changes that may take place in that organism in the absence of a gene. Our data suggest that the genetic manipulation used to generate the ApoE knockout has only eliminated the expression of ApoE S1 and ApoE S2 transcripts. We found
that both ApoE S3, a putative transmembrane protein with a sequence completely different than that known for the ApoE protein, and ApoE AS1, a protein potentially produced from an antisense transcript, are still expressed in the knockout. This is possible because the region of the gene responsible for these transcripts, in particular exon 4, has not been disrupted by the insertion of the neo gene in the knockout. The roles of these transcripts are currently unknown. In addition, further work must be done to determine whether they are translated in vivo.

An indication of a potentially important role for both the ApoE S3 and ApoE AS1 antisense messages in CNS disease comes from data in our mouse spinal cord injury model (16,17). In response to spinal injury, both of these transcripts are upregulated and follow the expression pattern of ApoE S1. However, as ApoE AS1 is 10-fold more upregulated than either ApoE S1 or S3 it might also be involved in influencing their translation. Again, it would be useful to know if either of the alternate mRNAs is translated in response to spinal injury.

ApoE is a lipoprotein, a class of proteins responsible for carrying cholesterol and other fats through the bloodstream as little packages and essential for the normal breakdown of these molecules. In particular, ApoE is a major component of specific lipoproteins called very low-density lipoproteins (VLDLs). A major function of VLDLs is to remove excess cholesterol from the blood and carry it to the liver for
Maintaining normal levels of cholesterol is essential for the prevention of cardiovascular diseases, including heart attacks and stroke. There are at least three slightly different versions of the ApoE gene. The major versions, or alleles, are called $e_2$, $e_3$ and $e_4$. The most common allele is $e_3$, which is found in more than half of the population. Researchers have found that most people with familial hypercholesterolemia, a condition that causes very high levels of cholesterol and an increased risk of heart attacks and strokes, have two copies of the $e_2$ allele. This allele seems to be one of several genetic factors that play a part in this disorder. In addition, ApoE allelic version $e_4$ is a risk factor for coronary artery disease. People who inherit at least one copy of the ApoE $e_4$ allele have an increased risk of developing type 2 Alzheimer’s disease, which first appears later in life. Studies have shown that people who inherit two copies of ApoE $e_4$ have the highest chance of developing the disease. It is not yet known how this allele affects a person’s risk of Alzheimer’s disease; however, it has been found that it increases the number of amyloid plaques, which are characteristic of the disease (9,19).

Finally, EST evidence for an ApoE alternative splice variant in human does exist (41). One EST corresponds to the last exon, which is different for ApoE S2 and S3. This has been validated by RT–PCR which showed that it is expressed in lung cancer but not as strongly as in normal tissue.

Table 1. Sequence variations of the human ApoE gene (accession no.: M10065) in relation to ApoE S1 and AS1 protein in intron 3 and exon 4

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<th>Position</th>
<th>Variation in sense strand</th>
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In conclusion, the ApoE protein may play a vital role in the occurrence of a diverse group of maladies. It now appears that the ApoE gene can be characterized by a complex expression pattern with multiple start sites in the sense and antisense direction. As they all have an open reading frame, these transcripts may be translated into proteins. Work must be done to determine whether the antisense transcript is involved in regulating expression post-transcriptionally, is translated into a novel protein or both. In addition, given the significance of exon 4 in the various allelic versions of ApoE, a knockout of this exon may give insight into its role.

MATERIALS AND METHODS

RT–PCR (mouse)

Tissue was dissected from CO2 euthanized 8-week-old female C57BL/6 mice or ApoE knockout mice (C57BL/6-Apoetm1Unc, Jackson Laboratory, Bar Harbor, ME, USA) and stored at −80°C until further use. RNA was isolated by homogenizing the pooled tissue from two animals in Trizol (Gibco-BRL) following the manufacturer’s instructions. RNA was treated 30 min with DNase (Promega) to remove any DNA that might have copurified. RNA concentration was determined by OD260 readings. Three micrograms of RNA was treated 30 min with DNase (Promega) to remove any DNA that might have copurified. RNA was isolated by phenol:chloroform and precipitated with alcohol.

RT–PCR (human)

Human liver RNA was obtained from Stratagene (catalog no. 504017). Enzymatic reactions were carried out as described earlier. For APOE S2, RT was carried out with primer 1R and PCR with primers 6F–5R or 7F–5R. For APOE AS1, RT was carried out with primer 3F and PCR with primers 8F–1R (Fig. 1).

Gene and sense–antisense-specific reverse transcription (mouse)

RNA was extracted either from exsanguinated liver or from spinal cord with Trizol, treated with DNase, re-extracted with phenol:chloroform and precipitated with alcohol. Samples from each time point were pooled equally (according to OD260) and reverse transcription was carried out on 0.75 μg for each time point in a 10 μl reaction as described earlier with the exception that gene-specific primers were used. To selectively amplify (i) ApoE exon 4 sense strand, we used primers 45R and 48R (for GADPH control) and (ii) the antisense, we used primers 45F and 48R (for GADPH control). Control reactions omitted reverse transcription. Reverse transcription was carried out at 42°C for 1 h. Samples were brought up to 100 μl with H2O and passed through Chroma Spin-100 size exclusion column (Clontech) to exclude unused primers, re-extracted with phenol:chloroform and precipitated with alcohol.

Real-time RT–PCR (mouse)

Real-time RT–PCR (mouse) (20) was carried out on a Lightcycler (Roche) (21) to determine the amount of ApoE S1 (RT primed with 45R, PCR primers 52F–41R), antisense in exon 4 (RT primed with 45F, PCR primers 46F–46R) as well as ApoE S3 (RT primed with 45R, PCR primers 44F–44R). The reaction was set up using the Faststart DNA Master Sybr Green I Kit (Roche) according to instructions in a 10 μl cocktail with a magnesium concentration of 2 mM. A period of 10 min at 95°C was used to denature and inactivate the Taq-start antibody. Cycling was carried out 45 times using 95°C for 10 s, 61°C for 10 s, 72°C for 20 s and the acquisition temperature was set at 85°C for 10 s. Levels are quantified on the basis of the fluorescence of intercalated SYBR Green I dye to dsDNA after each cycle. Quality control includes

Table 2. Primers to mouse ApoE (GenBank accession no. D00466.1) mouse GAPDH (M32599) and human ApoE (M10065)

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exponential of the curves and melting product analysis to ensure the amplification of only one template, as well as size analysis through gel electrophoresis.

**Northern hybridization (mouse)**

Total RNA was extracted with Trizol, treated with DNase and 10 μg run on denaturing 3.5% polyacrylamide urea (8 M) gels in 1 × TBE buffer. Electroblooting was done onto Hydrobond-N+ (Amersham-Pharmacia) in 0.25 × TBE with 150 mA, 50 V for 1.5 h. The RNA was checked with a UV light for integrity and evenness of transfer. RNA was immobilized onto the membrane through backing under vacuum at 80°C for 30 min. Sense and antisense-specific RNA probes were synthesized through T7 or T3 polymerase using Epicintress RiboScribe kit. Gel-purified PCR products generated with synthesized desalted by Integrated DNA Technologies TAAAGGG-3′ promoter served as templates for generating the probes. Location of the probes is indicated in Figure 1. T7 and T3 polymerase 0.5 U/l were carried at 37°C for 3 h followed by 15 min of DNase treatment at 37°C. Probes were purified thorough Chroma Spin-100 size exclusion column (Clontech) and an aliquot run on 2% agarose checking for size and labeling. Hybridization was carried out in UltraHyb (Ambion) hybridization buffer overnight at 68°C after 1 h of blocking in rotating chambers. Membranes were washed at 2 × 5 min at 65°C with 2 × SSC, 0.1% SDS followed by 2 × 15 min at 68°C with 0.1 × SSC, 0.1% SDS. Signal was collected on Phosphor Screens and scanned with a Storm scanner (Molecular Dynamics).

**Primers for RT and PCR**

Primers were designed using the software ‘primer 3 www.cgi v 0.2’ (22) applied to the ApoE genomic sequence (GenBank accession no. D00466.1). Specificity was determined by size and sequence verification for each product. The location of each primer pair can be seen in Figure 1. Primer sequences are shown in Table 2. When used to amplify templates for in vitro transcription of RNA probes, the T7 (5′-TAATAC GACTCACTATAGGG-3′) or T3 (5′-AATTAACCCCTCAC TAAAGGG-3′) promoter was added. Primers were synthesized desalted by Integrated DNA Technologies (Coralville, IA, USA).

**Spinal cord injuries**

For all experiments, female C57BL/6 mice (Jackson Laboratory) were used at an age of 8–10 weeks. Animals were injured using a previously described surgical model of complete spinal cord transection, including the meninges, the ‘cut dura’ model (16,17). In short, animals were anesthetized with ketamine and xylazine (100 and 15 mg/kg i.p., respectively). The skin was surgically scrubbed with chlorohexadine, and the hair at the surgical field was removed. A complete transection of the cord was carried out at level TH8–9 using a microscalpel (5 mm blade depth, 15°; catalog no. 37-7515; Roboz Surgical Instruments). Two sham, two unoperated controls and three animals per time point were used for real-time RT–PCR analysis from the injury site (TH7–10). For northern analysis, 10 μg of DNase treated, Trizol extracted total RNA from one animal per time point was used. Dissected spinal cord tissue was stored at −80°C until further use.

**Western blot (mouse)**

Liver and serum samples were collected from C57BL/6 and Apoe k/o mice (Jackson Laboratory) at an age of 4–5 months. For serum, blood was collected from the tail vein and left to coagulate for ~2 h at +4°C until separation. Liver was collected on wet ice, homogenized with a glass homogenizer and mixed with lysis buffer, containing proteinase inhibitor cocktail (CompleteTM from Boehringer Mannheim) in 1 × PBS with 0.1% SDS at +4°C. Total protein concentration of sera and liver samples was determined using a standard Lowry protein assay procedure (DC Protein Assay Kit, BioRad Laboratories, catalog no. 500-0116) and equilibrated to a total protein concentration of 10 mg/ml. Protein samples were mixed with 4× SDS gel loading buffer and boiled for 10 min, then loaded onto a 10% SDS–PAGE (for the liver samples) at 20–30 μg per well (lane) or loaded onto a 12% gel (for serum samples) at 20–30 μg/well. The gel was then electroblooted to Immobilon-P (Millipore) in 25 mm Tris–OH, 250 mm glycine, 10% methanol at 8 V, 30 mA overnight. The blot was blocked in PBS–Tween-20 (0.005%), 5% milk for 1 h. To detect ApoE S1 and ApoE S2, blots were incubated with a goat anti-apoE antibody (1:300) (sc-6384, Santa Cruz Biotechnology) overnight at +4°C. As a secondary antibody, an HRP-labeled mouse monoclonal anti-goat/sheep antibody was used (Sigma A-9452) for 1 h at RT at the following concentration: for liver, 1:5000; for serum, 1:7000. The blot was developed using the chemiluminescence kit from Boehringer Mannheim. The chemiluminescent signal was scanned and digitalized.

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**Conflict of Interest statement.** None declared.

**REFERENCES**