SREBP-1c expression in Schwann cells is affected by diabetes and nutritional status

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Abstract

Our previous work demonstrated that the sterol response element binding proteins (SREBP)-1 and SREBP-2, which are the key regulators of storage lipid and cholesterol metabolism respectively, are highly expressed in Schwann cells of adult peripheral nerves. In order to evaluate the role of Schwann cell SREBPs in myelination and functioning of peripheral nerves we have determined their expression during development, after fasting and refeeding, and in a rodent model of diabetes. Our results show that SREBP-1c and SREBP-2, unlike SREBP-1a, are the major forms of SREBPs present in peripheral nerves. The expression profile of SREBP-2 follows the expression of genes involved in cholesterol biosynthesis, while SREBP-1c is co-expressed with genes involved in storage lipid metabolism. In addition, the expression of SREBP-1c in the endoneurial compartment of peripheral nerves depends on nutritional status and is disturbed in type 1 diabetes. In line with this, insulin elevates the expression of SREBP-1c in primary cultured Schwann cells by activating the SREBP-1c promoter. Taken together, these findings reveal that SREBP-1c expression in Schwann cells responds to metabolic stimuli including insulin and that this response is affected in type 1 diabetes mellitus. This suggests that disturbed SREBP-1c regulated lipid metabolism may contribute to the pathophysiology of diabetic peripheral neuropathy.

Introduction

During the development of the rodent nervous system, the myelin membrane that surrounds peripheral nerves increases in surface as much as 10,000-fold in the first ten postnatal weeks (Webster, 1971). This dramatic increase in myelination requires synthesis of myelin proteins and is closely matched by synthesis of myelin membrane lipids (e.g. cholesterol, galactolipids and phospholipids) by Schwann cells (Jurevics and Morell, 1994; Morell and Jurevics, 1996; Yao and Bourre, 1985). Interestingly, maturation and maintenance of the myelin of the peripheral nervous system is accompanied by a significant, however less well understood, increase in free fatty acid synthesis in the endoneurium (Yao and Bourre, 1985).

In order to analyze the transcriptional changes associated with the process of myelination and myelin maintenance, we have recently performed microarray analysis of mouse peripheral nerve during development and have identified a large group of regulated genes involved in lipid metabolism (Verheijen et al., 2003). These genes can be divided into two clusters based on their functions and peaks of expression: (i) genes involved in cholesterol biosynthesis that are maximally expressed during myelination, and (ii) genes involved in metabolism of fatty acids and storage lipids, which are maximally expressed after the stage of active myelination (Verheijen et al., 2003). This temporally restricted expression of gene sets implicated in cholesterol and storage lipid metabolism may potentially be explained by the involvement of the SREBPs, the basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors known to regulate lipid metabolism in liver and adipose tissue. SREBP-1c and SREBP-2 preferentially govern the upregulation of genes involved in fatty acid and cholesterol metabolism respectively, while SREBP-1a activates both pathways (Horton et al., 2002). Whereas SREBP-1a is expressed ubiquitously at low levels (Horton et

al., 2002), the expression levels of SREBP-1c and SREBP-2 are differentially regulated. SREBP-2 expression is induced under conditions of sterol-depletion (Sato et al., 1996), whereas SREBP-1c expression in liver is repressed by fasting and induced by refeeding with a carbohydrate-rich diet (Horton et al., 1998). SREBP-1c expression is also under the control of insulin, glucose and fatty acids in several tissues, such as in liver and fat, where it is implicated in deregulated gene expression and complications due to diabetes mellitus (DM, (Becard et al., 2001; Guillet-Deniau et al., 2002; Sun et al., 2002)).

We have previously shown that SREBP-1c is expressed in Schwann cells of peripheral nerves and that local lipid metabolism is important for normal nerve function (Verheijen et al., 2003). This, and the observations by others that the action of SREBP-1c in multiple tissues is affected in diabetes, suggests that malfunction of SREBPs may also underlie the pathological changes associated with diabetic peripheral neuropathy (DPN). DPN is the most common secondary complication of DM with an average prevalence among diabetic patients of approximately 30% (Sima, 2003). Both axons and glial cells are thought to be increasingly affected by diabetes from the proximal to the distal end of the nerve (Dyck and Thomas, 1999; Sima, 2003). Some of the pathogenic elements suggested to be involved in type 1 diabetes mellitus (DM1) DPN may be due to glucose toxicity, ensuing from chronic hyperglycemia (e.g. activation of the polyol pathway, non-enzymatic glycosylation, oxidative stress and microvascular dysfunction/ischemia (Brownlee, 2001; Vincent and Feldman, 2004)), whereas others seem more related to insulin and C-peptide deficiency (e.g. altered neurotropism, Na+/K+-ATPase activity and nodal degeneration (Sima, 2003)). The contribution of mechanisms other than glucose toxicity is suggested by the results of the Diabetes Control and Complications Trial (DCCT), showing that extensive glycemic control reduced the incidence of DPN by 60% in DM1 patients, but did not completely prevent DPN (Tesfaye et al., 1996). Abnormal lipid metabolism was previously proposed as one of the contributing factors in the pathogenesis of DPN (Coste et al., 2003; Horrobin, 1997).

In order to understand the role of SREBPs in myelination and (dys)functioning of the peripheral nerve, we determined their expression during development, after fasting and refeeding and in a rodent model of diabetes. Importantly, our data revealed that the expression of the SREBP-1c isoform is regulated by food consumption and is disturbed in a rodent model of type 1 diabetes.

Results

Expression profiling of SREBPs in peripheral nerve

Using microarray measurements we have previously observed that the expression of SREBP-1 is upregulated in adult sciatic nerve only after the process of myelination has been completed (Verheijen et al., 2003). However, these measurements were performed on nerve samples containing both endoneurium and peri-epineurium. Also, it was not possible to distinguish between the expression of SREBP-1c and SREBP-1a, which differ only with respect to their first exon (Shimomura et al., 1997). In our current experiments, we isolated the nerves from P0, P4, P28 and P56 rats and separated the endoneurium from peri-epineurium at all time points except for P0. The importance of this separation can be demonstrated using SREBP-1c mRNA level as an example. We found that the peri-epineurial compartment already substantially contributes to the whole sciatic nerve SREBP1c expression at P4 (**Supplementary Fig. 1**). Semi-quantitative PCR analysis of SREBP-1a, -1c and -2 using cDNA prepared from isolated endoneurium shows that both SREBP-1c and SREBP-2 change their expression level over a wide dynamic range during development, while the expression level of SREBP-1a remains stable (**Fig. 1**). SREBP-1c is expressed maximally in adults (P56), while SREBP-2 is expressed maximally during myelination (P4). In order to compare the expression levels of different SREBPs in endoneurium and peri-epineurium we performed absolute quantitative PCR using adult (P56) nerve samples. As controls, mRNAs from liver and spleen were used, in which the expression level of SREBP-1c is respectively higher or lower than that of SREBP-1a (Shimomura et al., 1997). This analysis shows that both SREBP-1c and -2 mRNAs are the predominant SREBP transcripts present in endoneurium and peri-epineurium, whereas SREBP-1a mRNA is almost absent (**Fig. 2**).

Effect of fasting and refeeding on endoneurial SREBP-1c expression in mice

The substantial increase in SREBP-1c expression after the active process of myelination, which matches the expression of genes involved in storage lipid metabolism (Verheijen et al., 2003), led us to determine SREBP-1c transcriptional regulation. Previous studies have demonstrated that SREBP-1c expression in liver is repressed by fasting, whereas it is strongly upregulated by refeeding (Horton et al., 1998). To determine whether metabolic changes *in vivo* may also regulate SREBPs in cells of the peripheral nervous system, we isolated sciatic nerve endoneurium from mice fed *ad libitum*, fasted for 24 hours or mice fasted for 24 hours and subsequently refed for 9 hours. Indeed, SREBP-1c expression is repressed by fasting and strongly induced after 9 hours of refeeding while the expression of SREBP-2 remains stable (**Fig. 3a**). Importantly, the induction of SREBP-1c mRNA was accompanied by induction of SREBP-1c precursor protein (**Fig. 3b**). To establish whether a similar regulatory mechanism also applies to regulation of SREBP-1c in cells of the central nervous system, we measured its expression in optic nerve (CNS) as compared to sciatic nerve endoneurium (PNS) and liver, from mice either fasted for 24 hours or mice subsequently refed for 6 or 9 hours. In agreement with our previous observations SREBP-1c expression in endoneurium and in liver is repressed by fasting and already strongly induced 6 hours after refeeding ($p=4.0x10^{-7}$), and induction is even more pronounced after 9 hours of refeeding ($p=5.0x10^{-19}$, **Fig. 3c and 3e**). This increase seems to be functionally relevant since the induction of SREBP-1c in the endoneurium is accompanied by the induction of the downstream genes fatty acid synthase (Fasn) and stearoyl-coA desaturase (Scd1) (**Fig. 3d and 3f**). In contrast, expression of SREBP-1c in the optic nerve did not show any change after 6 hours of refeeding (**Fig. 3c**), whereas it showed a modest decrease after 9 hours of refeeding ($p=1.0x10^{-4}$, **Fig. 3e**).

Changes in SREBP regulated gene expression in endoneurium of diabetic BB/Wor rats

Our observation that the expression level of SREBP-1c in endoneurium is affected by metabolic changes suggests that SREBP-1c expression in Schwann cells of the peripheral nerve may be under the control of insulin and/or glucose, as was described for liver, muscle and fat (Becard et al., 2001; Guillet-Deniau et al., 2002; Sun et al., 2002). Therefore, we tested the possible deregulation of the SREBP-regulated transcriptional network in peripheral nerve endoneurium under type 1 diabetic conditions (insulin deficiency and hyperglycemia) in the BB/Wor rat, a DM1 animal model (Sima et al., 2000). At the age of 70-80 days, the BB/Wor rat spontaneously develops hyperglycemia, due to an immune-mediated destruction of insulin-producing pancreatic beta cells, and it requires daily insulin administration for its survival. A decrease in motor nerve conduction velocity (MNCV), which is a reliable marker for DPN, is detectable in BB/Wor rats already one week after the onset of diabetes (Sima et al., 2000; Vinik and Mehrabyan, 2004). We determined the endoneurial expression of SREBP-1c and its target genes after 2 weeks of diabetes when the reduction of MNCV is already substantial (**Fig 4a**). As shown in **Figure 4b**, SREBP-1c mRNA levels are reduced in diabetic endoneurium in contrast to mRNA levels of SREBP-2. In line with this, the reduced SREBP-1c mRNA level is accompanied by a reduction in the transcript levels of its target genes Fasn and Scd1. To exclude the possibility that the observed reduction in SREBP-1c mRNA level can be attributed to a loss of Schwann cells, we also determined the expression of the Schwann cell specific gene myelin protein zero (Mpz) and of apolipoprotein E (ApoE), a gene involved in lipid transport and with strongly induced expression in injured peripheral nerve (Leblanc and Poduslo, 1990; Verheijen et al., 2003). Mpz expression is not affected (**Fig. 4b**), strongly suggesting that the reduced expression of SREBP-1c, and its target genes, is a response that is specific to the diabetic state, and is not due to a loss of Schwann cells. The small increase in Apoe expression (**Fig. 4b**) may possibly reflect the beginning of myelin injury in the diabetic nerve.

The effect of fasting/refeeding on SREBP-1c regulated gene expression in a rat model of diabetes mellitus

Next, we determined whether the regulation of endoneurial SREBP-1c expression by nutritional status observed in mice, may also be observed in rats and furthermore, whether this response is affected in diabetic animals. Indeed, refeeding of non-diabetic rats led to an increase in endoneurial SREBP-1c (**Fig. 5a**), which was similar to the induction observed in mice (**Fig. 3a, c and e**). Short-term diabetes reduced the expression of SREBP-1c in fasted BB/Wor rats ($p=2.0x10^{-4}$), which is in agreement with our observation in diabetic BB/Wor rats fed *ad libitum* (**Fig. 4b**). Interestingly, refeeding did however not increase SREBP-1c expression in diabetic animals. The fasting/refeeding protocol therefore led to an even more pronounced difference in SREBP-1c expression level between control and diabetic animals ($p=7.0x10^{-4}$, **Fig. 5a**). In line with this, the expression of the SREBP-1c target genes Fasn and Scd1 also increased in refed control animals, whereas no induction of Fasn and Scd1 gene expression was observed in diabetic animals (**Fig. 5b**).

Insulin activates the SREBP-1c promoter in cultured Schwann cells

Our observations that the diabetic condition is associated with (i) reduced expression of endoneurial SREBP-1c, and (ii) blunted upregulation of endoneurial SREBP-1c expression by fasting/refeeding, suggest that insulin may affect SREBP-1c expression in Schwann cells of peripheral nerves. To test this we determined the action and regulation of SREBP-1c in isolated rat Schwann cells. SREBP-1c has been demonstrated to activate the Fas-promoter in liver and fat (Le Lay et al., 2002). **Figure 6a** shows that SREBP-1c also activates the FAS promoter in Schwann cells, since transfection of active SREBP-1c strongly activated a co-transfected FAS-promoter luciferase reporter (Amemiya-Kudo et al., 2002).

To determine whether depletion of insulin or hyperglycemia underlies the downregulation of the SREBP-1c transcriptional network in diabetic nerves, we evaluated the effect of insulin and/or high glucose on the activity of the SREBP-1c and FAS-promoter. We show that the activity of both promoters in Schwann cells is not affected by high glucose levels that are in the range of hyperglycemia observed in diabetes (25 mM) or at even higher levels (75 mM)(**Fig. 6b**). Nor did hypoglycemic levels of glucose (2.5 mM) have an effect on the SREBP-1c or the FAS promoter in Schwann cells. It should be noted that the same reporter constructs are activated by glucose in hepatocytes (Hasty et al., 2000). Importantly, treatment of Schwann cells with insulin did activate the SREBP-1c promoter, which again was not affected by the levels of glucose (**Fig. 6c**). In line with this, insulin-treatment also increases SREBP-1c precursor protein levels in Schwann cells (**Fig. 6d**). Taken together, these results suggest that it is the level of circulating insulin rather than that of glucose that determines the expression level of SREBP-1c in Schwann cells.

Discussion

We previously reported that SREBP-1 and SREBP-2 are highly expressed in Schwann cells of rodent adult peripheral nerve. Here, we show that the SREBP-1a gene is stably expressed at low levels and that the SREBP-2 gene follows the expression profile of genes necessary for cholesterol and myelin membrane biosynthesis. Most remarkably, we find that SREBP-1c expression in the nerve reaches its maximum long after the process of active myelination. We show that Schwann cell SREBP-1c expression is induced by insulin, which is in line with our observations that genes downstream from endoneurial SREBP-1c are induced by refeeding following fasting, and are also affected by type 1 diabetes.

SREBP expression in developing peripheral nerve

Our expression analysis of transcripts encoding different SREBP isoforms in developing peripheral nerve shows that the level of SREBP-1c transcript increases during development whereas SREBP-1a mRNA levels remain constantly low. These observations are in agreement with the measurements of Salles et al (Salles et al., 2003), who observe a small increase in the ratio of SREBP-1c:SREBP-1a expression in nerves isolated from five to forty day old mice. However, LeBlanc et al (Leblanc et al., 2005) proposed that both SREBP-1c and SREBP-1a are induced during postnatal development of the peripheral nerve. The discrepancy between these observations and our data may be explained partially by the fact that we have analyzed specifically gene expression in the endoneurial compartment of the developing sciatic nerve whereas others examined the whole nerve. Noteworthy, the peri-epineurial compartment, which is relatively rich in adipocytes (Verheijen et al., 2003), expresses all three SREBPs (cf. fig. 2).

SREBP-2 is the predominant form in adult sciatic nerve, expressed maximally during active myelination (P4-P10), together with other genes involved in cholesterol and myelin

membrane biosynthesis (Verheijen et al., 2003). This expression profile strongly supports an active role for SREBP-2 in myelination. The observed upregulation of SREBP-1c, once the process of myelination has been completed, may be related to the increase in expression of genes involved in fatty acid synthesis and storage lipid metabolism (Verheijen et al., 2003). This is in agreement with the previously reported increase in fatty acid synthesis in the endoneurium (Yao and Bourre, 1985).

Metabolic regulation of endoneurial SREBP-1c expression

The expression of SREBP-1c is known to be influenced by nutritional changes in liver, adipose tissue and skeletal muscle, which are all tissues primarily involved in lipid metabolism (Gosmain et al., 2005; Horton et al., 1998; Matsuzaka et al., 2004). Here we show that SREBP-1c is also nutritionally regulated in Schwann cells of the peripheral nerve. Furthermore, the increase in Schwann cell SREBP-1c expression is accompanied by an increase in expression of Fasn and Scd1, two genes known to be regulated by this transcription factor (Liang et al., 2002; Shimano et al., 1997). This strongly suggests that reduced SREBP-1c mRNA levels result in lower expression of its target genes in Schwann cells. On the other hand, our observations on SREBP-1c expression in cells of the central neural system (i.e. the optic nerve), suggest that it is non-responsive to changes in nutritional status, which is in line with very recent reports on SREBP-1c gene expression in the hypothalamus and cerebrum (Okamoto et al., 2006).

Our observations on diabetic rats show that decreased expression of SREBP-1c in Schwann cells is accompanied by reduced MNCV. However, no difference in MNCV between fasted and fasted/refed wild type animals could be detected (data not shown). This may be explained by the fact that downregulation of the SREBP-1c pathway in the fasting/refeeding protocol is short-term (<24 hrs), in contrast to the medium-term down-regulation (2 weeks) observed under diabetic conditions.

De-regulation of SREBP-1c expression in DPN

The observation that the endoneurial SREBP-1c transcriptional network is downregulated in a rat model of type 1 diabetes is in line with the long standing hypothesis that abnormal lipid metabolism may be involved in DPN (Horrobin, 1997).

Historically, research on DPN has focused mainly on the axon. The severity of neuropathy seems to correlate better with the reduced amplitudes of motor and sensory nerve responses than with the reduction in NCV (Krarup, 2003), suggesting that mainly axonal defects are responsible for the clinical symptoms. However, detailed studies in diabetic patients suggest that demyelination precedes axonal loss (Malik et al., 2005; Valls-Canals et al., 2002). In addition, distortion and disruption of paranodal myelin, so-called "axo-glial dysjunction", has been reported to correlate with reductions in NCV in type 1 DPN (Sima et al., 1988a; Sima and Brismar, 1985; Sima et al., 1986; Sima et al., 1988b) although its involvement in human DPN remained controversial (Giannini and Dyck, 1996; Sima, 1997; Thomas et al., 1996). Recent studies have demonstrated that these changes affect insulinopenic type 1 BB/Wor rats but not hyperinsulinemic type 2 BBZDR/Wor rats (Sima et al., 2001). Interestingly, allogenic transplantation of the pancreas (Sima et al., 1988b) and insulinomimetic C-peptide (Sima et al., 2004) prevents and improves "axo-glial dysjunction" and its underlying molecular aberrations. Observations in patients with inherited demyelinating peripheral neuropathies also show that Schwann cell defects often lead to axonal degeneration, which can occur without any sign of demyelination (Dewaegh et al., 1992; Frei et al., 1999; Giese et al., 1992; Hanemann and Gabreels-Festen, 2002; Martini, 2001). Taken together, these observations suggest that Schwann cells may be a primary target in DPN, followed by axonal damage. Our observations that SREBP-1c and its target genes are downregulated in Schwann cells, shortly after the onset of diabetes, are in line with this view.

Type 1 diabetes is associated with impaired polyunsaturated fatty acid (PUFA) metabolism by decreasing fatty acid desaturase activity, resulting in lower PUFA content in membrane phospholipids of multiple tissues, including the peripheral nerve (Coste et al., 2003; Horrobin, 1988, 1997; Ruizgutierrez et al., 1993). Dietary supply of PUFAs was demonstrated to improve the decreased nerve blood flow, nerve hypoxia and impaired NCV in a rodent type-1 DPN but also in humans (Coste et al., 2003; Horrobin, 1988, 1997; Jamal and Carmichael, 1990; Keen et al., 1993). In line with these observations, PUFAs have been demonstrated to be essential for the biosynthesis of vasodilatory and antiplatelet prostaglandins and necessary to modify the activity of axonal Na+/K+-ATPases (Horrobin, 1997; Sugimoto et al., 2000; Vreugdenhil et al., 1996). In addition, it should be noted that the metabolism of PUFAs requires peroxisomal fatty acid oxidation and that, importantly, several inherited metabolic diseases involving peroxisomal fatty acid oxidation are associated with peripheral neuropathy (Clayton, 2001). SREBP-1c has been demonstrated to mediate the insulin-induced transcription of the fatty acid desaturases stearoyl-coA desaturase (SCD1), delta-5 desaturase (D5D) and delta-6 desaturase (D6D) (Matsuzaka et al., 2002; Nakamura and Nara, 2002). While SCD1 is involved in the biosynthesis of mono unsaturated fatty acids (MUFAs), such as oleic acid, a major constituent of the myelin membrane, D5D and D6D are required for the metabolic conversion of gamma-linolenic acid into PUFAs and are implicated in reduced NCV of diabetic patients, as discussed above.

Our observation that diabetes leads to downregulation of the SREBP-1c transcriptional targets in Schwann cells provides an explanation for the reported changes in lipid metabolism in the diabetic nerve (Coste et al., 2003; Horrobin, 1997). Our observation that diabetes interferes with the upregulation of Schwann cell SREBP-1c expression by feeding suggests that Schwann cell SREBP-1c is under the control of circulating insulin rather than glucose, in agreement with a previous report that Schwann cells express the insulin receptor (Sugimoto et al., 2000). Our experiments in isolated rat Schwann cells suggest that the action of insulin on

Schwann cell SREBP-1c expression is at the level of the SREBP-1c promoter. However, the additional role of free fatty acids, which are elevated in insulin-deficient states due to unsuppressed lipolysis, cannot be ruled out (Jump, 2004).

Previous studies already suggested that factors from the adipocyte-rich epineurium, e.g. fatty acids and acylation stimulating protein (ASP), could, via the circulation, regulate lipid metabolism in the peripheral nerve (Chrast et al., 2004; Verheijen et al., 2003). Here we provide experimental data that add insulin to the list of such factors that may regulate lipid metabolism in Schwann cells of the peripheral nerve. The role of this regulatory process in the functioning of the nerve is unclear as yet, but its deregulation in the pathogenesis of DPN suggests its possible involvement in the pathological condition of peripheral nerves in type 1 diabetes.

In summary, our data suggests that SREBP-1c plays an important role in local regulation of lipid metabolism in peripheral nerve Schwann cells. We demonstrate that the expression of SREBP-1c and SREBP-2 in the developing endoneurium is actively and differentially regulated. We show that gene expression in Schwann cells of the peripheral nerve is affected by metabolic changes as well as by diabetes. This may have important consequences for our understanding and treatment of peripheral neuropathies associated with lipid metabolic disorders, including lipodystrophy and diabetes.

Experimental Methods

Materials:

Insulin was purchased from Gibco (Invitrogen, Breda, The Netherlands), cholesterol and 25-hydroxycholesterol from Steraloids Inc. (Newport USA), Heregulin-β EGF domain from Upstate (Millipore BV, Amsterdam, The Netherlands), forskolin was from Biaffin GmbH & Co KG (Kassel, Germany).

Animals:

Mice and rats were used in various experiments. All animals were housed in a controlled environment with a 12h light/12h dark cycle and free access to water and standard laboratory diet (except for fasting animals which had access to water only). Unless specified, both females and males were used.

SREBP developmental expression experiment:

Sciatic nerve endoneurium was dissected from wild-type (Sprague Dawley) rats at the postnatal age of P4, P28 and P56 days, as previously described (Verheijen et al., 2003). For the P0 developmental time point, the whole nerves were used as it is technically extremely difficult to reliably separate endoneurium from peri-epineurium at this stage. The quality of the separation of the endoneurium from peri-epineurium was evaluated by quantitative PCR using myelin protein zero as a marker of endoneurium and Acrp30 as a marker of the peri-epineurial compartment, as previously described (Verheijen et al., 2003). Based on these evaluations we estimated the level of endoneurial contamination by the peri-epineurium and vice versa to be <10%.

SREBP tissue expression experiment:

Endoneurium, peri-epineurium, liver and spleen were isolated from 56 days old wild type (Sprague Dawley) rats.

BB/Wor diabetic rats:

BB/Wor rats develop diabetes spontaneously at about 70 days of age. Body weight, urine volume, and glycosuria (Keto-Diastix; Bayer) were monitored daily to ascertain onset of diabetes and for titration of necessary daily insulin doses. After the onset of diabetes, diabetic rats received titrated doses (0.5–3.0 units/day) of protamine zinc insulin (Novo Nordisk) to maintain blood glucose levels at ~25 mmol/l glucose and to prevent ketoacidosis. Blood glucose levels were measured biweekly (Sima and Li, 2005). Motor nerve conduction velocity (MNCV) was determined in BB/Wor diabetic rats and age-matched non-diabetic control rats two weeks and eight weeks after the onset of disease in the sciatic-tibial conducting system as previously described (Sima et al., 2000); the animals were sacrificed and sciatic nerve endoneurium was isolated according to the previously published protocol (Verheijen et al., 2003).

Fasting and refeeding of BB/Wor diabetic rats:

Diabetic animals were treated with bovine insulin pellets (implanted s.c., release 1.5 – 2 IU/24 hr; Linplant Scarborough, Ontario, Canada) as described (Visser et al., 2003). Insulin pellets were removed from rats between 3-16 days of diabetes and subsequently, both diabetic and age-matched non-diabetic controls were either fasted for 24h and sacrificed (fasted group), or fasted for 18h and allowed to refeed for a subsequent 6h and then sacrificed (fasted – refed group). Sciatic nerve endoneurium was collected from each animal.

Fasting and refeeding in wild type mice:

Nine week old wild-type NMR1 strain female mice were used for the fasting – refeeding experiment. A group of 6 mice was fasted for 24 hours and then sacrificed (fasted group). Two groups of six mice were fasted for 24 hours and then refed for either 6 hours (6h

refeeding) or 9 hours (9h refeeding). Sciatic nerve endoneurium, optic nerve and liver were collected from each animal.

Total RNA preparation:

For all analyses, total RNA from sciatic nerve endoneurium, peri-epineurium and fat was isolated using Qiagen RNeasy lipid tissue kit (Qiagen) following the manufacturer's instructions. Total RNA from liver and spleen was isolated in TRIzol (Invitrogen) reagent and purified with the RNeasy kit (Qiagen). RNA quality was verified by agarose gel and/or by HDA-GT12 Genetic Analyzer (eGene) and the concentration was determined by ND-1000 Spectrophotometer (NanoDrop).

Quantitative RT-PCR:

250-500 ng of total RNA was subjected to reverse transcription using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. Resulting cDNA was used as a template for relative or absolute quantitative PCR (Q-PCR) as described below.

Relative quantitation:

Relative quantitation of expression of selected genes was performed using the ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems) and SYBR Green (Applied Biosystems) as DNA binding dye for the detection of PCR products. Primers were designed to prevent amplification of genomic DNA (see supplementary table 1 for a list of oligonucleotides used). The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. To detect and eliminate possible primer-dimer

artifacts, the dissociation curve was generated by adding a cycle of 95°C for 15 sec, 60°C for 1min and 95°C for 15 sec. All primer sets produced amplicons of the expected size and their identity was also verified by sequencing. The sample quantitation was performed using a standard curve established from a serial dilution of a mix of the samples. Results were normalized using the reference genes cyclophilin or ubiquitin.

Absolute quantitation:

The same methodology as for the relative quantitation was used except that a gene-specific standard curve was established for each quantified transcript. For this, PCR fragments containing the region to be amplified in the Q-PCR reaction were generated for each SREBP form using the cDNA of control rat liver (see supplementary table 2 for a list of oligonucleotides used). These PCR fragments were cloned into a TOPO vector (Invitrogen) according to manufacturer's instructions. Positive clones were selected, plasmid DNA was prepared using the QIAprep Spin Miniprep kit (Qiagen), and the concentration was determined by absorbance at 260 nm using the ND-1000 Spectrophotometer (NanoDrop). To obtain the number of moles per µl, we divided the obtained concentration by the weight of a mole of plasmids (mean weight of a nucleotide = 650 Da; each plasmid = 3973 nucleotides (TOPO vector) + length of PCR fragment). The number of plasmid copies was obtained by multiplying the number of moles by the Avogadro number. The plasmids were then diluted to different concentrations (from 10^7 to 10^2 copies per µl). These serial dilutions were used as a standard curve for the absolute quantitation.

Cell Culture, Transfections, Luciferase assays and Western Blotting

Schwann cells were isolated from sciatic nerves of Sprague Dawley rat pups shortly after birth (P0-P4) using immunoselection (Brockes et al., 1979), and cultured on Poly-L-lysine coated tissue plastic in DMEM containing 10 % fetal calf serum, 10 ng/ml neuregulin and 2 µM Forskolin. Transfections were done in DMEM for 3 hrs without serum but in the presence

of 2 μM forskolin and 10 ng/ml neuregulin on 90% confluent monolayers in 24 well plates using 0.5 μg DNA and 2.5 μl Geneporter Transfection Reagent (Gene Therapies Systems Inc., SD, USA). After transfection, the medium was changed with DMEM containing various depicted concentrations of FBS, glucose and insulin for 24 hours. Cells were lysed using Steady-Glow luciferase buffer according to the manufacturer's instructions (Promega, Leiden, The Netherlands). Luciferase activity was measured on a Wallac Victor 2 (Perkin Elmer). The same samples were subsequently used for beta-galactosidase measurements to control for transfection efficiency. Detection of the precursor form of SREBP-1c protein in Schwann cells, sciatic nerve endoneurium and liver extracts was done by Western Blotting as previously described (Hasty et al., 2000). Western blots were re-probed with mouse-anti β-actin antibodies to control for protein loading. Relative quantitation was performed using scanning densitometry using the Biorad GS-8000 Calibrated Densitometer and Biorad Quantity One software.

Supplementary data (web):

Supplementary table 1: Primers used for quantitative PCR

Rat transcript (GenBank accession number)	Primers	Amplicon length
Cyclophilin (AF071225)	Forward: 5'-ACGTGGTTTTCGGCAAAGTTC-3' Reverse: 5'-CCACCTTCCGTACCACATCCA-3'	52 bp
Krox20 (NM_053633)	Forward: 5'-GGAGGCCCCTTTGATCAGA-3' Reverse: 5'-TGTTGATCATGCCATCTCCAG-3'	54 bp
Mpz (NM_017027)	Forward: 5'-TTCACAAGTCTTCTAAGGACTCCTCG-3' Reverse: 5'-GCACTGGCGTCTGCCG-3'	51 bp
Srebp1a (XM_213329)	Forward: 5'-CGAGGTGTGCGAAATGGAC-3' Reverse: 5'-GAAGCATGTCTGATGTCGGTCA-3'	51 bp
Srebp1c (XM_213329)	Forward: 5'-ACGGAGCCATGGATTGCACA-3' Reverse: 5'-CAAATAGGCCAGGGAAGTC-3'	78 bp
Srebp2 (NM_001033694)	Forward: 5'-CGCTCGCATTTCACTGAAGTAG-3' Reverse: 5'-GGCATAGAAGACGGCCTTCAC-3'	83 bp
Ubiquitin (XM_344046)	Forward: 5'-AGTGCGGAAAACTGGAAGCC-3' Reverse: 5'-GGACTGGATTACTTGGTCAGTCTTG-3'	51 bp

Supplementary table 2: Primers used for cloning

Rat transcript	Primers	Amplicon length
Srebp1a	Forward: 5'-ATGGACGAGCTACCCTTCG -3' Reverse: 5'-CATCAAATAGGCCAGGGAAGTC-3'	138 bp
Srebp1c	Forward: 5'-ATCAGCGCGGACGCTGTA-3' Reverse: 5'-CATCAAATAGGCCAGGGAAGTC-3'	174 bp
Srebp2	Forward: 5'-GATGATGCAGCTGTGCGCTC -3' Reverse: 5'-AATGACAGAAGGAGTTCTGTT-3'	164 bp

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Figures

Figure 1. SREBP expression in developing rat peripheral nerve.

Relative mRNA levels of SREBP-1a, SREBP-1c and SREBP-2 were determined in whole sciatic nerve at P0 and in sciatic nerve endoneurium of P4, P28 and P56 rats, using quantitative PCR. For each time point, the mRNA levels are presented as fold increase over the mRNA levels at P0. The data represent the mean ± SD of triplicate measurements.

Figure 2. Tissue distribution of different SREBP mRNA isoforms.

The expression of SREBP-1a, -1c and -2 was measured by quantitative PCR using cDNA prepared from P56 rat sciatic nerve (endoneurium and peri- epineurium), liver and spleen. For each tissue, mRNA levels are presented as a percentage of the expression of the SREBP isoform with the highest mRNA level. The data represent the mean ± SD of triplicate measurements.

Figure 3. Effect of fasting and refeeding on SREBP expression in mice.

a) The expression of SREBP-1c and SREBP-2 was measured in the endoneurial compartment of sciatic nerve isolated from wild type mice fed *ad libitum*, fasted for 24 hours (Fasted) and fasted for 24 hours and subsequently refed for 9 hours (Fasted/Refed) using quantitative PCR. For both transcripts the average of the *ad libitum* samples was set arbitrarily to 1 and all other measurements were appropriately normalized. Each bar represents the mean and standard deviations of three measurements using the cDNA prepared from a pool of six animals for each condition. b) The level of SREBP-1c protein expression in the endoneurial compartment of sciatic nerve and in the liver isolated from wild type mice fasted for 24 hours (F) and fasted for 24 hours and subsequently refed for 12 hours (F/R) was evaluated by Western blotting using antibodies specific for SREBP-1c (detecting SREBP-1c precursor protein at around 125

Kd and an unspecific band (*) of approximately 140 Kd). The relative intensity of the SREBP-1c band after correction for loading differences by measuring the amount of β-actin is denoted below the band. The value for the fasted sample was arbitrarily set to 1 and the F/R measurement was appropriately normalized. c-f) Expression of SREBP-1c mRNA was subsequently measured in the endoneurial compartment of sciatic nerve, optic nerve and in the liver isolated individually from six wild type mice after 24 hours fasting (white bars) and subsequent 6-hour (c) or 9-hour (e) refeeding (black bars) using quantitative PCR. For each tissue the average of the fasted samples was set arbitrarily to 1 and all other measurements were appropriately normalized. Each bar represents the mean of three measurements from one animal with standard deviations. The significance of the difference (p-value) in the SREBP-1c expression between fasted and fasted-refeeded samples was calculated using the Student's t-test. mRNAs from the samples used in experiment (c) and (e) were pooled to measure the level of Fasn and Scd1 (genes regulated by SREBP-1c) (d and f). For each measurement the average of the fasted sample was set arbitrarily to 1 and the other measurement was appropriately normalized. Each bar represents the mean of three measurements with standard deviations.

Figure 4. Changes in SREBP regulated gene expression in endoneurium of diabetic BB/Wor rats.

a) Motor nerve conduction velocity (MNCV) measurements in control (white bars) and BB/Wor rats at 2 weeks after the onset of diabetes (black bars). The data represent the mean ± SD of triplicate measurements. b) The mRNA levels of SREBP-1c, Scd1, Fasn, SREBP-2, Mpz and ApoE were measured by quantitative PCR in endoneurium from control (white bars) and BB/Wor rats at 2 weeks after the onset of diabetes (black bars). For each gene, the value is presented as a percentage of control levels. Each bar represents the mean and standard deviations of three measurements using the cDNA prepared from the pool of three animals.

Figure 5. SREBP-1c mRNA expression in the endoneurium of short-term diabetic BB/Wor rats put through fasting and refeeding.

a) The expression of SREBP-1c was measured using quantitative PCR in the endoneurium of sciatic nerve isolated from control (white bars) or short-term diabetic BB/Wor rats (black bars) after 24h fasting (Fasting) or 18h fasting and subsequent 6h refeeding (Fasting-refeeding). For graphical representation of relative SREBP-1c levels, the average of the control fasted samples was arbitrarily set to 1 and all other measurements were appropriately normalized. Each bar represents the mean of three measurements from one animal with standard deviations. The significance of the difference (p-value) in the SREBP-1c expression between control and diabetic samples was calculated using the Student's t-test. b) mRNAs from the samples used in experiment (a) were pooled to measure the levels of Fasn and Scd1. The data were normalized as in figure 5a. The expression of mRNA for the cyclophilin gene was used to standardize the amount of cDNA in each reaction.

Figure 6. Regulation of SREBP-1c expression in cultured rat Schwann cells.

a) SREBP-1c activates the FAS promoter in Schwann cells. Cultured rat Schwann cells were transfected with a Fas-promoter luciferase construct together with increasing amounts of a CMV-driven expression construct for an active form of SREBP-1c. The data represent the mean ± SD of triplicate samples. Inset: Western blot with SREBP-1 antibody revealing the expression of transfected active mature SREBP-1c (arrow). b) Activity of the SREBP-1c and the FAS-promoter in Schwann cells as a function of glucose concentrations. Schwann cells were transfected with a FAS- or a SREBP-1c promoter luciferase reporter construct, as depicted. Subsequently, cells were cultured for 24 hrs in DMEM with 10% FBS and different concentrations of glucose as depicted. c) SREBP-1c promoter activation by insulin. Schwann cells were transfected with a SREBP-1c promoter luciferase reporter construct, and cultured

for 24 hrs in DMEM with 0.1% FBS with depicted concentrations of glucose, and with or without depicted concentrations of insulin. d) SREBP-1c precursor protein expression in Schwann cells is induced by insulin. Schwann cells were cultured for 18 hours in DMEM with 0.1% FBS and subsequently treated with or without insulin (300 ng/ml) for 24 hrs, as depicted. Shown are Western blots using samples from 2 independent experiments, probed with antibodies specific for SREBP-1c (detecting SREBP-1c precursor protein around 125 Kd) and β-actin (protein loading control). The relative intensity of SREBP-1c band after correction for loading differences by measuring the amount of β-actin is denoted below the band. The mean of values for "control" samples was set arbitrarily to 1 and the measurement of band in "insulin" samples was appropriately normalized.

Supplementary Figure 1. Quantitation of SREBP-1c mRNA expression in endoneurial and peri-epineurial fractions of P4 sciatic nerve. mRNA levels are presented as a percentage of the expression of the SREBP-1c in the complete nerve.

Figure

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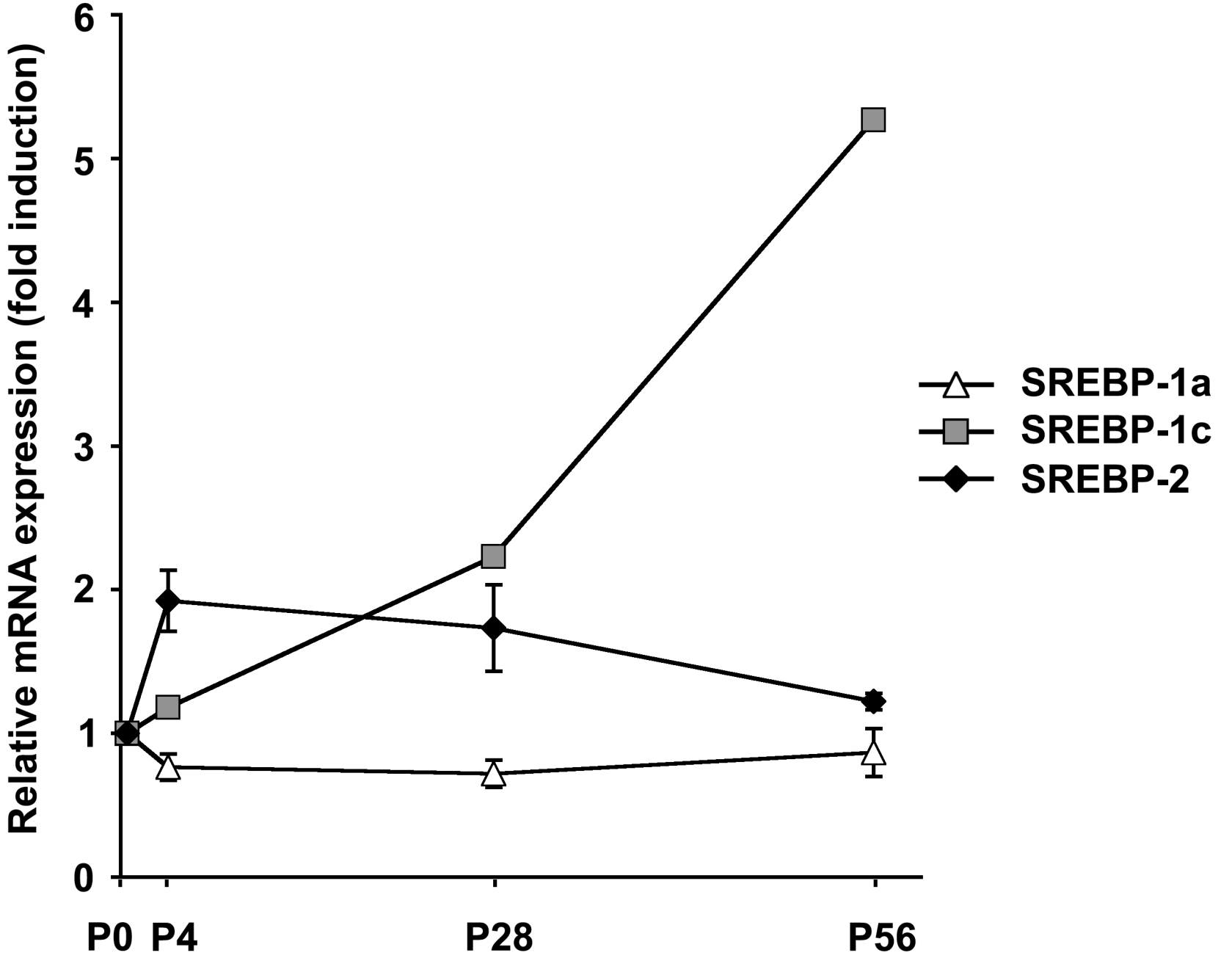

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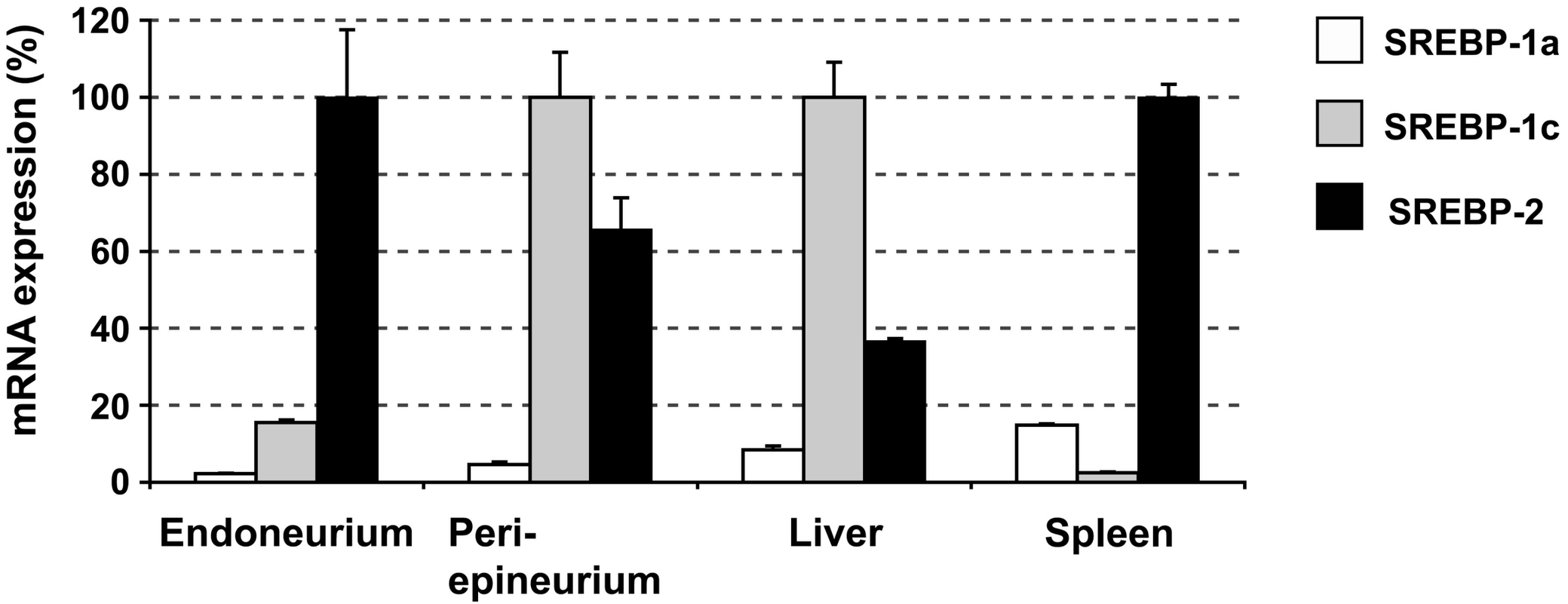

Figure
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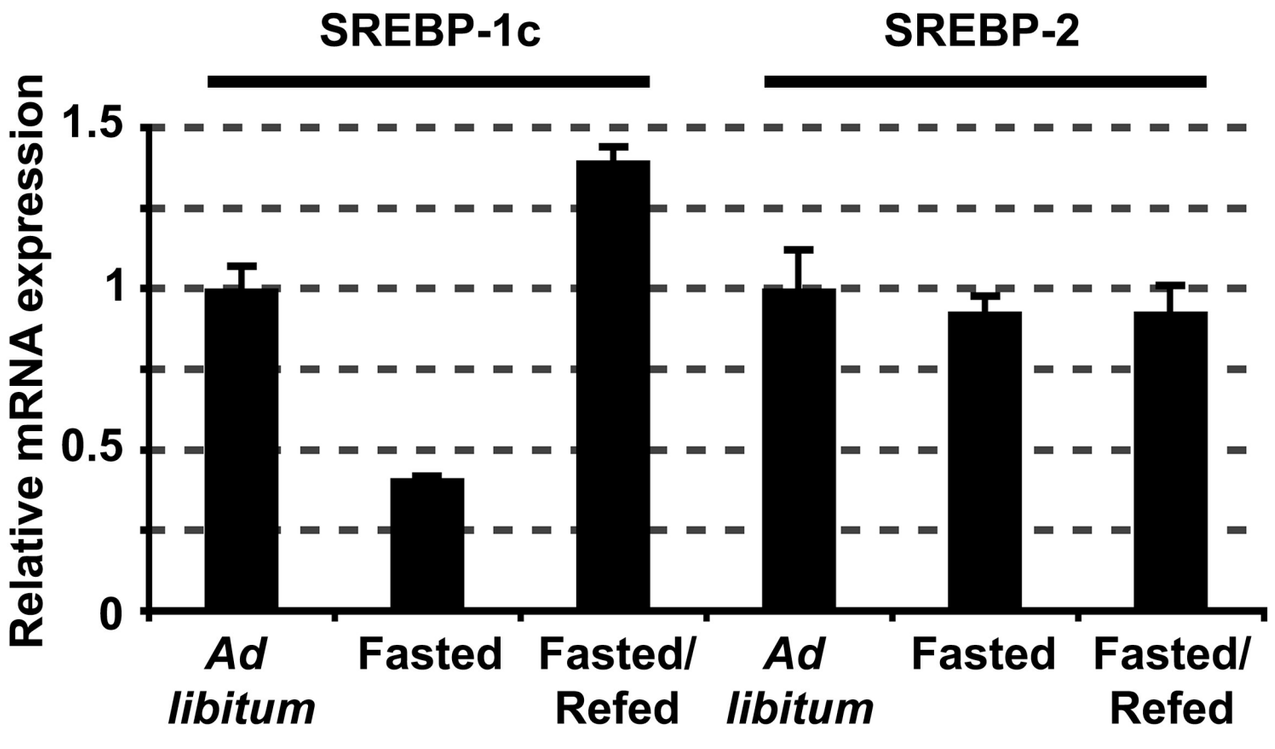

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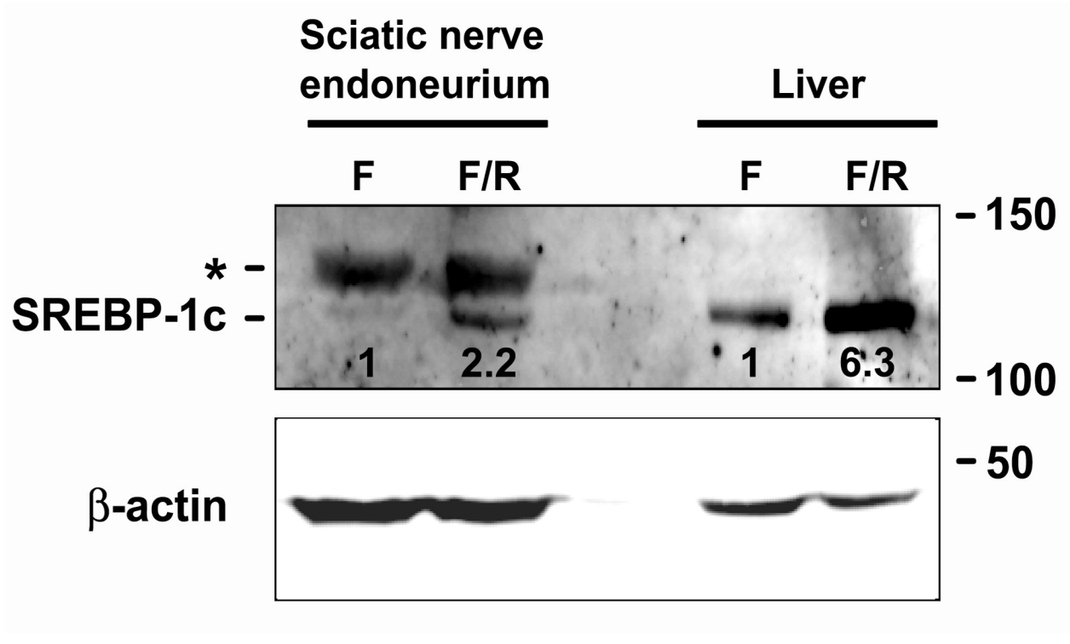

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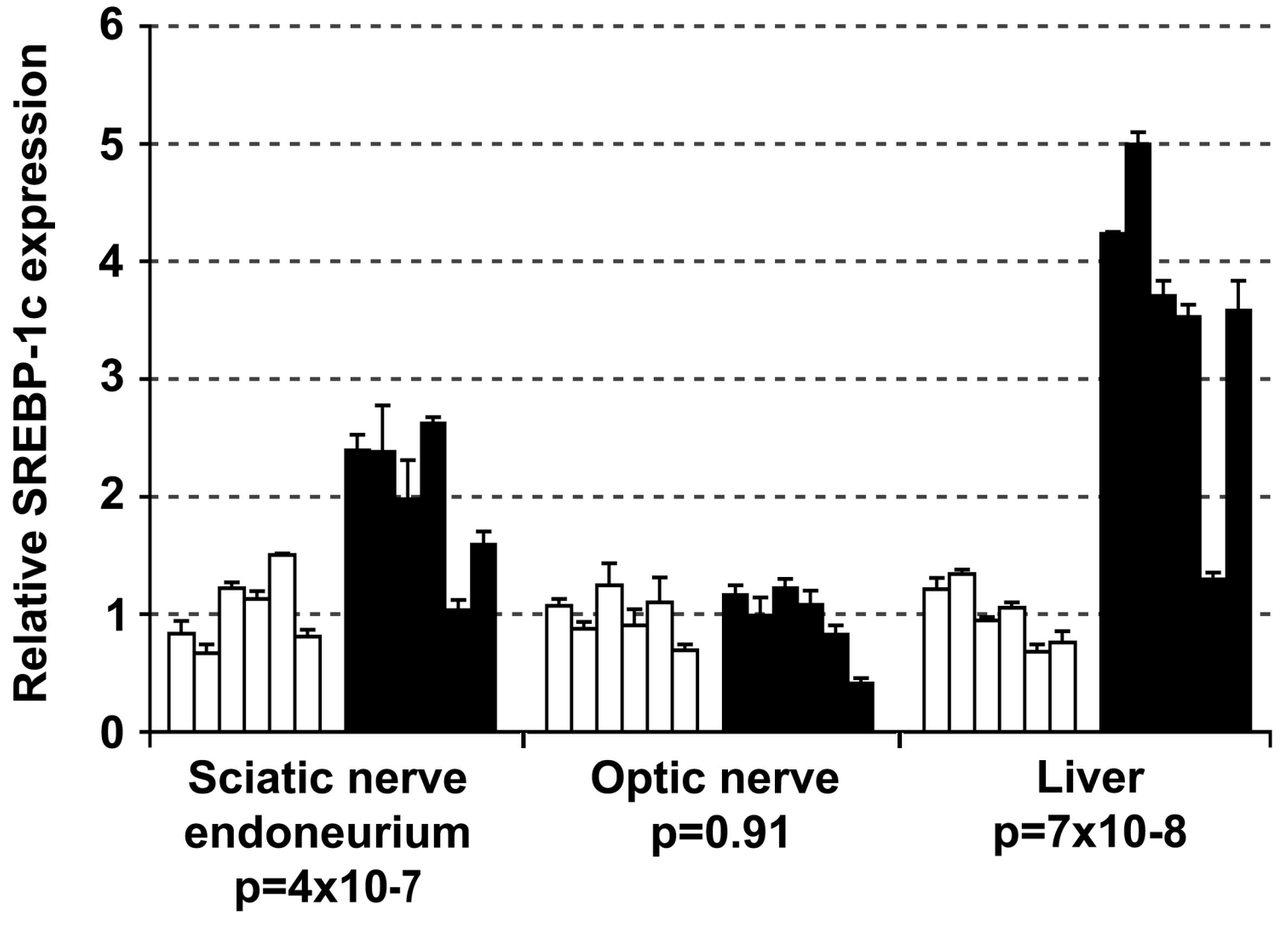

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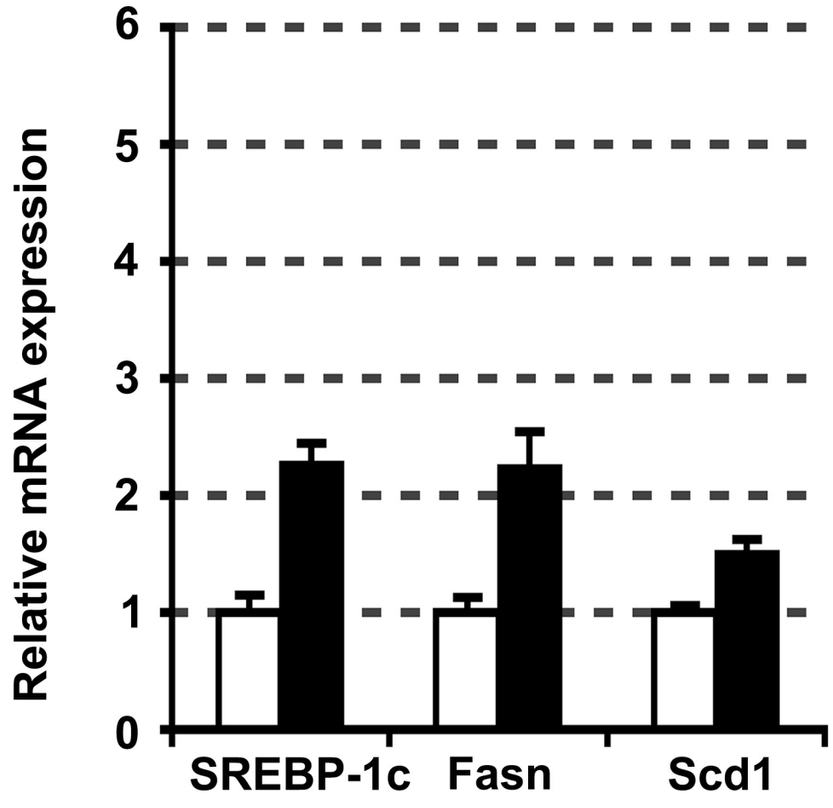

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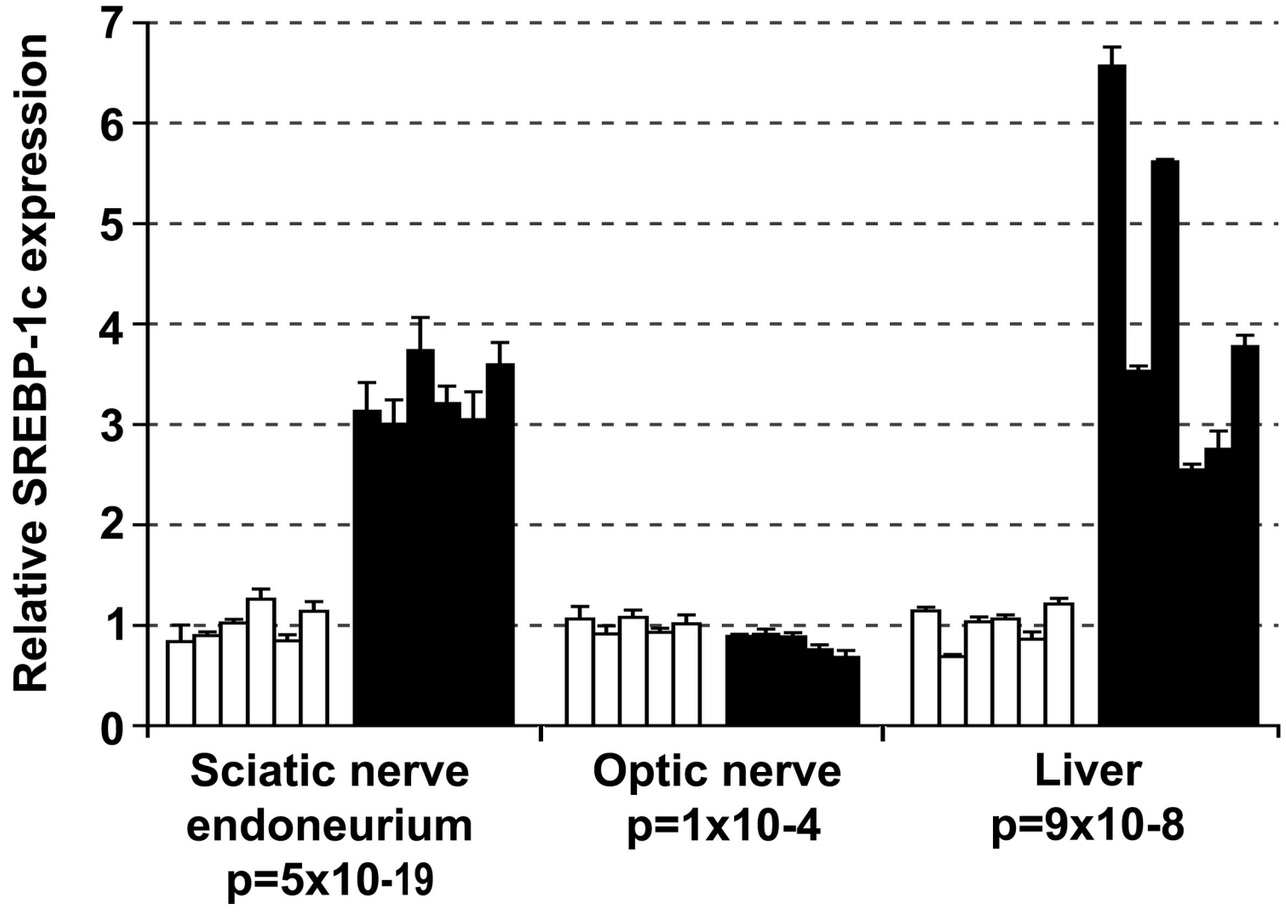

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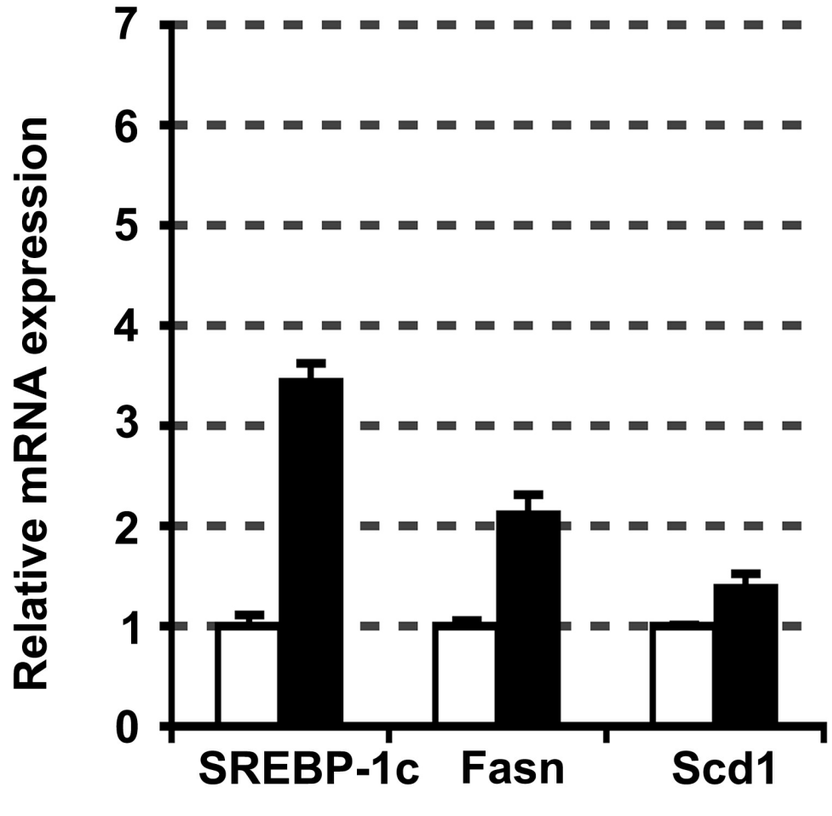

Figure

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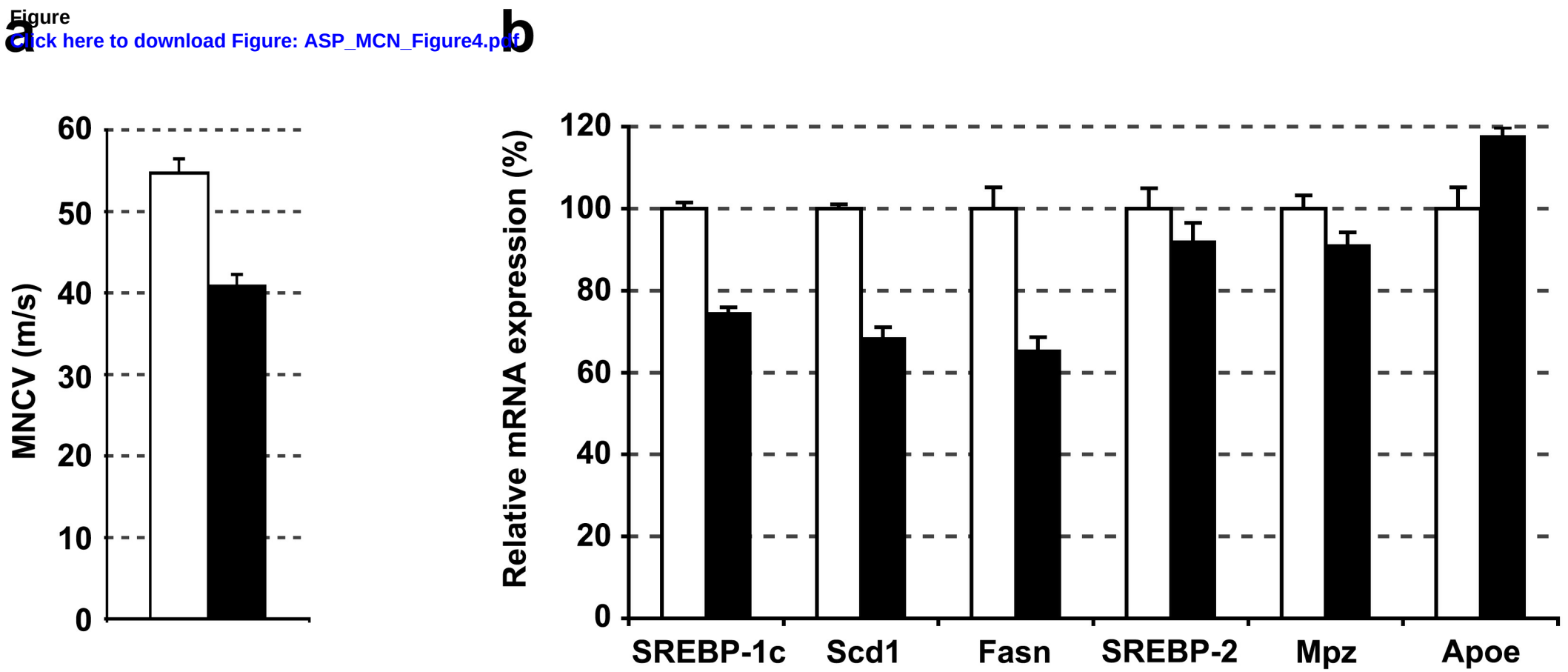

Figure
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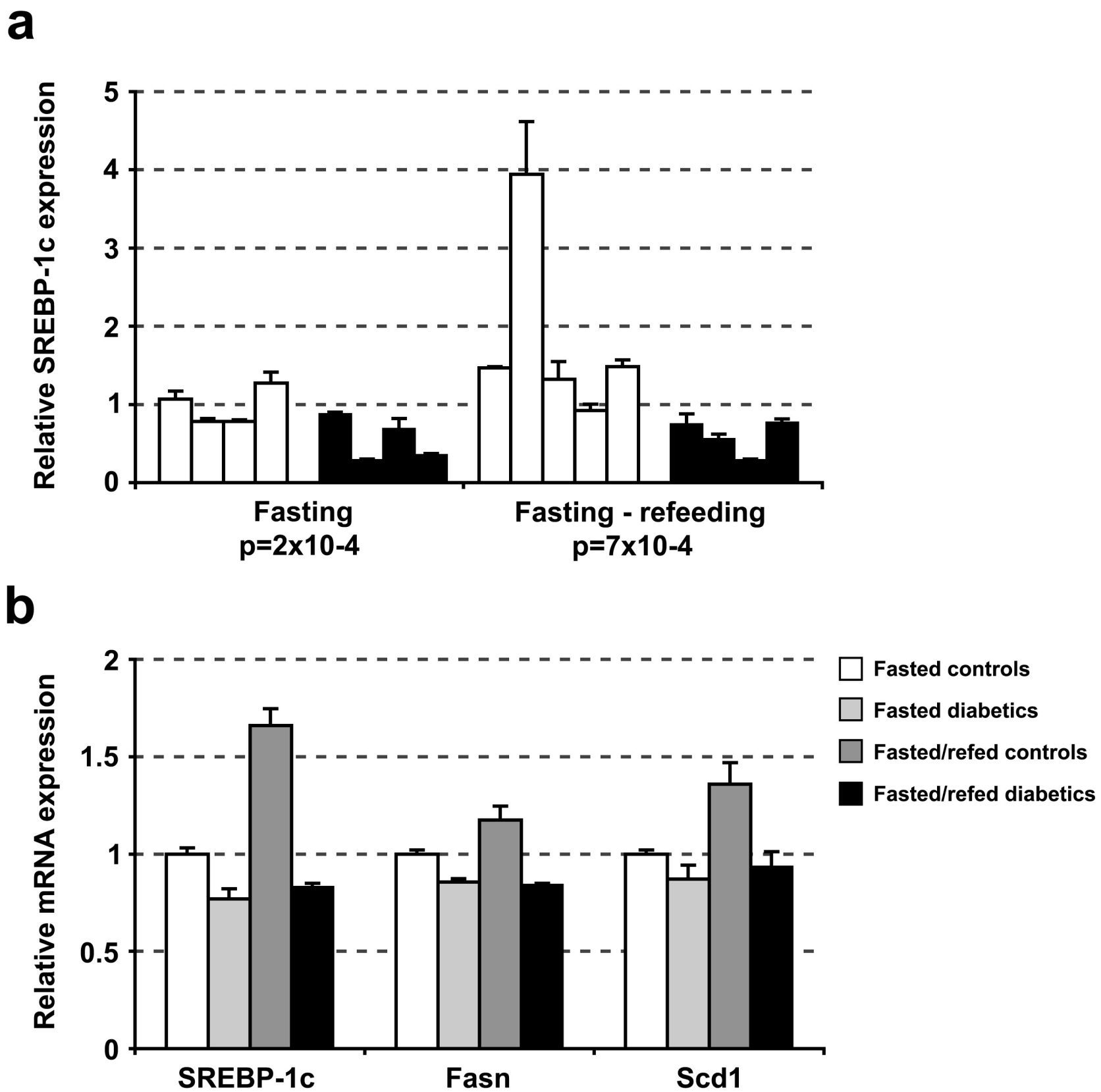

Figure
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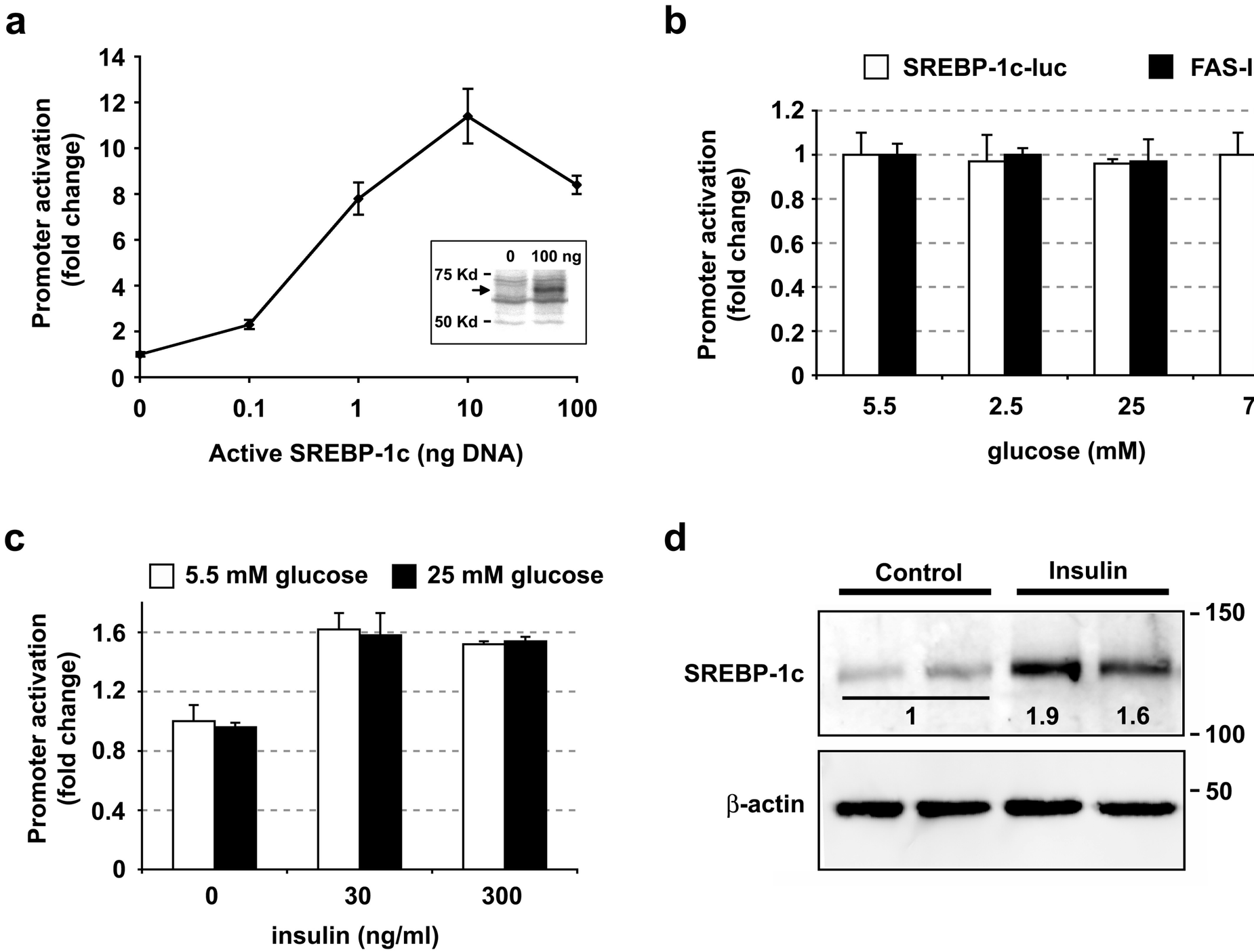

Figure

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