The p75 neurotrophin receptor appears in plasma in diabetic rats—characterisation of a potential early test for neuropathy

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Abstract

Aims/hypothesis. This study tested the premise that immunoreactivity representing the p75 neurotrophin receptor (p75^{NTR}) appears in plasma of diabetic rats in association with the early stages of neuronal dysfunction or damage. We also examined whether treatment beneficial to neuropathy might reduce the p75^{NTR} immunoreactivity.

Methods. Plasma proteins were fractionated by SDS-PAGE and immunoblots exposed to p75^{NTR} antibody, using receptor protein from cultured PC12 cells as an external standard. Rats were made diabetic with streptozotocin for various periods and exsanguinated. Plasma glucose, HbA$_1$c and plasma proteins were determined. We also studied plasma samples from diabetic mice lacking the gene coding for p75^{NTR}, as well as the effect of sciatic nerve crush on healthy male Wistar rats.

Results. Plasma p75^{NTR} immunoreactivity began to exceed normal levels at 8 weeks after induction of diabetes, and was significantly raised at 10 ($p<0.05$) and 12 weeks ($p<0.001$). Treatment between 8 and 12 weeks with insulin, fidarestat (an aldose reductase inhibitor), nerve growth factor and neurotrophin 3 all normalised the plasma p75^{NTR} immunoreactivity. Plasma from p75^{NTR} (–/–) mice contained no such immunoreactivity, though it was present in plasma from wild-type mice. Following nerve crush, p75^{NTR} immunoreactivity appeared in plasma of non-diabetic mice, indicating that this can be a result of nerve trauma.

Conclusions/interpretation. These observations suggest that plasma p75^{NTR} immunoreactivity may serve as an early indicator of neuronal dysfunction or damage in diabetes. The time course of its appearance relates well to that of early neuropathy and its response to interventions that are neuroprotective suggests that it might mirror neurological status.

Keywords Nerve growth factor · Neuropathy · Neurotrophins · p75^{NTR} · Plasma marker · Rats · Receptor shedding

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Introduction

The generic phenomenon of receptor shedding, with consequent appearance (either de novo or in increased titre) in plasma, has been studied in several disease states. Most of the relatively small amount of published work is recent (later than 1995) and concentrates on receptors for cytokines and adhesion molecules. Thus, TNF-α receptors are shed in association with neoplasia [1, 2], osteoarthritis [3], retinal diseases (not including diabetic retinopathy) [4] and asthma [5]. Other cytokine receptors are shed in inflammatory bowel disease [6, 7] and osteoarthritis [8]. Receptors for adhesion molecules are shed in relation

to leucocyte activation [9, 10] and in neoplasia [11]. The extent to which these observations are suggestive of mechanisms varies; nevertheless, they may have diagnostic potential purely on pragmatic grounds.

No systematic studies are described in the literature on shedding of neurotrophin receptors. There are two receptors involved in transduction of the neuronal response to nerve growth factor (NGF). Neurotrophin selectivity is conferred by the high-affinity receptor trkA, but binding and internalisation of NGF into the neurone are facilitated by, if not actually dependent on a second, low-affinity receptor, neurotrophin receptor of apparent molecular weight 75 M_r (p75^{NTR}; see [12] for review). This latter receptor is directly analogous in structure and cellular disposition to the p75 TNF-α receptor, prompting speculation that shedding behaviour exhibited by the TNF variant (referred to above) may be shared by the NGF p75 receptor.

The p75^{NTR} was purified from tissue sources some years ago, antibodies were raised and a truncated form (p50), expressed particularly by Schwann cells, was found to contain a similar epitope to the mature receptor [13]. This same antibody was also used to demonstrate p75-like immunoreactivity in the urine of diabetic patients diagnosed with neuropathy [14]. Details of this last observation are scant, in that the antibody used was not monoclonal, there was not an acceptable fractionation of the proteins before immunocharacterisation, relationships between plasma and urine levels of immunoreactivity were not determined and neurological characterisation of the patients was either rudimentary or under-reported. Nevertheless, the observation has enormous potential. The aim of the present study was to determine whether circulating p75^{NTR} immunoreactivity can be detected in plasma from rats with experimental diabetes. That done, the secondary aims were characterisation of the immunoreactivity and examination of the effects, in diabetic rats, of several interventions that might be expected to ameliorate neurological stress or damage. Thus, we sought to lay a foundation for the development of plasma p75^{NTR} immunoreactivity as a surrogate marker for small fibre neuropathy in diabetes.

Methods

Induction and monitoring of diabetes in rats; drug treatments. Male Wistar rats were used in all experiments (Charles River Laboratories, Margate, Kent, UK). On arrival body weights were approximately 250 g. Animals were housed for a week to allow settling and fasted overnight prior to induction of diabetes. Diabetes was induced using a single intraperitoneal injection (50 mg/kg body weight) of streptozotocin (STZ) (Sigma-Aldrich Company, Gillingham, Dorset, UK) dissolved in 0.9% physiological saline immediately prior to use. Diabetic status was confirmed 3 days after induction by measurement of glucose levels in tail blood using glucose test strips (BM-Accutest; Roche Diagnostics, Welwyn Garden City, Herts., UK). Rats were given free access to water and chow and main-

tained for up to 12 weeks. Rats were killed by anaesthesia and exsanguinated in accordance with UK Home Office legislation; blood was handled as described below.

With the exception of fidarestat (see below) all treatments were begun 8 weeks after induction of diabetes and maintained for the final 4 weeks. At the 8-week time point diabetic animals were allocated to treatment groups after weighing to preclude bias and achieve sub-groups with similar mean pre-treatment weights. Normalisation of glycaemia with insulin was achieved by subcutaneous implantation of two Linplant pellets (Linshin Canada, Scarborough, Ont, Canada) under isoflurane anaesthesia. The implants gradually dissolve providing a sustained daily release of approximately 2 IU/implant. The human recombinant neurotrophins, NGF (a gift from Genentech, San Francisco, Calif., USA) and neurotrophin 3 (NT-3; a gift from Regeneron, Tarrytown, N.Y., USA) were given at the doses shown in the relevant figure three times per week (Monday, Wednesday, Friday) by subcutaneous injection. Fidarestat (a gift from Sanwa Kagaku Kenkyusho, Tokyo, Japan) treatment was begun at the end of week 2; animals received a daily oral dose of 2 mg/kg for 10 weeks.

Collection of plasma. Rat blood was obtained by exsanguination from the right ventricle under halothane anaesthetic in accordance with the Animals (Scientific Procedures) Act 1986. Samples were centrifuged (Megafuge 1.0; Heraeus Sepatech, Osterode/Harz, Germany) at 1000 *g* for 15 minutes. The plasma layer was transferred to a 4-ml internally threaded cryovial (Alpha Laboratories, Eastleigh, Hampshire, UK) and stored at −40 °C until usage.

Plasma glucose determination. Hyperglycaemia was confirmed post-mortem using an ultra-violet (UV) D-glucose detection kit (Boehringer Mannheim, London, UK) adapted to 96-well plates. D-glucose is phosphorylated to glucose-6-phosphate in the presence of hexokinase and ATP. This is further oxidised by NADP catalysed by glucose-6-phosphate dehydrogenase to form gluconate-6-phosphate and NADPH. The increase in NADPH is measured utilising its absorbance of light at 340 nm.

Glycated haemoglobin determination. After removal of plasma, red cells from rat blood were washed three times with 0.9% isotonic saline. The cleaned cells were haemolysed, and glycated haemoglobin was measured using a spectrophotometric assay (kit 441; Sigma-Aldrich) following manufacturer's instructions. A fresh cation-exchange resin column was used for each sample.

Fractionation of plasma proteins by SDS-PAGE. In initial studies, it was assumed that plasma albumin would interfere with measurement of p75^{NTR}, since the latter was expected to be present in minor amounts compared to albumin. Thus the first method employed removed albumin using commercial affinity columns. Plasma was diluted 1+4 with 0.02 mol/l di-sodium hydrogen orthophosphate and 5 ml loaded on to a Bio-Rad Econo-pac Blue Cartridge (Bio Rad, Hemel Hempstead, Herts., UK), prepared in accordance with the manufacturer's instructions. The sample was left on the column to equilibrate for 5 minutes and then plasma minus albumin eluted from the column with 5 ml 0.02 mmol/l di-sodium hydrogen orthophosphate. Of this eluate, 80 µl were added to 720 µl of 0.02 mol/l di-sodium hydrogen orthophosphate plus and 200 µl SDS and the mixture boiled for 5 minutes.

Subsequently it was discovered that the p75^{NTR} band could be effectively separated from albumin by PAGE (see Fig. 1), so the affinity column chromatography was abandoned and plasma samples were diluted directly with SDS. Samples
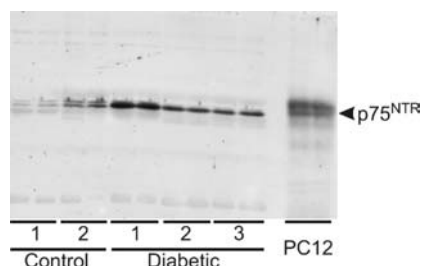
Fig. 1. Rat plasma western blots from SDS-PAGE, showing immunoreactivity to the p75^{NTR} antibody. Plasma extracts are duplicated in adjacent lanes, with samples from 2 control (non-diabetic) rats, 3 rats with STZ-diabetes of 12 weeks duration and a lysate from PC12 cells, which express the p75^{NTR}

(20 µl) were then run on 10% polyacrylamide gels in a running buffer comprising 0.025 mol/l Tris, 0.192 mol/l glycine, 0.1% (w/v) SDS, pH 8.3 at 120 V for 45 to 60 minutes. Proteins were then transferred to the nitrocellulose membranes and blocked for 1 hour at room temperature in Tris buffered saline containing Tween (150 mmol/l NaCl, 10 mmol/l Tris-HCL pH 7.6, 0.05% Tween 20) + 10% milk substitute.

To enable comparison between gels, an external standard was always included in triplicate, using the two outer lanes, plus the centre lane. This comprised a standard amount of lysate from PC12 cells in culture, which express p75^{NTR} at a very high level. This rat adrenal phaeochromocytoma (PC12) cell line (ATCC [CRL 1721]) was obtained from the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de). Cells were grown in RPMI 1640 medium without glutamine, enriched with 10% heat-inactivated horse serum and 5% fetal calf serum. To this was added 2 mmol/l glutamate and 1% penicillin-streptomycin (all components supplied by Gibco Life Technologies, Paisley, UK). Cells were grown in suspension and maintained at 37 °C in an environment consisting of 95% air and 5% CO_2. At infrequent intervals large cultures were prepared and the cells lysed to produce several hundred identical aliqots for use with plasma gels. Lysates were stored for up to 1 year at –70 °C.

The membranes were incubated overnight at 4 °C with anti-human p75^{NTR} antibody at 1:5000 dilution in TBS Tween + 0.1% sodium azide (20 ml). Membranes were washed (3×15 minutes) at room temperature in TBS Tween and incubated with secondary antibody, i.e. Anti rabbit (New England Biolabs, Hitchin, Herts., UK) 1:5000 dilution in TBS Tween for 1 hour at room temperature (20 ml). This was followed by 3 further washes (each 15 minutes) in TBS Tween. Finally, membranes were exposed to standard Lumiglo (New England Biolabs) chemiluminescence enhancement and exposed to X-ray film for 10 to 30 seconds.

Special animal studies. In one experiment plasma samples from diabetic mice lacking the gene coding for p75^{NTR} were compared with those from wild-type mice. Plasma samples from *exon-3* hypomorphic mice (*p75^{NTR}exonIII–/–*) were a kind gift from Professor Johannes Jakobsen (Dept. Neurology, Århus University, Denmark). *Exon III* encodes the ligand-binding site of p75^{NTR}, deletion of this exon results in a knock-out phenotype unable to respond to neurotrophins [15, 16]. These transgenic mice continue to produce p75^{NTR} isoforms, generated from a splice variant of the *p75^{NTR}* gene, which results in incomplete ablation of the wild-type phenotype [17]. *Balbc* mice were used as a wild-type control.

In a separate study unilateral sciatic nerve crushes were performed on healthy male Wistar rats to determine whether
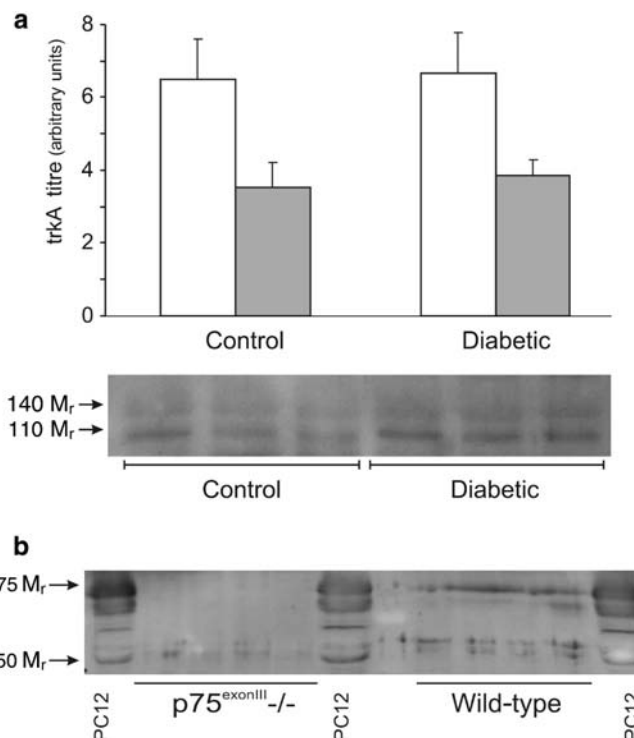

Fig. 2 a. Immunoreactivity to the trkA antibody in plasma from control and diabetic rats. As in nervous tissue, 110 (open bars) and 140-M$_r$ (grey bars) components were present, but only in trace amounts and no differences were seen between control and diabetic rats. **b.** Plasma from p75(–/–) and wild-type diabetic mice showing p75^{NTR} immunoreactivity in 75 and 50-M$_r$ fractions. No 75-M$_r$ IR was found in the (–/–) mice and the 50-M$_r$ fraction was much reduced

such axonal trauma could result in the appearance of p75^{NTR} immunoreactivity in plasma. Rats were anaesthetised with isoflurane (initial anaesthesia achieved using 5% isoflurane in oxygen and maintained by 1.5% isoflurane in oxygen). Left sciatic nerves were crushed at mid-thigh level for 15 seconds using watchmaker forceps. This was repeated once more at the same site. Animals were allowed to recover and were maintained for 2 to 14 days before being killed and exsanguinated.

Results

p75NTR immunoreactivity in plasma gels. Figure 1 shows a typical gel from rat plasma, illustrating the separation of p75^{NTR} immunoreactivity (IR). In spite of the potential conflict with 70 M$_r$ albumin, p75^{NTR} IR was easily resolved and the bands scanned. In most samples, two reactive bands were present, neither of which was present in blots exposed to secondary antibody only. The increase in titre in samples from diabetic rats is clearly illustrated in this gel; for quantification of this effect, see below.

Figure 2a shows that traces of trkA IR were also present in rat plasma; it was not seen in human plasma. There were no differences in titre in plasma for either the 110 M$_r$ or the full-length 140 M$_r$ trkA IR
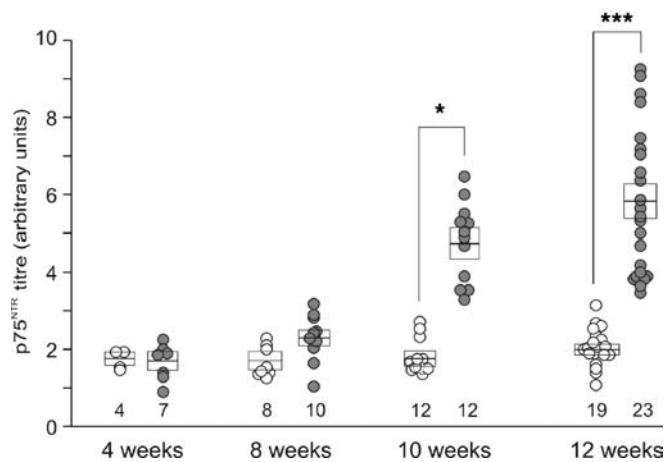
Fig. 3. The time-course of appearance of p75^{NTR} IR in plasma of diabetic rats (filled circles) compared to controls (open circles). Circles represent individual animals, horizontals are group means, boxes indicate ±1 SD. The values below indicate the numbers of rats per group. * $p < 0.05$; *** $p < 0.001$

between control rats and rats 12 weeks after induction of diabetes with STZ.

Figure 2b shows a comparison of the p75 region of immunoblots from plasma taken from wild-type *Balbc* (*p75+/+*) and *p75−/−* mice, both with STZ-induced diabetes of 4 weeks duration. There was no IR in the p75 region of the −/− mice, but a clear doublet was present in this region of wild-type mice.

Time course of effects of diabetes in rats. Figure 3 shows data for individual animals, together with means and SEMs for groups of rats with STZ-induced diabetes of 4 to 12 weeks duration. No increase in plasma p75^{NTR} IR was associated with 4 weeks diabetes, but significant increases were present at 10 and 12 weeks. The beginning of this trend was visible at 8 weeks after induction of diabetes.

This time course study served as a baseline for subsequent testing of interventions. In each study, animals were untreated for the first 8 weeks of diabetes, but treatment was then implemented for the last four weeks of a 12-week diabetes protocol.

Effects of insulin. Figure 4a shows the effect of maintaining tight glycaemic control on plasma p75^{NTR} IR (parameters of glycaemic control, see Table 1). Note that insulin treatment in the last 4 weeks of a 12-week protocol normalised plasma glucose and glycated haemoglobin, and significantly increased body weight at the end of the study. Plasma p75^{NTR} IR was also normalised in the insulin-treated group.

Effect of aldose reductase inhibition. Inhibition of aldose reductase with fidarestat had no effect on body weight or plasma glucose (Table 1), but normalised sciatic nerve sorbitol and fructose (Table 2). The treatment also normalised plasma p75^{NTR} IR (Fig. 4b).
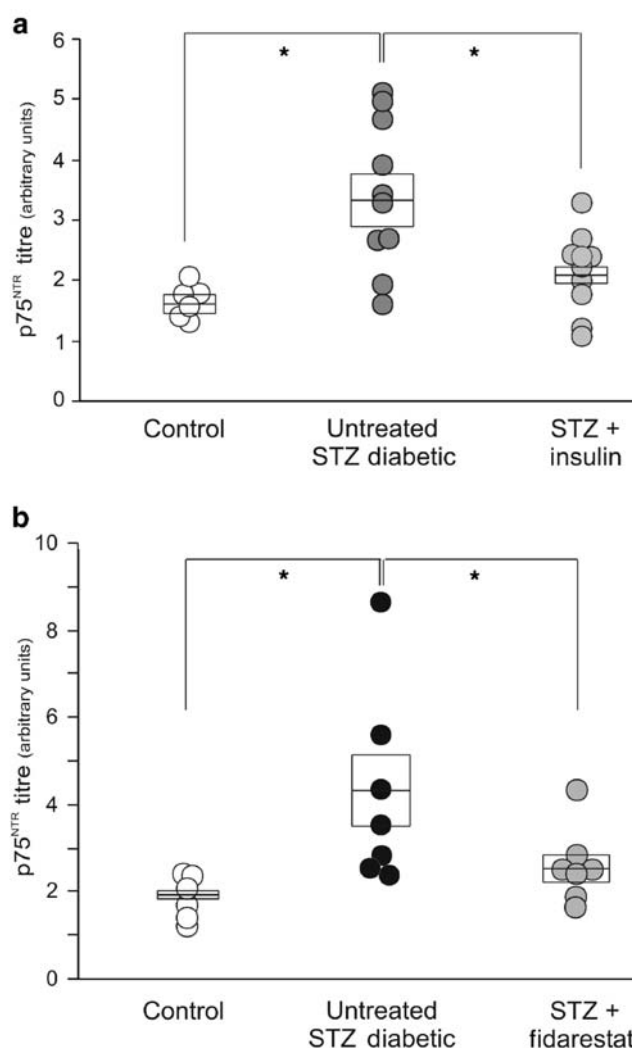

Fig. 4. Effects of insulin (**a**) and the aldose reductase inhibitor, fidarestat (**b**), on appearance of p75^{NTR} IR in plasma of diabetic rats. Key to symbols, see legend Figure 3. * $p < 0.05$

Effect of neurotrophins. Treatment with either NGF or NT-3 had no effect on the indices of diabetes, i.e. body weight and either plasma glucose or glycated haemoglobin (Table 1). However, as Figure 5 shows, both neurotrophins reduced and normalised the p75^{NTR} IR titre in plasma in a dose-dependent manner.

Effect of nerve crush without diabetes. The central premise for this work was that metabolic stress applied to neurones or Schwann cells could give rise to shedding of the p75^{NTR} receptor, leading to its appearance in plasma. Indirect support for this hypothesis was sought by inflicting nerve damage in the form of a crush injury to one sciatic nerve. After this operation, rats were killed after 2, 7 or 14 days, with sham operations conducted on control animals for the 7 and 14-day time points. Plasma from these samples was gel-fractionated, blotted and exposed to the p75^{NTR} antibody as shown in Figure 6. There was clear immunoreactivity at the 14-day time point, with an upward

Table 1. Indices of diabetes in rats with insulin implants or with NGF, NT-3 or aldose reductase inhibitor treatment, compared with untreated control and diabetic rats

	Body weight (g)	Plasma glucose (mmol/l)	Glycated haemoglobin (%)
Control (*n*=6)	458±15	6.8±1.0	2.3±0.3
Untreated diabetic (*n*=10)	291±42	34.3±6.4	4.8±0.9
Insulin-treated diabetic (*n*=10)	409±38	7.0±1.3	2.4±0.4
Control (*n*=6)	634±42		2.4±0.3
Untreated diabetic (*n*=10)	421±49		5.8±0.6
Diabetic + NGF 0.1 mg/kg (*n*=10)	416±33		5.7±0.3
Diabetic + NGF 0.5 mg/kg (*n*=10)	396±41		5.2±0.6
Control (*n*=9)	619±14	7.0±0.4	
Untreated diabetic (*n*=10)	342±11	33.0±1.1	
Diabetic + NT-3 1 mg/kg (*n*=10)	347±20	35.3±2.8	
Diabetic + NT-3 5 mg/kg (*n*=10)	387±14	34.0±1.7	
Control (*n*=9)	631±20	7.0±0.3	
Untreated diabetic (*n*=10)	359±8	28.9±1.1	
Diabetic + fidarestat (*n*=10)	362±14	31.6±1.2	

Duration of diabetes was 12 weeks, insulin implants were present for the last 4 weeks. NGF or NT-3 were given at the doses cited by subcutaneous injection 3 times per week for the last 4 weeks. Data are mean ± 1SD; blank cells indicate that the variable was not measured. For all variables diabetic rats were sig-nificantly different than the other two groups ($p<0.001$ by one-way ANOVA with Student–Neuman–Keuls range tests), except for insulin-treated diabetic rats, which were not different from controls, but were significantly different ($p<0.001$) from untreated diabetic rats

Table 2. Effects of diabetes and fidarestat treatment on polyol pathway metabolites in the sciatic nerve

	Glucose	Fructose	Sorbitol	*myo*-Inositol
Control (*n*=9)	6.9±1.2	4.7±1.0	0.9±0.4	13.3±3.0
Untreated diabetic (*n*=10)	36.1±2.1	14.7±0.8	6.2±0.7	5.2±0.8
Diabetic + fidarestat (*n*=10)	39.6±1.9	7.5±0.6	0.6±0.2	11.9±2.6

Data are means ±SEM in nmol/mg dry tissue. For all variables untreated diabetic rats were significantly different than controls and significantly different than diabetic rats treated with fidarestat ($p<0.01$ by one-way ANOVA with Student–Neuman–Keuls range tests), except for glucose

trend at 7 days. Neither sham operation was associated with increased appearance of p75^{NTR} in plasma.

Discussion

It is clear from these studies that p75^{NTR} IR appears in plasma in rats with STZ-induced diabetes and that the titre increases with increasing duration of the disease. The IR is specific, in that incubation of blots without primary antibody gave no product in the 75-M_r region of the blot and product was absent from the plasma of *p75^{NTR}* (–/–) mice. In a pilot study of plasma from human volunteers, p75^{NTR} IR was present and the titre was higher in diabetic subjects. So far this latter observation has not been quantified nor has a possible association with neuropathy been explored; this is the subject of on-going investigation. Nevertheless, our pilot studies with human plasma underline the relevance of the rat data presented here.

The main object of these studies was to validate the specificity and time course of appearance of p75^{NTR}, as defined above, but then to explore a series of inter-ventions that might influence the titre in plasma of diabetic rats. Treatment with insulin was obviously essential to differentiate between diabetes as a prime cause of p75^{NTR} appearance and other potential influences, such as non-specific effects of STZ. Prevention of appearance by insulin was perfectly clear. This suggests, but does not prove that hyperglycaemia is a cause. The fact that the aldose reductase inhibitor, fidarestat, prevented the appearance of p75^{NTR} IR in plasma strengthens the above suggestion. Exaggerated flux through aldose reductase and the polyol path-way—the target of an aldose reductase inhibitor—is driven directly by intracellular glucose resulting from elevated extracellular glucose (see [18] for recent review).

The observation that the p75^{NTR} was present in the plasma of diabetic wild-type mice, but absent from the plasma of the p75^{NTR} (–/–) mouse also argues against STZ as a causative agent and strengthens the conclusion that the plasma immunoreactivity reflects "real" p75 receptor protein.

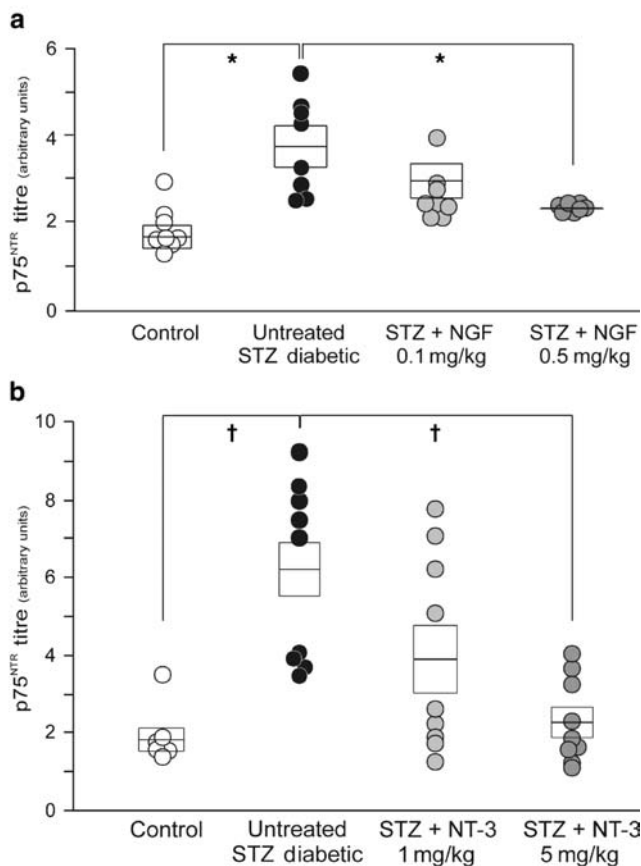The effect of fidarestat may also indicate that plasma p75^{NTR} IR serves as a marker of efficacy for

Fig. 5. Effects of the neurotrophins NGF (**a**) and NT-3 (**b**) on appearance of p75^{NTR} IR in plasma of diabetic rats. Key to symbols, see legend to Figure 3. * $p<0.05$; † $p<0.005$

Fig. 6. Effects of a unilateral sciatic nerve crush injury on appearance of p75^{NTR} IR in plasma of normal (i.e. non-diabetic) rats. * $p<0.05$

agents that ameliorate at least some of the factors responsible for diabetic neuropathy. For example, it is well established that aldose reductase inhibitors prevent nerve conduction deficits in experimental diabetic neuropathy [19] and attenuate these deficits in the clinic [20]. The effects of the neurotrophins support this. Both NGF and NT-3 normalised the plasma level of p75^{NTR} at the higher of the two doses used. The lack of effect at the lower doses suggests that there is a pharmacological (i.e. dose-related) effect. Since both of these molecules bind to p75^{NTR} and this binding is implicated in their capacity to signal through the trk receptors [21, 22, 23], the loss of p75^{NTR} receptors into plasma may reflect the deficits in both NGF and NT-3 that are clearly present in experimental diabetes [24, 25, 26].

It has long been known that the p75^{NTR} is not specific for nervous tissue [27] and its production in neoplasias, especially prostate cancers, is well established [28, 29, 30]. Hence, it cannot be assumed that the p75^{NTR} immunoreactivity detected in plasma is certain to have originated in nervous tissue. Absolute proof of this would require generation of a transgenic animal carrying a mutant p75^{NTR} on neurone- and Schwann-cell-specific promoters. Rather than go to such lengths, we performed the nerve crush study, which
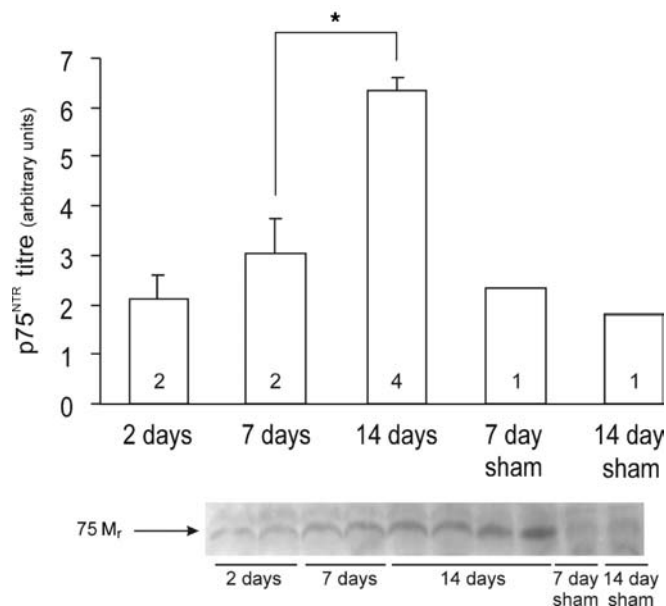
does show that nerve trauma can cause a measurable elevation of plasma p75^{NTR} immunoreactivity. This, taken together with the effect on this immunoreactivity of neuronally-targeted agents, such as NGF and NT-3, strongly suggests that the plasma p75^{NTR} immunoreactivity does originate from peripheral nerve and might indicate an abnormality therein.

Other studies have examined the status of p75^{NTR} in peripheral nerve in diabetes. In diabetic rats, increased expression of p75^{NTR} immunoreactivity was observed in sciatic nerve without signs of degeneration [31]. In contrast a human study observed increased p75^{NTR} immunoreactivity in teased fibres only in patients with established neuropathy and fibre degeneration [32]. However, another study failed to detect abnormalities in p75^{NTR} immunoreactivity in human nerve in association with degeneration and failure of regeneration [33]. Notwithstanding this latter observation, there are grounds for suggesting that maintenance/expression of p75^{NTR} in nerve in diabetes is abnormal, so that the increase in plasma titre reported here probably has its origins in peripheral nerve.

This study concentrated on effects of interventions, but a thorough demonstration of a relationship between the plasma p75^{NTR} immunoreactivity titre and diabetic neuropathy would require close correlation with structural and functional indices of the condition. This can only be done on a limited basis in rodents; hence a clinical study addressing these issues is now in progress.

for fidarestat and to Johannes Jakobsen and Yun Jiang for plasma from mice. The authors have no conflicts of interest in respect of this work.

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