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TrkAd5: A novel therapeutic agent for treatment of inflammatory pain and asthma

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Abstract

Elevated levels of nerve growth factor have been linked to the onset and persistence of many pain related disorders and asthma. Described here are the design, expression, refolding and purification of a monomeric (non strand-swapped) form of the binding domain of the nerve growth factor receptor (designated TrkAd5). We have shown that TrkAd5 produced recombinantly binds nerve growth factor with picomolar affinity. TrkAd5 has been characterized using a variety of biophysical and biochemical assays, and is shown here to be stable in both plasma and urine. The palliative effects of TrkAd5 are demonstrated in animal models of inflammatory pain and allergic asthma. We conclude that TrkAd5 will prove effective in ameliorating both acute and chronic conditions where nerve growth factor acts as a mediator, and suggest a role for its application *in vivo* as a novel therapeutic.

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Introduction

Many persistent pain states are associated with increased levels of nerve growth factor (NGF) (Woolf et al., 1994; McMahon et al., 1995) and the application of exogenous NGF can produce rapid and prolonged behavioral hyperalgesia in humans (Petty et al., 1994; Dyck et al., 1997) and in animal pain models (Lewin et al., 1993; Andreev et al., 1995). In addition, patients suffering from allergic diseases, including asthma, have increased serum levels of nerve growth factor (Bonini et al., 1996; Frossard et al., 2004).

Increased endogenous nerve growth factor levels may cause hyperalgesia by both neurogenic and non-neurogenic means. Importantly nerve growth factor causes a rapid sensitization of nociceptive sensory neurons (Shu and Mendell, 1999) via TrkA receptors (Averill et al., 1995) resulting in sensitization of the capsaicin vanilloid receptor, TRPV1 (Chuang et al., 2001), and downstream intracellular signalling (Shu and Mendell, 1999; Chuang et al., 2001; Bonnington and McNaughton, 2003). In addition the TTX-resistant voltage-gated sodium channel Nav 1.8 (Kerr et al., 2001) and the purinergic receptor P2X3 (Ramer et al., 2001) are modulated by nerve growth factor; both are expressed on primary sensory neurons and are associated with sensitization to pain.

We initially showed that the nerve growth factor binding site on the TrkA receptor corresponds to the immunoglobulin-like domain 4 and 5 (Holden et al., 1997). Further to this we showed that all of the binding activity resided in the d5 domain (TrkAd5). This domain was expressed with a histidine-tag in *E. coli*, purified and the structure determined (Robertson et al., 2001). At the concentrations required for

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crystal formation the protein folds as a strand swapped dimer, where the A strand from each monomer is transposed such that the domain is incapable of binding nerve growth factor. Here we demonstrate the production and purification of a non histidine-tagged TrkAd5 and describe a novel refolding strategy which results in high concentrations of monomeric protein. In addition we show that the protein is biologically active *in vitro*, is stable in blood and urine and is able to be lyophilized and reconstituted with full activity. We further demonstrate an extremely potent effect *in vivo* in an animal model of inflammatory pain and an *in vitro* model of allergic asthma. Conventional pharmaceuticals do not provide effective therapy for conditions such as interstitial cystitis and asthma, and we conclude that TrkAd5 represents a novel therapeutic agent for these disease states and many others in which raised nerve growth factor levels play a role in the etiology of the disease. TrkAd5 is currently being developed for use in clinical trials and has been designated ReN1820 (ReNeuron Ltd).

Methods

Expression vector construction.

Thirteen primers were designed to incorporate silent mutations within the first thirty codons of the ORF of TrkAd5 and their theoretical mRNA secondary structures determined by analysis using the MFOLD structure prediction program (M. Zuker, Pasteur Institute). From the results generated, six were chosen and used as forward primers in a PCR reaction with the wild type pET24a(+)TrkAd5 as template. The six mutant PCR products were ligated into the pET24a(+) vector (Novagen) at the NdeI/XhoI sites, then amplified by transformation into a non-expression host, XL1 blue. Purified, mutant constructs were confirmed by sequencing (MWG-Biotech),

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then transformed into BL21(DE3) *E.coli* for expression studies. Samples of uninduced/induced cultures from single colonies were subjected to SDS-PAGE analysis.

Production and purification of recombinant TrkAd5

5 L of 2YT medium, 50 µg/ml kanamycin, was inoculated with a 25 ml overnight culture of *E. coli* BL21(DE3) pET24a(+)TrkAd5 7 at 37 °C. The culture was induced by the addition of 5 ml 1 M isopropylthio-beta-D-galactoside (IPTG). Cells were harvested by centrifugation and pellets stored frozen at –80 °C until required. Inclusion bodies were purified and processed as described previously (Robertson et al., 2001). Inclusion body pellets were stored overnight at –80 °C, and the pellet subsequently re-suspended in 50 ml Solubilisation Buffer (8 M urea, 40 mM Tris pH 8.5, 100 mM NaCl, 50 mM DTT, 1 mM EDTA) and rocked on a platform for 3 h at room temperature (RT). Solubilised inclusion bodies were loaded on a Qsepharose Waters AP-5 column, pre-equilibrated with Solubilisation Buffer containing 10 mM DTT. TrkAd5 was step eluted using Solubilisation Buffer containing 200 mM NaCl. The protein peak was collected and incubated at RT for 14-21 days. Urea solubilised TrkAd5 was refolded and purified on a HiLoad Superdex 200 XK26/70 column, pre-equilibrated with elution buffer (20 mM Tris pH 8.5, 100 mM NaCl). Peaks corresponding to the monomer fraction were pooled. For animal studies, endotoxins were removed from TrkAd5 using a 20 ml Detoxigel column (Pierce) and found to be 1.71 endotoxin units (EU)/mL by Limulus assay (Biowhittaker). The TrkAd5 fractions were concentrated to 0.8-1 mg protein/ml, 10% (v/v) glycerol added and the protein filter sterilized, snap frozen in liquid nitrogen, and stored at –80 ºC.

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PC12 cell neurite outgrowth

The ability of TrkAd5 to sequester nerve growth factor was assessed in the rat phaeochromocytoma cell line, PC12, by noting the reduction in neurite outgrowth using the method previously described (Allen et al., 2001; Robertson et al., 2001). This method is routinely used to visually assess activity. However quantification of TrkAd5 activity in PC12 cells is carried out using the apoptosis assay as described below.

PC12 cell apoptosis assay

PC12 cells were plated out on collagen coated 96-well plates in 100 µl of serum-free media at a cell density of 1 x 10^4 cells per well, after washing three times in serum-free DMEM (containing 1 % (v/v) penicillin/streptomycin solution and 2 mM L-glutamine). Nerve growth factor and/or TrkAd5/Tris buffer were added at required concentrations with 10 x DMEM (Sigma D-2554) used to make up the total volume per well to 200 µl and incubated at 37 °C with 5 % CO_2 for 3 days. Metabolic turnover was assessed by addition of 40 µl per well of premixed CellTiter 96®AQueous assay reagents (Promega). Plates were incubated for up to 4 h. Optical density was measured at 492 nm.

***In vitro* nerve growth factor competition ELISA**

Binding of TrkAd5 to nerve growth factor was assessed using a competitive ELISA format. 96-well plates (Nunc Maxisorp) were coated with 50 µl per well of a 1:1000 dilution of nerve growth factor capture antibody (Sigma) in 50 mM sodium carbonate solution pH 9.6 and incubated overnight at 4 °C. Non-specific binding sites were blocked by addition of 200 µl of blocking buffer (50 mM sodium carbonate

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containing 1 % (w/v) BSA, and 5 % (w/v) sucrose) per well and incubation at RT for 2 h. Washing was with TBS-T (containing 0.1 % (v/v) Tween 20). Standards (50 µl) of human recombinant nerve growth factor-Beta (Sigma) (0-1 ng/ml) were prepared with and without TrkAd5, and pre-incubated at RT for 10 min before addition to plate for 1 h at RT. 50µl of 0.1 µg/ml biotinylated anti-human nerve growth factor monoclonal antibody (R&D Systems) in TBS-T with 1 % (w/v) BSA was added to each well and the plate incubated at RT for 1 h. 50 µl of 0.5 µg/ml of streptavidin alkaline phosphatase polymer (Zymed Laboratories) in TBS-T with 1 % (w/v) BSA was added per well and incubated at RT for 1 h. 50 µl of 4-methyl umbelliferyl phosphate (4-MUP, Sigma) substrate solution was added per well. Fluorescence readings were measured on a Fluoroskan II (LabSystems) at excitation/emission wavelengths of 355/460 nm after 10, 20 and 30 min.

Mass spectrometric characterization of TrkAd5 and N-terminal sequencing

Molecular weight of proteins was measured accurately using MALDITOF mass spectrometry as previously described (Robertson et al., 2001).

A sample of TrkAd5 was run on a 15% tricine gel (Schagger and von Jagow, 1987), then transferred onto ProBlott membrane (Applied Biosystems) using standard western blotting techniques. Protein bands were visualized following staining with 0.1 % (w/v) Serva Blue G. N-terminal sequencing was carried out using an Applied Biosystems 492 cLC protein sequencer using standard cycles.

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Shelf-life stability at 4 °C

Sterile filtered TrkAd5 (0.5 mg/ml in 20 mM Tris pH 8.5, 148 mM NaCl, 10 % (v/v) glycerol) was stored at 4 ºC. At specific time points, 100 µl of sample was prepared for HPLC analysis by the addition of 40 µl protein standard (insulin 1 mg/ml) and 1860 µl of HPLC solvent A (4.4 % acetonitrile, 0.1 % TFA (trifluoroacetic acid v/v)). The prepared sample was injected onto a Supelco LC-304 C5 column, equilibrated at a flow rate of 1 ml/min using 100 % solvent A for 10 min, then eluted over 50 min with a linear gradient ending at 100 % solvent B (95.5 % acetonitrile, 0.1% TFA (v/v)). TrkAd5 had an over all retention time of 39 min against the insulin standard of 32 min.

Sterile filtered TrkAd5 was stored at 4 °C for up to 3 months. The end point sample was also tested using the PC12 cell apoptosis assay and *in vitro* nerve growth factor competition ELISA.

Lyophilisation

TrkAd5 was dialyzed against three changes of 5 % (w/v) sucrose, snap frozen in liquid nitrogen, lyophilized, and stored at -80 ºC.

Iodination of TrkAd5 and stability time courses

TrkAd5 was iodinated as previously described (Robertson et al., 2001). 50,000 cpm was used per time point for the stability time course. Protein stability was studied over 1920 min and ten time points taken. Radiolabelled TrkAd5 was added to 3 ml rat serum, human serum or human urine; and a thousand-fold amount of unlabelled TrkAd5 added as a carrier protein. At each time point 100 µl samples were taken,

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snap frozen on dry ice and stored at -80 ºC until the assay end point. Samples were then thawed and 100 µl of 15% TCA added. Following 20 min incubation on ice, samples were spun at 2,500 rpm, the supernatant removed and the pellets counted.

Chemically induced cystitis study

The method used was similar to that described elsewhere (Dmitrieva et al., 1997). All animal studies were carried out in accordance with the declaration of Helsinki. Briefly, twenty-one female Wistar rats (190-220 g) were anaesthetized with urethane (1.25 g/kg, ip), which produced a stable level of anesthesia lasting the entire experiment. The carotid artery was cannulated. Body temperature was measured and maintained close to 37 ºC. The bladder was catheterized transurethally with a 1.1 mm polythene catheter. A ventral midline laparotomy was performed enabling complete bladder emptying to be confirmed.

Bladder motility was assessed by slow filling of the bladder with normal saline through a transurethral catheter, at 0.05 ml/min for 14 min (bladder volume was increased from 0 to 0.7 ml in this period), rate of filling used was within the physiological range. In normal animals, during filling, bladder pressure increased gradually for approximately 5 min, beyond which a series of regular micturition contractions were elicited, typically 2-5 within the 14 min period of measurement. Only animals with no visible signs of bladder inflammation had clear urine and showed normal baseline cystometrograms were chosen for further experimentation.

After control determinations, animals were subjected to one of two treatments and cystometrograms were subsequently undertaken at times 1, 3 and 5 h. Briefly 0.5 ml

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of a turpentine/olive oil mixture (50:50) was instilled into the bladder for 1 h after which the turpentine was drained. This treatment produced a sterile inflammatory immune response with invasion of immune cells and development of hyper-reflexia. The inflammation started within 1 h of turpentine installation and progressively increased over the next few hours. To facilitate the analysis of treatment for each animal the slope of the regression line was calculated for each outcome measure and compared between the saline and TrkAd5 by unpaired t-tests.

In vitro organ bath studies

Organ bath studies were carried out as described previously (De Vries et al., 2001). Male Hartley guinea pigs (44-66 g: Harlan CPB, The Netherlands) were used in all experiments. The Animal Care Committee of the Utrecht University approved the animal studies. The animals were sacrificed by cervical dislocation and isolated tracheal rings (three cartilage segments per ring) were placed in an isometric organ bath set-up, containing warmed (37 ºC) Krebs solution (pH 7.4, with 8.3 mM glucose) gassed with 95 % O2/ 5 % CO2. The experiment started with four washout periods lasting 15 min each. During these washouts a tension was applied of 2000, 2000, 4000 and 2000 mg, respectively. A histamine concentration response curve (10^{-8} – 10^{-3} M) was subsequently conducted to measure contractility of the tracheal rings. Nerve growth factor was applied at a concentration of 20 ng/ml 30 min before the start of the response curve. TrkAd5 at 7.64 µM was applied 5 min before addition of nerve growth factor.

In a second set of experiments, guinea pigs were sensitized to ovalbumin (grade V; Sigma) by injections of a gel containing 20 µg ovalbumin/ml, and 200 mg

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$Al(OH)_3$/ml as adjuvant. Six injections were placed on the same day: one injection of 0.5 ml intraperitoneally, one of 0.1 ml nuchally, two injections of 0.1 ml axillarly, and two of 0.1 ml inguinally. Control animals were not sensitized and received instead a vehicle gel, not containing ovalbumin. Fourteen days after the sensitization procedure tracheal rings were isolated. The experiment started with four washout periods lasting 15 min each. During these washouts a tension was applied of 2000, 2000, 4000 and 2000 mg, respectively. TrkAd5 (7.4 μM) was added and the response to ovalbumin challenge (1 mg/ml) was then measured. 60 min later, following two 15 min washouts, the histamine response curve was measured.

Results

Design of construct.

TrkAd5 without extraneous amino acids was required for potential clinical application. The TrkAd5 construct was therefore re-cloned to eliminate the histidine tag and any vector-derived amino acid sequence. This resulted in a lack of expression probably due to the presence of positioning of the AUG start site at the base of a long stem-loop structure (**Fig. 1a**), which has the potential to interfere with the AUG translation initiation codon (Tessier et al., 1984). Predicted secondary structure of the expression construct was re-designed using MFOLD (M. Zuker, Pasteur Institute), such that the mRNA structure was altered, whilst maintaining the amino acid sequence of TrkAd5 (**Fig. 1b**). In total six constructs were designed, incorporating silent mutations within the first twenty to thirty nucleotides of the ORF, predicted to alter the mRNA structure to make both the ribosome binding site and the translational start more accessible. One construct was designed for optimal prokaryotic codon

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usage. Recombinant proteins were then expressed (**Fig. 1c**). Of the constructs created, five produced high yields of protein of the correct molecular weight. One of these constructs d5#7, whose MFOLD structure can be seen in **Fig. 1b**, was chosen for further analysis. The construct designed for optimal codon usage did not express protein (**Fig. 1c** lane 7)

Protein expression, purification and refolding.

Inclusion body preparations were isolated and processed from 5 L bioreactor cultures of d5#7. Final purification on a S200 Superdex column allowed rapid refolding and separation of the monomer away from any aggregate and/or dimeric species. The effects of incubating the protein for different times in urea show that extended incubation effectively converts aggregate and dimer into monomeric protein (**Fig. 2**). The monomeric state of the protein was also assessed by native gel silver stain analysis with over 95 % homogeneity of the sample (data not shown).

MALDI-TOF analysis and N terminal sequencing

Monomeric fractions were shown by MALDITOF to have a molecular weight of 13593 Da (**Fig. 3**). This corresponds to the calculated molecular weight of TrkAd5 with the initiating methionine removed and with the addition of one carbamylation adduct, which occurs during extended incubation in urea. N terminal sequencing of TrkAd5 showed the first nine residues to be PASVQLHTA, corresponding to the predicted amino acid sequence.

The nerve growth factor sequestering ability of TrkAd5.

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PC12 cells differentiate and extend processes in a dose-dependent manner upon addition of nerve growth factor (**Fig. 4a**). TrkAd5 was shown to inhibit nerve growth factor-induced neurite outgrowth in PC12 cells (**Fig. 4b**) and to exacerbate the apoptotic response in a nerve growth factor-dependent cell survival assay, in which serum is withdrawn from PC12 cells (**Fig. 4c**). At a concentration of 5 ng/ml nerve growth factor, addition of TrkAd5 resulted in a dose-related reduction in cell survival to background levels, with an approximate IC_{50} of 1 µM. Both assays confirm the *in vitro* sequestration of nerve growth factor by TrkAd5.

A competition sandwich ELISA assay was designed for rapid assessment of binding capability of the active monomer. Pre-incubation of standard solutions of nerve growth factor with various concentrations of TrkAd5 (0-4.5 µM) gave a dose dependent reduction in fluorescence signal (**Fig. 4d**) with an IC_{50} of approximately 0.05 µM.

Stability and lyophilisation

To determine any effect lyophilisation may have on the bioactivity of the TrkAd5, the protein was lyophilized, stored overnight at -80 ºC and then reconstituted to its original concentration. This procedure showed no adverse affects on the bioactivity of the protein as determined using the apoptosis assay (**Fig. 4c)**.

The shelf life stability of TrkAd5, stored at 4 ºC was determined over a period of 75 days. Samples were assayed by HPLC with no appreciable degradation observed (data not shown). As a prerequisite to animal pharmacokinetic studies, the *in vitro* stability of radiolabelled TrkAd5 was measured, using TCA precipitation, over a 32 h time

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course in human and rat serum and human urine (**Fig. 5**). After 32 h in human and rat serum, 90 % of the protein was still intact. The protein was less stable in urine with a half-life of approximately 32 h.

Effect of TrkAd5 in a model of chemically induced acute cystitis

The ability of TrkAd5 to reduce inflammatory changes was investigated in an established rat chemically induced cystitis paradigm (Dmitrieva et al., 1997; McMahon and Abel, 1987). Experimental inflammation was induced by turpentine infusion and followed by injection of either saline (iv) or TrkAd5 (200 µg iv, equivalent to 14.8 nmols) 1 hour later at the onset of bladder irritation. Cystometrograms were taken for up to 5 h after the instillation of the irritant, and bladder responsiveness was assessed by micturition threshold, number of contractions, and the total duration of contractions (TCT). At 1 h after irritation of the bladder both groups of animals treated with either saline (n=13) or TrkAd5 (n=8) developed similar degrees of hyper-reflexia, bladder reflexes were exaggerated and showed decreased micturition threshold, increased number of contractions and increased TCT. Saline treated animals thereafter continued to show progressive hyper-reflexia with bladder reflexes increasing from 216 % at 1 h to 329 % of control at 5 h. In the TrkAd5-treated animals there was a reversal of progression of hyper-reflexia from 194 % to 138 % of control (unpaired t-test p=0.0006) (**Fig. 6a**). In saline treated animals total time of contraction increased from 278 % to 410 % of control; however, in TrkAd5 treated animals, contraction times were reduced from 214 % to 158 % of control (unpaired t-test p=0.0102) (**Fig. 6b**). Similarly micturition threshold was reduced from 68 % to 51 % of control in saline treated animals, but increased

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from 67 % to 85 % of control in TrkAd5 treated animals (unpaired t-test p=0.0275) (**Fig. 6c**).

Effect of TrkAd5 on the responsiveness of guinea pig tracheal smooth muscle

The effect of TrkAd5 on the nerve growth factor-induced hyperresponsiveness of isolated guinea pig tracheal rings was investigated in an organ bath set-up. As previously demonstrated (De Vries et al., 2001), incubation with 20 ng/ml nerve growth factor induced a hyper-responsiveness following application of histamine. We show here that the addition of 7.64 µM TrkAd5 prior to nerve growth factor exposure was able to block the concentration-related hyperresposiveness to histamine, presumably by sequestration of nerve growth factor (**Fig. 7a**). Addition of TrkAd5 alone had no effect compared to controls (**Fig. 7a**). In a second series of experiments, the effect of TrkAd5 on ovalbumin challenge was investigated in tracheal rings isolated from guinea pigs which had been ovalbumin sensitized with ovalbumin two weeks previously (**Fig. 7b**). TrkAd5 was shown to inhibit this direct allergen-induced tracheal contraction (**Fig. 7b)**. In addition, TrkAd5 was also shown to inhibit the hyper-responsiveness to histamine 60 min after ovalbumin challenge (**Fig. 7c**).

Discussion

Asthma and inflammatory pain states are known to be associated with increased levels of endogenous nerve growth factor. For example, nerve growth factor is up regulated in the joints of human arthritic patients (Aloe et al., 1992a), mRNA levels of nerve growth factor and TrkA are increased in samples from patients with chronic pancreatitis (Zhu et al., 1999) and increased bladder nerve growth factor levels are

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associated with painful conditions including interstitial cystitis (Lowe et al., 1997). A related animal model exists for each of these conditions, with studies suggesting nerve growth factor has a pivotal role in nociceptor sensitization in each case (Braun et al., 1998; De Vries et al., 1999; Aloe et al., 1992b; Toma et al., 2000; McMahon and Abel, 1987; Dmitrieva et al., 1997). Neurotrophins levels have been shown to be raised in the urine (Okragly et al., 1999) and bladder (Lowe et al., 1997) of women suffering with interstitial cystitis. Studies with a cyclophosphamide (CYP) induced bladder inflammation model have demonstrated alterations in micturition reflex (Yoshimura and de Groat, 1999), probably mediated by neurotrophins produced in the bladder during cystitis (Vizzard, 2000). Thus inflammation-induced changes in neurotrophins may sensitize afferent and postganglionic nerves; resulting in bladder over activity, and may be involved in neuroplasticity of the lower urinary tract pathways (Vizzard, 2000; Qiao and Vizzard, 2002; Okragly et al., 1999; Lowe et al., 1997).

Studies in animal pain models in which TrkA immunoadhesins are administered have shown that sequestration of endogenous nerve growth factor is able to block the hyperalgesia associated with inflammation (McMahon et al., 1995; Zahn et al., 2004). However, these are large molecules and are unlikely to be clinically useful. Previously we have shown that a histidine-tagged TrkAd5 domain reversed the electrophysiological correlates of complete Freund's adjuvant (CFA) inflammation in the guinea pig (Djhouri et al., 2001). We describe here the design, expression, refolding and purification of a small non-tagged monomeric 13.5 kDa domain of the TrkA receptor, capable of sequestering nerve growth factor in an *in vivo* model of

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cystitis and an *in vitro* model of allergic asthma and in reversing symptoms associated with these conditions.

The protein purification protocol involved prolonged incubation in 8 M urea. Aggregation is a major problem of refolding protein from *E. coli*. This extended incubation in urea (14-28 days) may be beneficial for the refolding of other recombinant proteins in *E. coli* where aggregation is an issue. Endotoxins were removed prior to *in vivo* studies and the protein was tested *in vitro* for the ability to neutralize the effect of nerve growth factor in two PC12 cell bioassays: prevention of neurite outgrowth and nerve growth factor-induced rescue from apoptosis. In a previous study (Robertson et al., 2001) we have shown that this nerve growth factor binding ability is unique to TrkAd5 since the related domain TrkAd4 does not bind or sequester nerve growth factor. In addition we describe here a competition ELISA in which TrkAd5 was able to sequestrate nerve growth factor with an IC_{50} of 40nM. Using surface plasmon resonance TrkAd5 binds nerve growth factor with an affinity of 94pM, which is with a tenfold greater affinity than to NT-3, and with over a thousand fold greater affinity than to BDNF or NT-4 (unpublished data).

The effectiveness of TrkAd5 in modulating bladder excitability was assessed in an established *in vivo* model (McMahon and Abel, 1987; Dmitrieva et al., 1997) of acute inflammatory cystitis. In our study, animals infused with turpentine into the bladder showed features typical of cystitis in humans, with clear changes in bladder activity recorded. Systemic injection of TrkAd5 proved effective in reversing all symptoms. Thus TrkAd5 is, to date, unique in being the only molecule to reverse the symptoms in this chemically induced model of cystitis, suggesting a clear clinical role.

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The importance of nerve growth factor as a factor in the development of human allergic disease and asthma was initially suggested by the detection of increased serum levels of nerve growth factor in patients with these conditions (Bonini et al., 1996). A number of different cell types in the lung are known to be involved in production of nerve growth factor including: macrophages, T-cells, mast cells, fibroblasts and epithelial cells (Burgi et al., 1996; Nilsson et al., 1997; Ehrhard et al., 1993). In asthmatics, nerve growth factor has been detected in bronchoalveolar lavage fluid (Undem et al., 1999) with increase in levels with allergen challenge, and in fluid and bronchial biopsies from ovalbumin-sensitized and challenged mice (Virchow et al., 1998). Nerve growth factor pre-treatment of airways induces bronchial hyper-responsiveness *in vivo* in guinea pigs and *in vitro* on tracheal segments (De Vries et al., 1999; De Vries et al., 2001).

Using isolated guinea pig tracheal rings, as previously demonstrated (De Vries et al., 2001), incubation with nerve growth factor induced an increased dose-related histamine hyper-responsiveness. We show here that addition of TrkAd5 prior to nerve growth factor exposure was able to block this hyper-responsiveness. Interestingly, in guinea pigs previously ovalbumin sensitized, TrkAd5 was able to moderate the direct allergen-induced tracheal contraction upon ovalbumin challenge. Moreover, 60 min following this ovalbumin challenge, the histamine-induced hyper-responsivity was inhibited in the rings which had received one dose of TrkAd5, preceding the ovalbumin challenge. This significant finding indicates that TrkAd5 is able to modify the airway late hyper-responsiveness to histamine. Presumably effects are due to the ability of TrkAd5 to sequester endogenous nerve growth factor, and it is of note that

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administration of TrkAd5 causes the contractile response to histamine to be lower than control after ovalbumin challenge.

Overall, these studies indicate that the TrkAd5 protein is stable in blood and urine and show potent effects in animal models of cystitis and allergic asthma. Further studies will address pharmacokinetic issues and the clinical use of this protein for these and other disease states.

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We would like to thank Dr Annick de Vries (University of Edinburgh, Scotland) for all her advice on nerve growth factor in relation to asthma.

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Footnotes

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Legends for Figures

Figure 1 Introduction of silent mutations into the 5' end of WT TrkAd5 facilitates protein expression. Predicted mRNA structures of the WT (**a**) and mutant TrkAd5#7 (**b**) pET24a(+) constructs showing increased access to the translation initiation ATG sites (boxed). (**c**) SDS-PAGE analysis of mutant constructs (Lanes 2-8) showing protein expression upon IPTG induction in five out of six clones. Lane 1 shows an un-induced control, Lane 3 shows construct d5#7 and Lane M molecular weight markers.

Figure 2 Conversion of aggregate and dimeric TrkAd5 into monomer with extended incubation in urea. Overlay of elution profiles obtained from a Superdex 200 column when refolding of TrkAd5 was performed at various time points over a 14-day period following solubilisation in 8 M urea. Refolding on day 1 (dotted line) shows the majority of TrkAd5 eluting as an aggregate species at approximately 55 minutes. On day 4, (light gray) there is a decrease in aggregate with a corresponding increase in dimeric and monomeric species. On day 11 (dark gray) there is a further decrease and by day 14 (black), the majority of TrkAd5 elutes at approximately 85 minutes as a monomeric species.

Figure 3 MALDITOF analysis of TrkAd5. A typical trace obtained for monomeric TrkAd5 showing a molecular weight of 13593 Da.

Figure 4 Effect of TrkAd5 on PC12 cell survival bioassays. Photomicrographs of PC12 cells show neurite extension two days (**a**) after addition of 1 ng/ml nerve growth factor to the growth media (**b**) when 4.5µM TrkAd5 was added in addition to nerve

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growth factor. (**c**) Graph showing the effect of increasing TrkAd5 concentration on percentage survival of PC12 cells when grown in incomplete media supplemented with 5 ng/ml nerve growth factor. TrkAd5 prepared according to methods (solid circles) and TrkAd5 lyophilized and reconstituted (open circles). (**d**) Graph showing the effect of increasing TrkAd5 concentration in a nerve growth factor capture ELISA. Error bars are SD of triplicates.

Figure 5 Stability of TrkAd5 in serum and urine. The histogram shows percentage degradation of TrkAd5 with time as calculated by TCA precipitation of radiolabelled TrkAd5 in rat serum, and human serum and urine. Results are mean values of triplicates.

Figure 6 Effect of TrkAd5 on bladder reflex hyper-excitability in a chemically induced rat model of acute cystitis. (**a**) Number of bladder contractions (**b**) Total contraction time and (**c**) Micturition threshold are shown for the control state and then 1, 3 and 5 h after instillation of turpentine into the bladder lumen. Animals were treated (iv) with either saline or 200 µg TrkAd5 1 h after onset of bladder irritation.

Figure 7 Effects of TrkAd5 on guinea pig tracheal responsiveness. The effect of TrkAd5 on nerve growth factor-induced tracheal hyperresponsiveness to histamine is shown in (a). The effect of TrkAd5 on tracheal contractions induced by ovalbumin challenge (1 mg/ml) of tracheal rings isolated from ovalbumin-sensitized guinea pigs is presented in (b), whereas (c) shows the influence of TrkAd5 on hyperresponsiveness to histamine of these tracheal rings (n=3, * $P<0.05$ compared with control).

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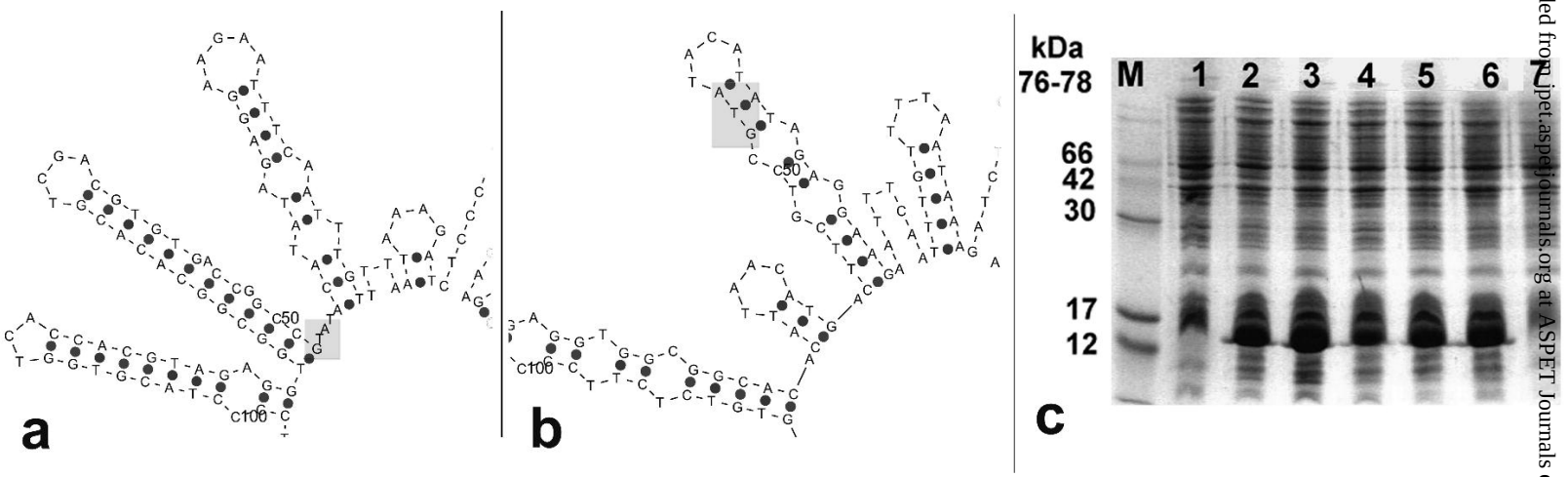

Figure 1

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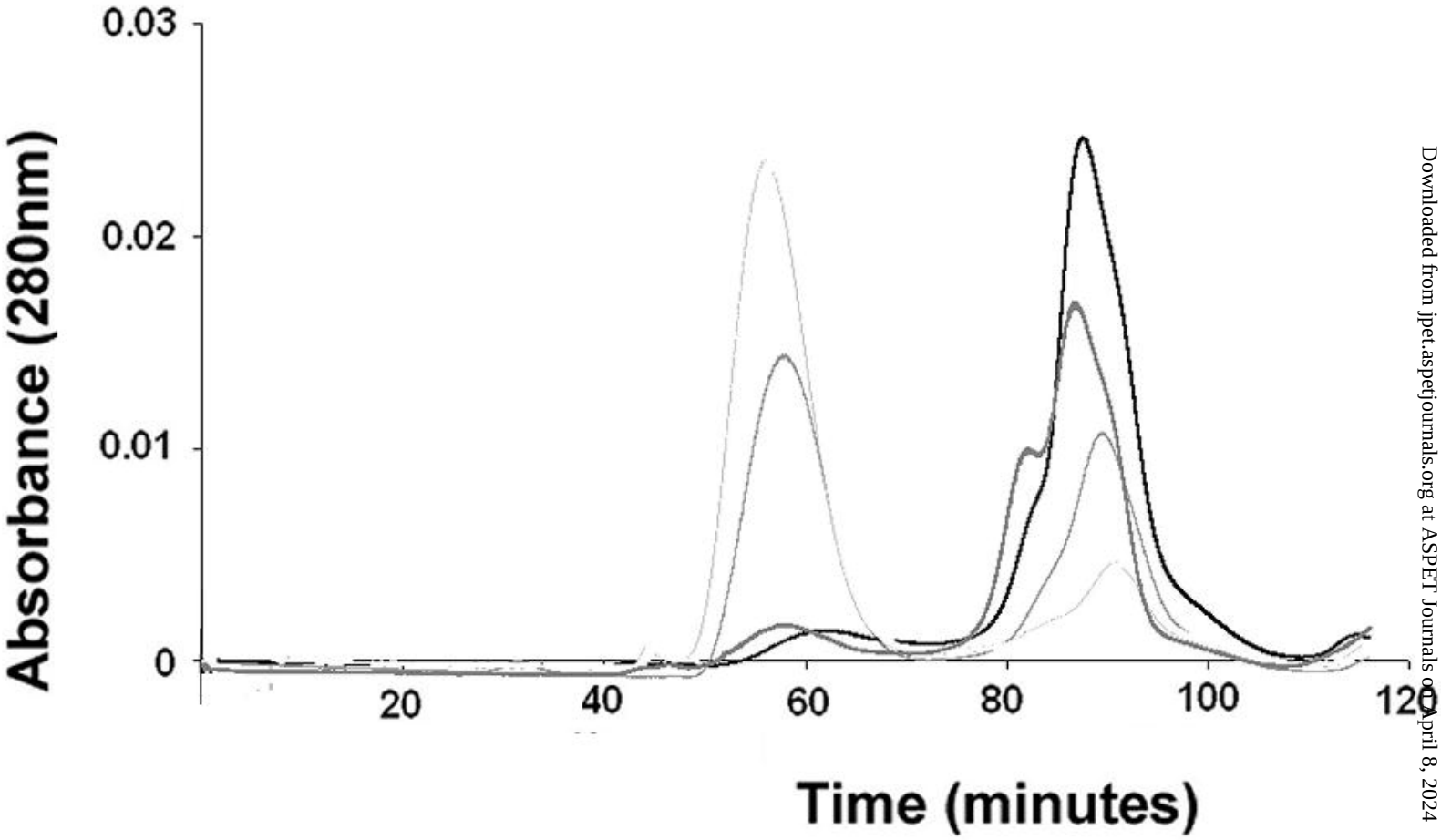

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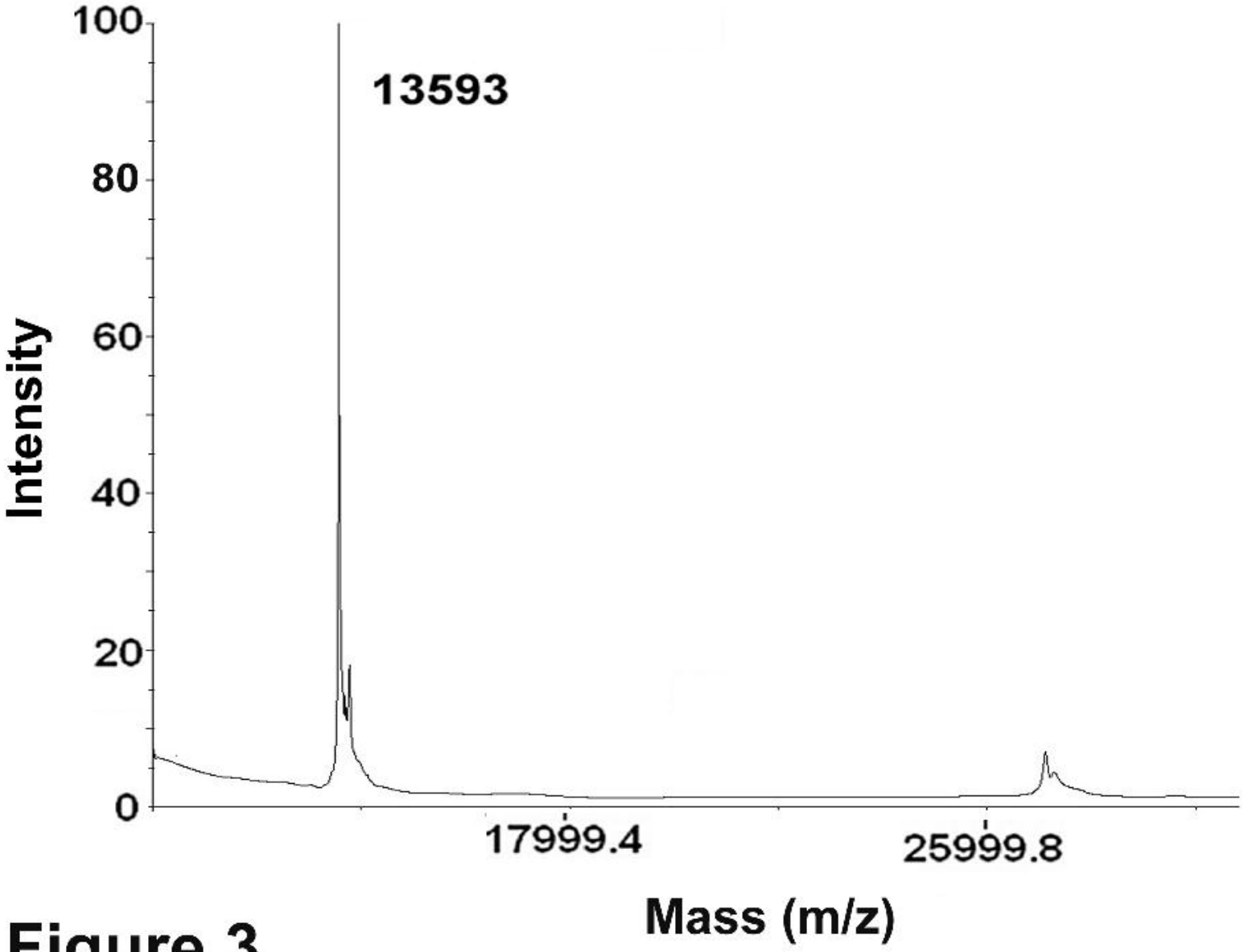

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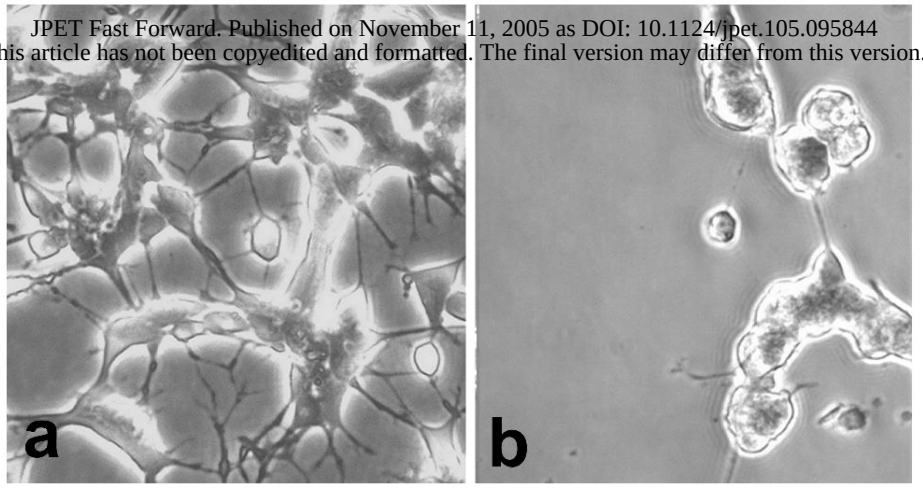

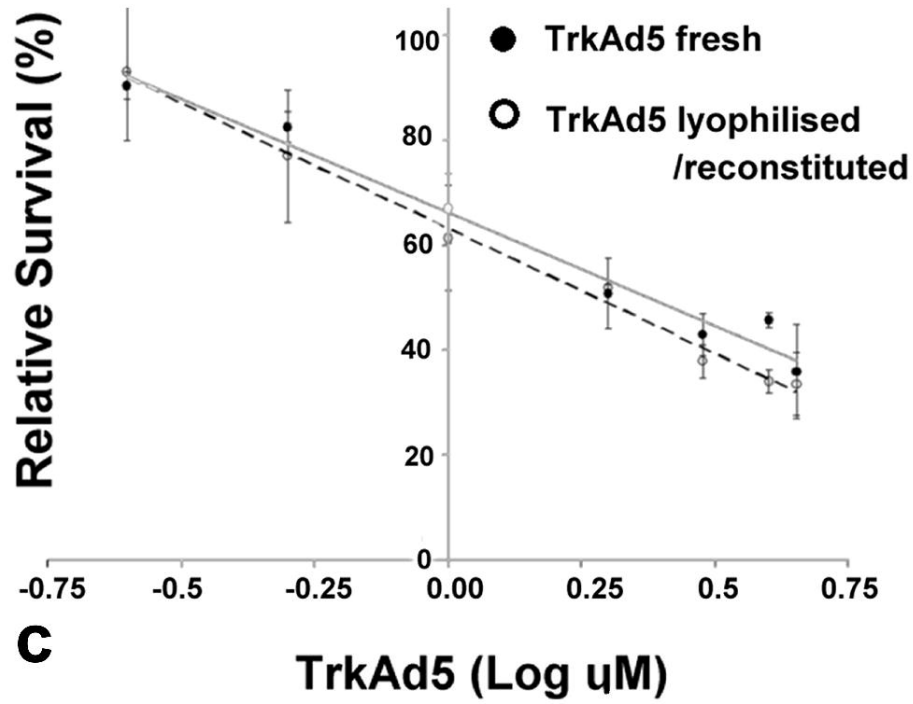

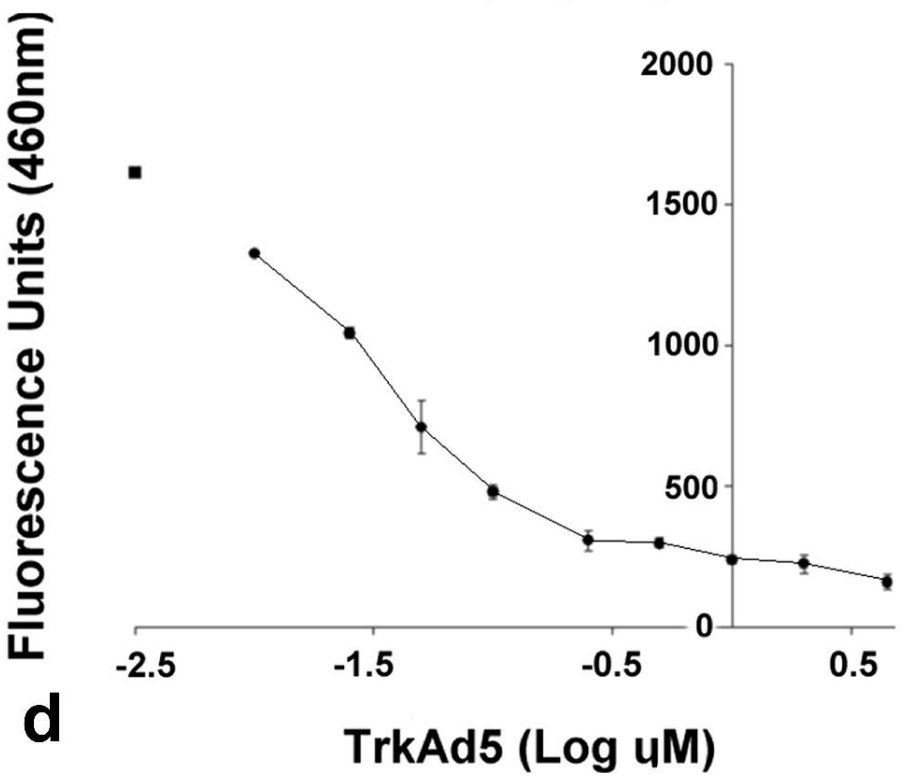

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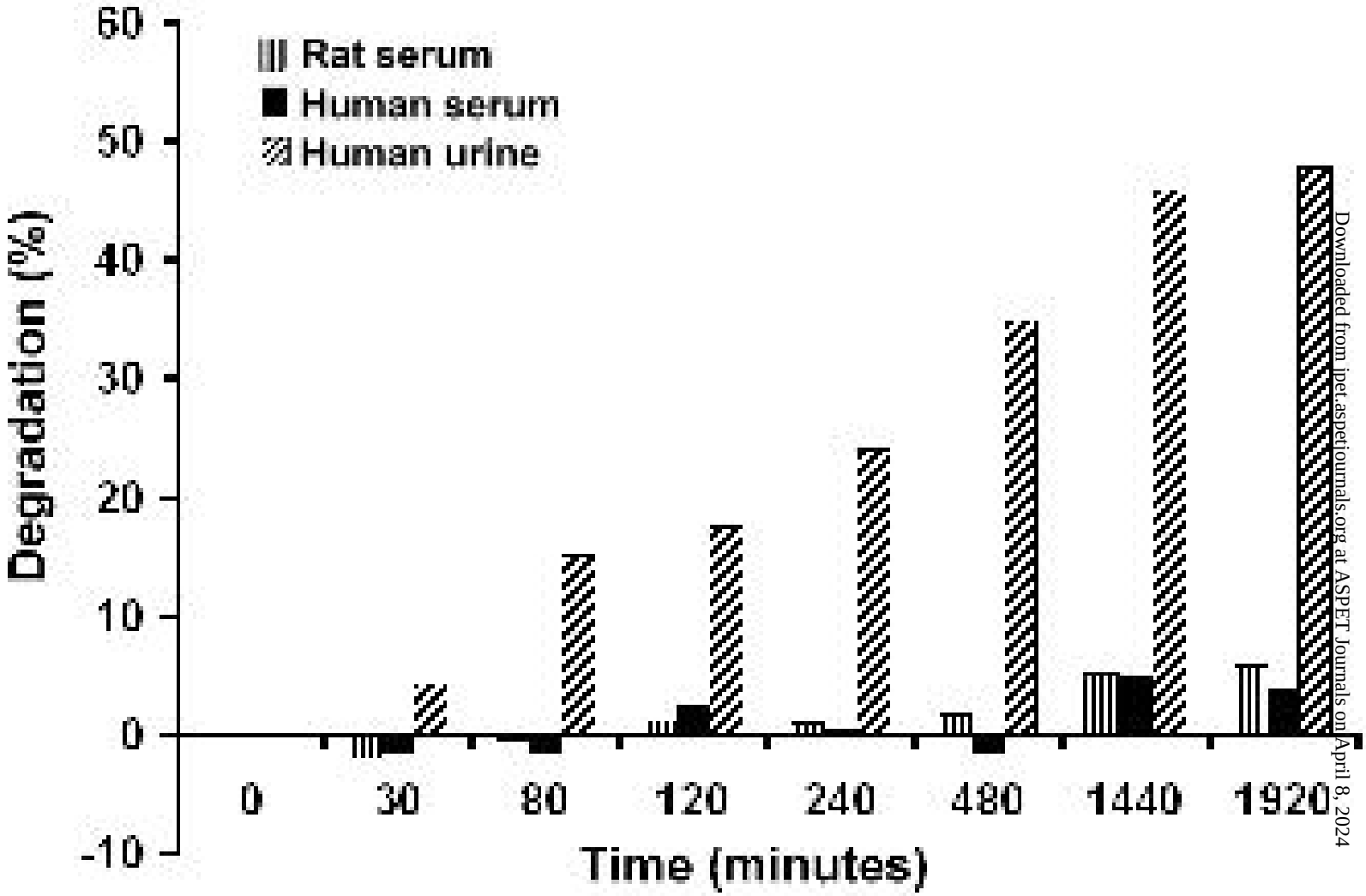

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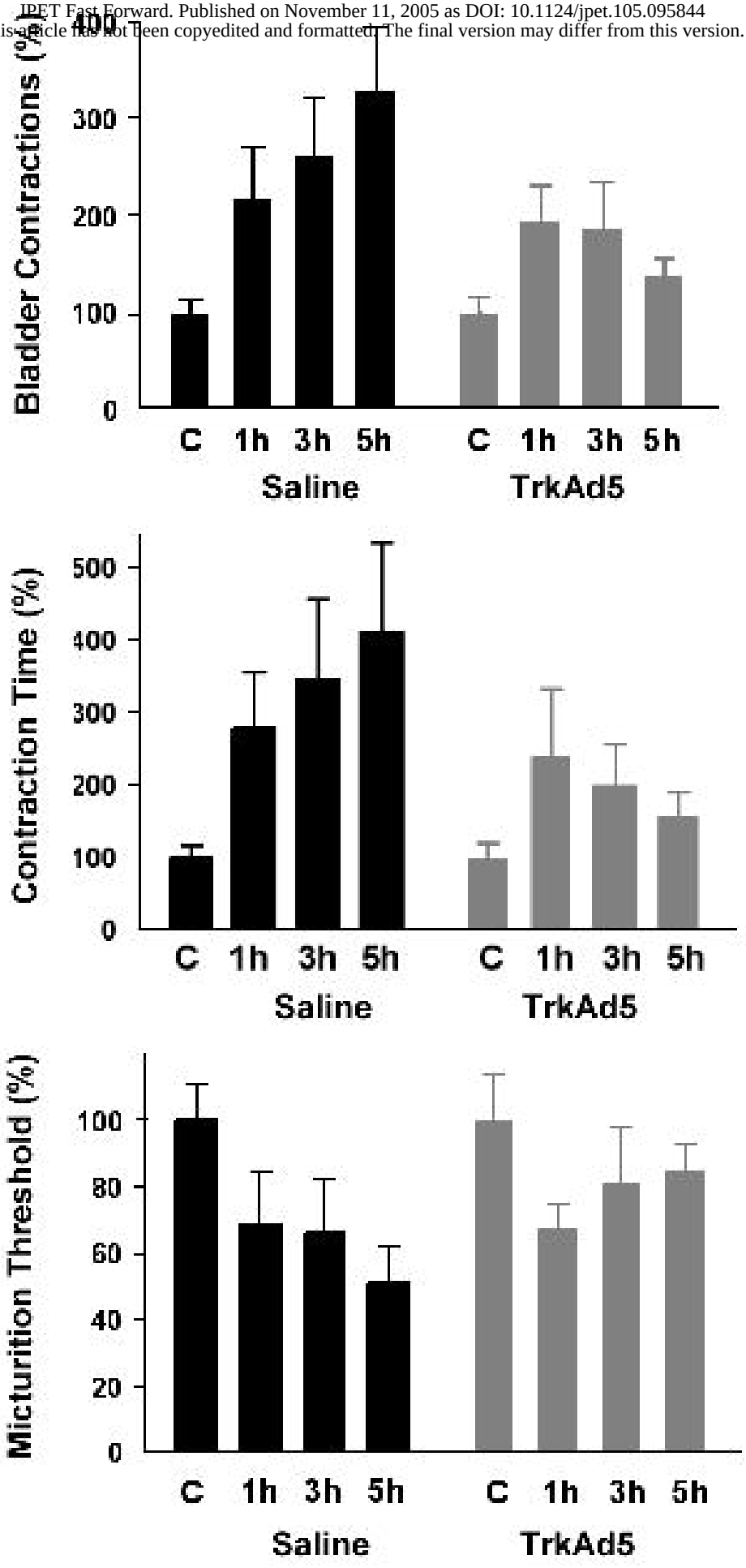

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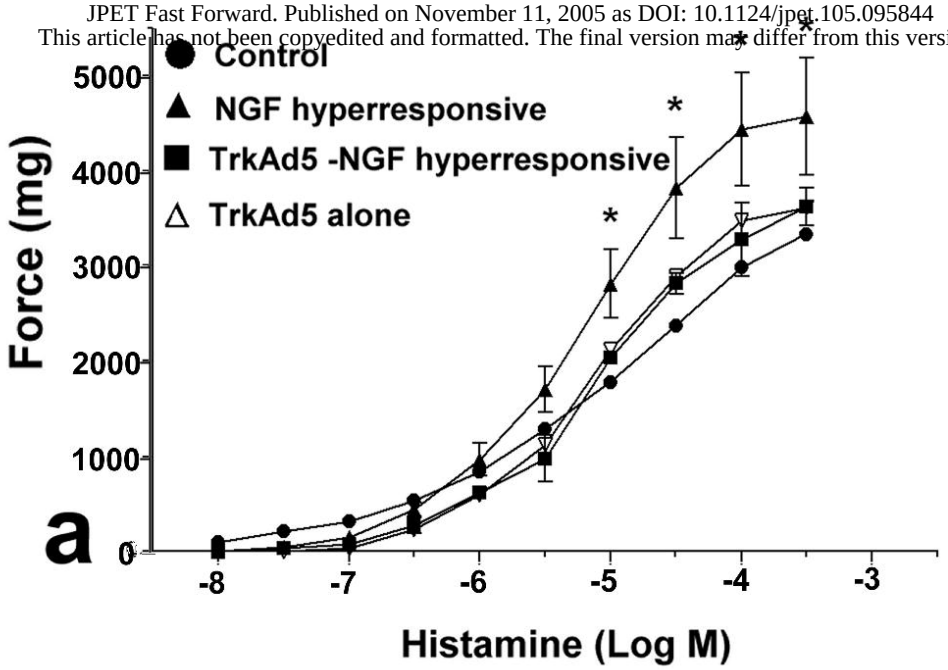

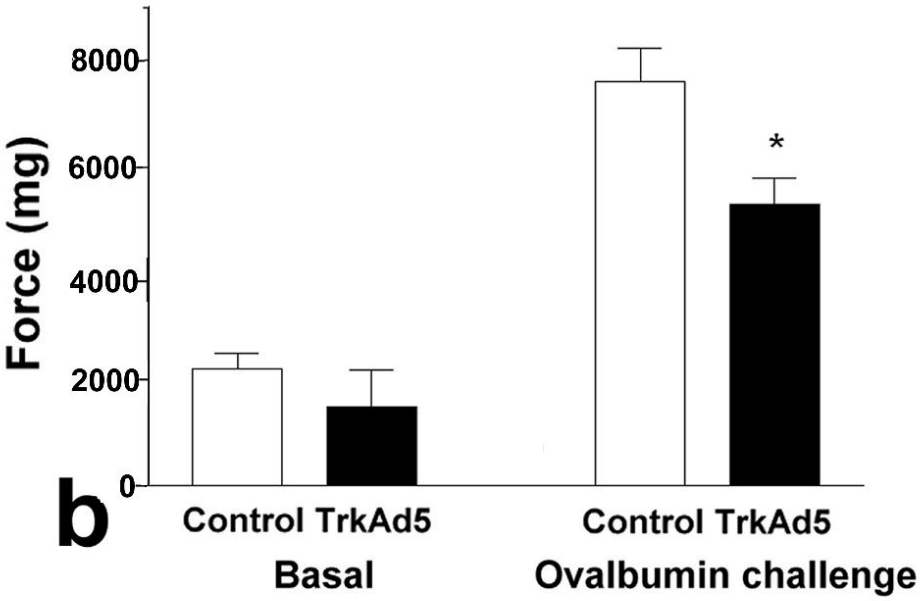

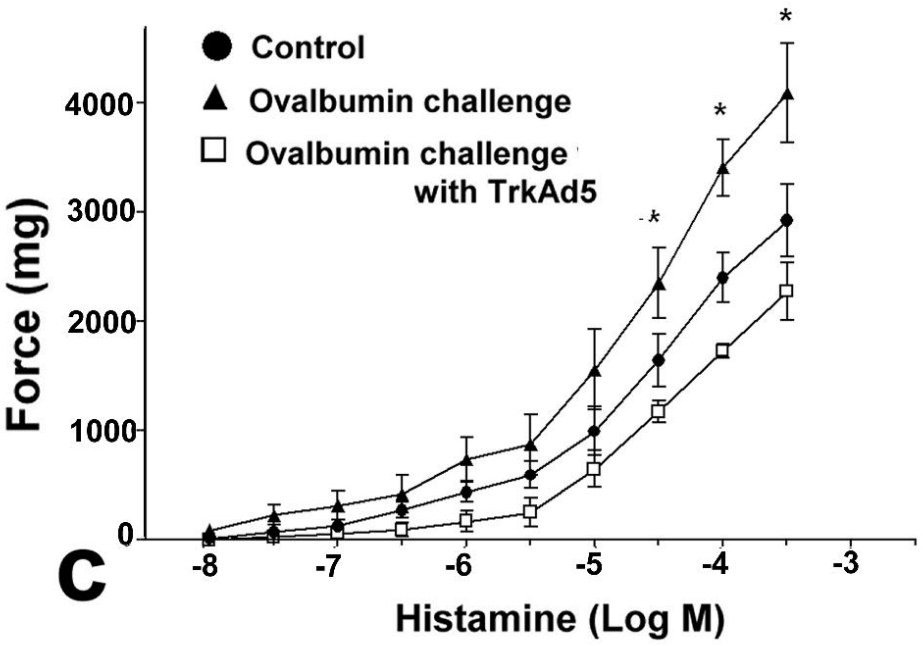

Figure 7

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