PHARMACOLOGIA

Anti-trkb Antibodies as Pharmacological Tools to Study the Function of the TrkB Receptor and its Role in the Regulation of Food Intake

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ABSTRACT

Background: In the present experiments we studied the function of the TrKB receptor and its role in the regulation of food intake by using an immunological approach. **Results:** In a first series of experiments rats were immunized against an extracellular domain of the TrkB receptor in order to induce specific Abs. Immunized rats showed a significant increase in body weight and partial protection against Lipopolysaccharide (LPS) induced anorexia. In a second series of experiments we produced a monoclonal antibody (mAbD2) and its scFv fragment (scFvD2) against the same peptide antigen. The anti-TrkB Abs, mAbD2 and scFvD2 were characterized *in vitro* in PC12 cells expressing the rat TrkB receptor or in neurons prepared from mouse embryonic stem cells. In the presence of anti-TrkB Abs, their Fabs or mAbD2 and scFvD2 a decrease in the maximum efficacy of brain-derived neurotrophic factor (BDNF) on TrkB phosphorylation and an increase in its internalization rate were observed. By contrast, anti-TrkB Abs and their Fab influenced neither neurotrophin-4 induced TrkB receptor phosphorylation nor internalization. Intracerebroventricular injection of anti-TrkB Abs or scFv D2 in rats lead to a significant increase in food intake while no effects on other parameters were observed. **Conclusion:** Anti-TrkB Abs acted as highly selective non-competitive antagonists of BDNF. Studies with intact Abs and their Fab fragments or with a mAb and its scFv derivative showed that neither the activation of the TrkB receptor nor its dimerization by bivalent Abs was a prerequisite of its internalization. The observation that active immunization of rats with a TrkB peptide conferred significant protection against LPS-induced anorexia while passive immunization increased food intake suggests that blockade of the TrkB receptor may have therapeutic potential in the treatment of anorexia and cachexia.

Key words: Brain-derived neurotrophic factor, internalization, biologics, appetite, anorexia, cachexia

INTRODUCTION

The tropomyosin-related kinase B (TrkB) receptor and its endogenous ligand Brain-derived Neurotrophic Factor (BDNF) are components of an important central pathway involved in the regulation of eating behavior in mammals. In previous studies we and other authors have demonstrated that BDNF and its TrkB receptor are downstream mediators of the melanocortin-4 receptor (MC4R) in rodents (Xu *et al.*, 2003; Nicholson *et al.*, 2007; Bariohay *et al.*, 2009). The clinical relevance of the TrkB/BDNF system is obvious from the fact that genetic dysfunction of either TrkB or BDNF leads to hyperphagia and obesity in humans (Yeo *et al.*, 2004).

Trk receptors are activated by neurotrophins including nerve growth factor (NGF), BDNF, neurotrophin 3 and 4 (NT3, NT4). These neurotrhins interact with their receptors with different affinities (Barbacid, 1995). While the three Trk receptor subtypes that have been identified in mammals (TrkA, TrkB and TrkC) were long thought to regulate neuronal

development it has recently become apparent that TrkA or TrkC, but not TrkB create a ligand-dependent state for neuronal survival (Chao, 2003; Nikoletopoulou *et al.*, 2010). Neurotrophins initiate their cellular responses by specific binding to Trk receptor subtypes. NGF is specific for TrkA, whereas BDNF and NT4 are specific for TrkB. NT3 primarily activates TrkC but also TrkA and TrkB receptors with less efficiency. Neurotrophins induce Trk dimerization and autophosphorylation at specific tyrosine residues in the cytoplasmic domain. This creates docking sites for adaptor proteins that activate PI3K/Akt, MEK/ERK, and PLCα signaling pathways (Huang and Reichardt, 2003). Formation of a Trk/neurotrophin complex also initiates internalization which appears to be required for some biological functions of neurotrophins (Grimes *et al.*, 1996; Zheng *et al.*, 2008).

In a previous study, we generated functionally active antibodies (Abs) against the melanocortin-4 receptor (MC4R) using a peptide-based approach for immunization (Peter *et al.*, 2007). Active immunization against the MC4R generated a mild obese phenotype in rats which increased their body weight mainly through an

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increase in fat mass. After passive immunization the Abs induced an increase in food intake in rats. Moreover we could demonstrate that active or passive immunization protected against lipopolysaccharide (LPS)-induced anorexia. From these observations we concluded that such an immunological approach made it possible to produce a phenotype and to generate pharmacological tools for the further investigations of the physiological and pathophysiological role of a target. If positive, such studies may result in the identification of novel treatments (Hofbauer *et al.*, 2008).

MATERIALS AND METHODS

Peptide: A peptide corresponding to the first loop of the D5 domain of the TrkB receptor (Banfield *et al.*, 2001) (B1 peptide) (TITFLESPTSDHHWCIPFTV, residues 288-307) and a control (CO) (CANISREERREVFLSVPGL) peptide with an unrelated sequence (Peter *et al.*, 2007) were synthesized (Neimark and Briand, 1993).

Animals: Male Sprague-Dawley rats, 6 weeks old at the beginning of the experiments (initial body weight approximately 200 g), were obtained from Charles River (Charles River Laboratories, L'Arbresle, France), and kept at a 12 h light/dark cycle (lights on from 6:00 a.m. to 6:00 p.m.) in a room with constant temperature (22°C) and humidity (50%).

Rats were housed individually one week before the beginning of an experiment and were given free access to tap water and standard laboratory chow (NAFAG 3432, 3.0 kcal g^{-1}, 61.6% of total calories from carbohydrate, 24.8% from protein and 13.6% from fat, Nafag Ecosan, Gossau, Switzerland). All experiments were performed in accordance with the Swiss regulations for animal experimentation.

Active immunization in rats: Rats were divided into 3 groups: a sham treated control group (sham, n = 10), a group immunized with the B1 peptide (n = 12) and a group immunized with the CO peptide (n = 10). Peptides were applied subcutaneously (s.c.) behind the neck (25 μg peptide/rat in 0.2 mL complete Freund's adjuvant for the first injection). Thirty and 60 days later rats received booster injections of peptides in incomplete Freund's adjuvant. In order to estimate the efficacy of the immunization procedure, blood was collected before each injection by tail bleeding under isoflurane anaesthesia, and the presence of anti-peptide Abs was assessed by ELISA test. Body weight and food intake were recorded once per week at 9:00 a.m.. At the end of the experiments rats were killed by decapitation under isoflurane anesthesia and blood was collected in EDTA treated tubes.

Production of monoclonal antibodies: Balb/c mice were immunized with 25 μg of the free B1 peptide emulsified in complete Freund's adjuvant and injected subcutaneously (s.c.). Four weeks later a booster injection of 25 μg in incomplete Freund's adjuvant was given. Another four weeks later 10 μg peptide dissolved in NaCl 0.9% was injected i.v. three days before harvesting the spleen cells for fusion. Fusion was performed with polyethylene glycol 1500 (Sigma, Saint Louis, MO, USA) at a ratio of 2 splenocytes for 1 SP2O myeloma cell. Hybridomas were cultivated in 96 well plates precoated with peritoneal macrophages of C57BL/6 mice 1000 cells/well. Total 5×10^5 cells were distributed per well in Isocoves Modified Dulbecco's Medium (IMDM) supplemented with 10% heat inactivated fetal calf serum, 200 mM glutamine, 100 mM sodium pyruvate, 1% penicillin streptomycin (Omnilab, Mettmenstetten, Switzerland), 3% hypoxanthine, aminopterine, thymidine (HAT, Gibco, Lucerne, Switzerland) in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Secreting clones were screened by enzyme immunoassay and subcloned by limiting dilution (Oi *et al.*, 1980). The isotype of the selected mAbs was determined using MonoAbiD kit according to manufacturer's instructions (Zymed Lab, Paris, France).

Determination of the immune response by direct ELISA: B1 and CO peptides (2 μM) or recombinant TrkB receptor (0.01 μM) were adsorbed with carbonate buffer (Na$_2$CO$_3$: 15 mM, NaHCO$_3$: 35 mM, pH: 9.6), on 96-wells microtiter plates (BD Biosciences, San Jose, CA, USA), 50 μL/well, by incubating for 1 h at 37°C. Plates were saturated with Phosphate Buffered Saline (PBS) (Na$_2$HPO$_4$: 10 mM, NaCl 150 mM, KCl: 27 mM, pH 7.4) supplemented with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) and 0.05% Tween 20 (Fluka, Buchs, Switzerland) (PBS-T BSA) for 1 h at room temperature (RT). Serial dilutions of rat sera or hybridoma culture supernatants were added to the plates and incubated for 1h at RT. Plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with goat anti-rat immunoglobulin G (IgG) horseradish peroxidase conjugated or goat anti-mouse IgG horseradish peroxidase conjugated (Jackson Immunoresearch Laboratories, San Diego, CA, USA), diluted 1/5000, for 1 h at RT. After washing the plates with PBS-T and PBS, enzymatic reactions were carried out at RT by adding 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of 0.04% H_2O_2. Reactions were stopped after 15 min by the addition of HCl (1 N). Optical density was measured at 450 nm by using a microplate reader Multiscan RC (Labsystem, Kilsyth, Victoria, Australia).

Inhibition ELISA: Purified anti-TrkB Abs were pre-incubated with increasing concentrations (2×10^{-12} to 2×10^{-6} M) of the B1 peptide or the recombinant TrkB in solution. The mixture was submitted to the standard ELISA procedure described above.

Purification of Abs: Anti-TrkB Abs/mAb or anti-CO Abs from rat sera were affinity-purified on B1 or CO peptide respectively coupled by their N-terminus end to activated CNBr-Sepharose 4B column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Sera diluted 2 times in PBS were loaded on the column at 4°C. The Abs were eluted with 0.2 M glycine pH 2.7, collected in tubes containing 1 M Tris buffer pH 8.0, subsequently dialyzed against PBS overnight at 4°C and finally stored at -20°C.

Fab preparation: Fab fragments were obtained by papain (Fluka) digestion of the affinity purified anti-TrkB Abs in a 1/100 ratio papain/Abs. After 8 h at 37°C the reaction was stopped by iodoacetamide 30mM (Sigma). Fabs were dialysed against PBS and purified by affinity chromatography on CNBr-Sepharose 4B (GE Healthcare) coupled to B1 peptide. Purity of the Fabs was assessed by silver stained SDS-PAGE gel.

Cell culture: Embryonic stem cells were differentiated and cultured as previously described (Bibel *et al.*, 2004). PC12 cells expressing rat TrkB (rTrkB) (Sommerfeld *et al.*, 2000) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 10% horse serum, 5% fetal calf serum (Bioconcept, Allschwil, Switzerland) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) in a humidified atmosphere containing 7% CO_2 at 37°C.

Immunocytofluorescence: PC12 cells expressing rat TrkB were fixed for 15 min with 2% paraformaldehyde in PBS. Slides were saturated with PBS supplemented with 1% BSA. Anti-TrkB or anti-CO peptide Abs (7 μg mL^{-1}) were applied on cells for 1 h at RT. After 2 washes with PBS, goat anti-rat Alexa fluor 594-conjugated (1/500, Molecular Probes, Junction City, OR, USA) was allowed to react with the fixed primary Ab for 1 h at RT. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (1 μg mL^{-1}, Molecular Probes) was used for nuclear staining. The same magnification (400x) and exposure time (500 msec) were used for each slide.

Immunoprecipitation: PC12 cells expressing the rTrkB receptor were lysed in cold RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40 0.5% sodium deoxycholate, 0.1% SDS) supplemented with anti-protease cocktail (Roche, Mannheim, Germany) for 1 h on ice. After a centrifugation of 30 min at 10 000 g protein concentration was determined by using BCA kit (Pierce Chemical, Rockford, IL, USA). 100 μg of TrkB membrane preparations were incubated with 25 μg purified anti-TrkB Abs or 2.5 μg of commercial anti-TrkB Abs (sc-11, Santa Cruz, Heidelberg, Germany) overnight at 4°C. The mixture was immunoprecipitated with 30 μL of protein G. The samples were loaded on a 10% polyacrylamide gel and transferred onto nitrocellulose membranes. The presence of TrkB receptor was detected using anti-TrkB mAb 1/500 (BD Biosciences).

Cloning of cDNA encoding the variable domain of the mAbs: Total RNA was prepared from 10^7 freshly subcloned hybridoma cells using the RNAnow kit (Biogentex Inc., Seabrook, TX, USA) and first strand cDNA was synthesized using iScript™cDNA Synthesis kit (Biorad, Hercules, CA, USA). The V_H and V_L chain domains were amplified by PCR using IgG primer set (Novagen, Gibbstown, NJ, USA). The 50 μL PCR mixtures contained 50ng hybridoma cDNA, 20 pmol of each appropriate primer, 250 μM of each dNTP, $1 \times Taq$ buffer (Sigma) and 1U *Thermophilus aquaticus* (*Taq*) polymerase. Amplification included 50 cycles of 1.5 min at 94°C, 2.5 min at 55°C and 3 min at 72°C in a thermocycler (PTC-150, MJ Research, Waltham, MA, USA). The amplified DNAs were ligated into the pGEMT vector (Promega, Madison, WI, USA) and the recombinant plasmids purified using miniprep kit (Qiagen, Hombrechtikon, Switzerland). The DNA sequences of the cloned V_H and V_L inserts were determined using the ABI PRISM Cycle Sequencing kit (Applied Biosystem™, Carlsbad, CA, USA) and M13 Forward and Reverse primers. The sequences of the V genes were determined in two independent batches of RNA preparations to ensure accuracy.

Construction of the scFv genes: scFv proteins were created by joining their V_H and V_L genes together by PCR splicing with overlap extensions using oligo-nucleotides that encoded a 15 amino-acid linker $(G_4-S)_3$ between the C-terminal of the V_H and the N-terminal of the V_L gene. The ends of the D2 mAb variable gene were modified by PCR using as primers, D2V$_H$Rev (5' ATG GCC ATG GAT GAA GTG AAG CTG GTG GAG TCT G3') which encodes the N-terminal wild type sequence of the V_H containing a *NcoI* site and V$_H$For (ACC ACC GGA TCC GCC TCC GCC TGA GGA GAC GGT GAC TGA 3') which encodes the C-terminus of the VH and a part of the linker. D2V$_L$Rev (5'GGAGGCGGATCCG GTGGTGGCGGATCTGGAGGTGGCGGAAG CATTTCCCAGGCTGTTGT GACTCA3') and V$_L$For, containing a *XhoI* site which encodes 6 His residues (5' GCA ATT CCT CGA GTT AGT GAT GGT GAT GGT GAT GTA GGA CAG TCA GTT TGG TTC C 3') were used to amplify and modify the V_L domain. The scFv gene was inserted in frame with the PelB sequence on the expression vector pET22b (EMD Biosciences, Darmstadt, Germany) between the *NcoI* and *XhoI* sites.

Expression of scFv: *Escherichia Coli* Rosetta bacterias transformed with pET22b(+)-D2 were grown in 500 mL of medium 2xYT (bactotryptone 1.6%, bactoyeast extract 1%, NaCl 0.5%, pH 7.0) containing ampicilline 0.15 mM (Applichem, Darmstadt, Germany) and chloramphenicol 0.1 mM (Gerbu Biotechnik, Gaiberg, Germany) until an OD_{600nm} of 0.6 at 37°C with agitation at 200 rpm. The

expression of scFv was induced by adding 1mM IPTG (Applichem) at RT during 4 h.

Periplasmic extraction: 500 mL of bacteria cultures were centrifuged (10 min, 10,000 g, 4°C) and the pellet was resuspended in 200ml of TES (Tris 30 mM, EDTA 1 mM, sucrose 2%, pH 8.5). After centrifugation (10 min, 10,000 g, 4°C), the pellet was resuspended in 50 mL $MgSO_4$ 5 mM. After a last centrifugation (10 min, 10,000 g, 4°C) the supernatant corresponding to the periplasmic extract (PE) was collected. This PE was dialyzed in wash buffer NiNTA (imidazole: 20 mM, Na_2HPO_4: 50 mM, NaCl: 300 mM, pH 8.0) overnight at 4°C.

Purification of scFv: The scFvs were purified from the PE on NiNTA columns according to the manufacturer's instruction (Qiagen). After elution, purified scFvs were dialysed in PBS (Na_2HPO_4 10 mM, NaCl: 150 mM, KCl: 27 mM, pH 7.4) overnight at 4°C. The scFvs were then purified by immunoadsorption as described previously in the *Purification* of Abs section. The concentrations were determined with Micro BCA Protein Assay kit (Pierce). Quality and purity of the purified scFv fractions were assessed by SDS-PAGE analysis using 12.5% acrylamide gels followed by staining with Coomassie brilliant blue (Biorad) and western blot. For western blot analysis, the proteins were transferred from the gels onto a nitrocellulose transfer membrane a mini trans-blot system (Biorad) in transfer buffer (Tris-HCl: 25 mM, glycin: 190 mM, methanol: 20%, pH: 8.3). The membranes were soaked 1h in PBS-T supplemented with 5% non fat milk powder and 0.1% Tween 20. This was followed by 1 h incubation with anti-His$_6$ Ab conjugated to HRP 1/2000 (Sigma). The Ab was diluted in the blocking solution PBS-T milk. The proteins on the membranes were revealed by the classical procedure of the ECL reagents (Amersham Bioscience).

TrkB phosphorylation assay: TrkB expressing PC12 cells (Sommerfeld *et al.*, 2000) were transferred to 6-well culture plates, pretreated with poly L-lysine (Sigma-Aldrich, St-Louis, MO, USA), 3 days before the experiments. PC12 cells or neurons were washed for 4h with Dulbecco's Modified Eagle Medium (Sigma-Aldrich) and preincubated for 30 min at 37°C with 10nM of purified anti-TrkB Abs/Fab or anti-CO Abs or 30 nM of mAb D2 or 300 nM of scFv D2 in PBS supplemented with 0.1% BSA (Sigma). Effective concentrations were determined in dose finding experiments. Cells were then treated with increasing concentrations of BDNF or NT4 (Anawa, Allschwil, Switzerland) (10^{-10} M to 10^{-8} M) for 15 min at 37°C. Alternatively, cells were preincubated 30 min at 37°C with increasing concentration of anti-TrkB Abs ($0.3.10^{-10}$ M to 10^{-7} M) in PBS supplemented with 0.1% BSA (Sigma) and then treated with BDNF (10^{-8}M) for 15 min at 37°C. Cells were washed with PBS, collected in tubes and the pellets were lysed with cold

RIPA buffer (50 mM Tris pH8, 150 mM NaCl, 1% NP-40 0.5% sodium deoxycholate, 0.1% SDS) supplemented with anti-protease (Roche) and anti- phosphatase (1/100, Sigma P2850 and P5726) for 1h on ice. After a centrifugation of 30 min at 10000 g, 15 µg of supernatants (determined by using the BCA kit, Pierce Chemical) were loaded on 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The presence of phospho-TrkB was detected using anti-phospho TrkB Abs 1/100 (Cell Signalling, Billerica, MA, USA) and the signal was normalized using anti-TrkB mAb 1/500 (BD Biosciences). Western blot films were digitalized and resulting images were quantified using scion Image Beta 4.03.02 (downloaded from www.markosweb.com/www/scioncorp.com). Results are expressed as the percentage of maximum of the ratio between phospho-and total Trk protein. The maximum is defined the signal obtained in cells treated only with BDNF or NT4 at 10^{-8}M in 3 independent experiments.

TrkB internalization assay: TrkB internalization assay was performed as described by Du *et al.* (2003). Briefly, PC12 cells expressing rTrkB receptor were pretreated with or without 10 nM of anti-TrkB Abs/Fabs during 30 min at 4°C. Then cells were stimulated with BDNF or NT4 (10^{-8} M) for 15 min at 4°C. Cells were washed three times with cold PBS. The cell surface proteins were labeled with 0.5 mg mL^{-1} NHS-SS-biotin (Pierce Chemical Co.) in PBS containing 10 mM Ca^{2+} and 10 mM Mg^{2+} for 3 min at 37°C, and then washed extensively with ice-cold PBS. Internalization was initiated by switching to warm media supplemeted with HEPES 1mM (37°C) for 30 min. The remaining, biotinylated surface proteins were debiotinylated by washing with glutathione buffer (50 mM reduced glutathione, 100 mM NaCl, 1 mg mL^{-1} BSA, 1 mg mL^{-1} glucose, and 50 mM Tris, pH 8.6) for 3 times 30 min at 4°C. The cells were washed two more times with PBS and harvested with the lysis buffer (see TrkB phosphorylation assay). The internalized, biotinylated proteins (100 µg) were precipitated by immobilized streptavidin, separated by SDS-PAGE, and subjected to Western blot using a monoclonal anti-TrkB Ab (1:500; Cell Signaling). Western blot films were digitalized and resulting images were quantified using scion Image Beta 4.03.02 (downloaded from www.markosweb.com/www/scioncorp.com).

Glucose and insulin tolerance test: The day before glucose or insulin tolerance tests rats were submitted to 50% food restriction. The following day rats received glucose via intraperitoneal (i.p.) injection at 10 mg kg^{-1} at 9:30 a.m. Blood was collected by tail bleeding at t = 0, 15, 30, 60 and 120 min after i.p. injection. Plasma glucose (RTU kit, Biomérieux, Marcy l'Etoile, France) and plasma insulin (rat insulin RIA kit, Linco, St. Charles, MO, USA) were measured. The same experiment was performed for the insulin tolerance test with an ip

injection (0.45 U mL^{-1} in 2 mL kg$^-$) with plasma glucose measurements.

Intracerebroventricular (icv) cannulation: Male Sprague-Dawley rats (275 to 325 g) were anaesthetized with isoflurane in medicinal oxygen (4% for induction and 2% for maintenance of anaesthesia). A stainless steel cannula (26 gauge, 10 mm long) was implanted into the third cerebral ventricle using the following coordinates, relative to the Bregma: -2.3 mm anteroposterior, 0 mm lateral to the midline, and -7.5 mm ventral to the surface of the skull. The guide cannula was secured in place with 4 stainless steel screws and dental acrylic and cement, and a stylet was inserted to seal the cannula until use.

Temgesic® (Essex Chemie AG, Lucerne, Switzerland) (0.03 mg kg^{-1}) was given subcutaneously for 2 days post-surgery. Seven days after recovery from surgery, accuracy of the cannula placement in the third ventricle was tested by measuring the dipsogenic response (immediate drinking of at least 5 mL water in 15 min) to an icv injection of 20 pmol of angiotensin II in 5 µL injection volume.

Icv injections: Purified anti-TrkB Abs or anti-CO Abs were slowly (1 min) injected icv at 9:00 a.m. at a dose of 1 µg in a volume of 2 µL using a Hamilton syringe. Following the injection of Abs, food intake was continuously recorded during the following 3 days using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany) at one hour intervals.

Injection of lipopolysaccharide: Lipopolysaccharide (LPS) (Sigma) was injected intraperitoneally (i.p.) at a dose of 75 µg kg^{-1} at 5:30 p.m., i.e., 30 min before the beginning of the dark phase. Food intake was measured 1, 2, 4 and 24 h post injection.

Data analysis: All data are expressed as Mean±SD or SEM as indicated. Data were analysed by two-way ANOVA repeated measures with Bonferroni *post-hoc* test or by Student t-test using Graph pad Prism 4 software.

RESULTS
Immune response: All rats immunized against B1 or CO peptides developed IgG Abs against the respective antigens. No cross-reactions between antigenic peptides were detected using direct ELISA procedures.

Phenotype after active immunization: Rats which developed anti-TrkB Abs gradually and significantly increased their body weight more than control rats (505±44 g vs. 476±27 g, p<0.05, Student's t-test at the end of the experiments day 80 post-immunization). Neither glucose tolerance nor insulin tolerance test revealed significant changes in glucose metabolism of immunized rats. At day 80 post immunization, rats

received an i.p. injection of LPS. Food intake of rats immunized against the B1 peptide was 36% and significantly higher (p<0.05, two-way ANOVA with repeated measures, Fig. 1) than in control rats. No differences in metabolic parameters or in organ weights were found at the end of the experiments (Table 1).

Selectivity of Abs: The selectivity of the Abs was studied by immunocytofluorescence, immunoprecipitation and inhibition ELISA. As shown in Fig. 2a, anti-TrkB Abs labelled PC12 cells expressing the rTrkB whereas anti-CO Abs did not. Immunoprecipitation using membrane preparations from PC12-cells demonstrated an interaction between anti-TrkB Abs and TrkB (Fig. 2b). Inhibition ELISA confirmed this interaction and showed that anti-TrkB Abs have apparently 1000x higher affinity for the B1 peptide than for the TrkB receptor (Fig. 2c).

Pharmacological properties of the anti-TrkB Abs vs. BDNF: The pharmacological activity of the anti-TrkB Abs was studied in PC12 cells expressing rTrkB and in neurons prepared from mouse ES cells. In experiments, cells were treated with increasing concentrations of BDNF in the presence or absence of anti-TrkB Abs. The phosphorylation of TrkB which reflects its activation was studied by Western blot experiments (Fig. 3a). In the presence of 10 nM of anti-TrkB Abs, the maximum effect of BDNF on TrkB phosphorylation was significantly decreased in both cell types (p<0.001, F-test; Fig. 3b). The presence of anti-CO Abs did not influence the maximum effect. In a second set of experiments, cells were treated with increasing concentrations of anti-TrkB Abs in the presence or absence of BDNF. TrkB phosphorylation significantly decreased in a concentration dependent manner (Fig. 3c). Internalization of TrkB was studied in PC12 cell expressing rTrkB. Treatment with BDNF increased the internalization of TrkB (Fig. 3d). The presence of anti-TrkB Abs induced a 2-fold increase in the internalization of TrkB under basal conditions and in the presence of BDNF (Fig. 3d).

Pharmacological properties of the anti-TrkB Fab vs BDNF: Fab fragments prepared from purified anti-TrkB Abs had the same effect on PC12 cells. In the presence of

Table 1: Plasma parameters of immunized rats against TrkB, immunized rats against control peptide (CO) and Sham rats

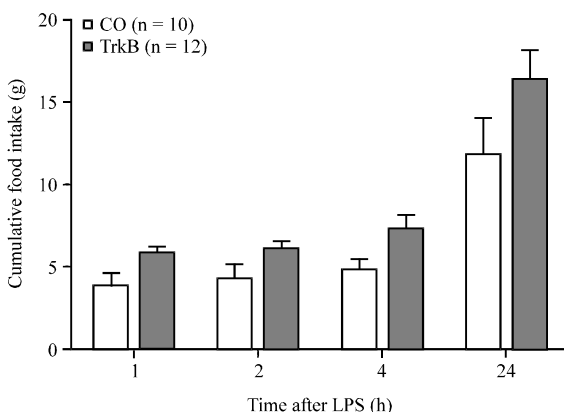| Parameters	TrkB (n = 12)	CO (n = 10)	Sham (n = 10)	Significance
Glucose (mM)	7.1±1.9	6.7±1.7	7.3±1.4	ns
Triglycerides (mM)	0.6±0.3	0.7±0.3	0.6±0.1	ns
FFA (mM)	0.3±0.1	0.3±0.1	0.3±0.3	ns
Insulin (ng mL^{-1})	0.6±0.7	0.8±0.8	0.6±0.3	ns
Leptin (ng mL^{-1})	2.6±1.4	2.6±1.6	2.0±0.6	ns
IL-6 (pg mL^{-1})	95.7±49.5	114.6±82.7	72.7±137.5	ns
TNFα (pg mL^{-1})	19.0±34.4	19.7±14.2	13.3±16.6	ns

Values are Mean±SD, ns: not significant Student's t-test

Fig. 1: Cumulative food intake of rats immunized with the B1 peptide and of control rats after an ip injection of LPS (75 µg kg^{-1}). Rats immunized with B1 peptide showed a significant (*: $p < 0.05$, two-way ANOVA with repeated measures) protection from LPS-induced anorexia as compared with control rats

10 nM of anti-TrkB Fab, the maximum effect of BDNF on TrkB phosphorylation was significantly decreased ($p < 0.01$, F-test; Fig. 4a). Fab fragments induced a 2.5-fold increase in the internalization of TrkB under basal conditions and internalization in the presence of BDNF (Fig. 4b).

Pharmacological properties of the anti-TrkB Abs/Fab vs NT4: In these experiments PC12 cells were treated with increasing concentrations of NT4 in the presence or absence of anti-TrkB Abs. The presence of anti-TrkB Abs did not influence phosphorylation of TrkB induced by NT4 (Fig. 5a). Moreover, neither anti-TrkB Abs nor anti-TrkB Fab influenced the internalization of TrkB induced by NT4 (Fig. 5b).

Effects after passive immunization: Purified anti-TrkB Abs or anti-CO Abs (1 µg) were injected into the third ventricle of rats. Rats which received anti-TrkB Abs showed a significant increase in food intake over 72 h as compared with control rats ($p < 0.05$, two-way ANOVA
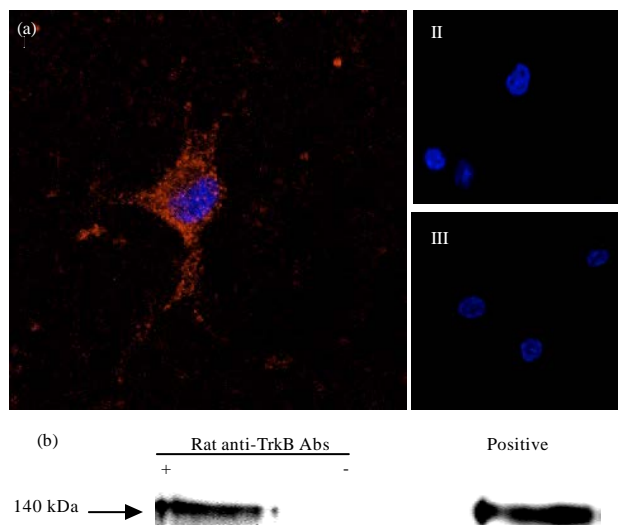

Fig. 2(a-c): Immuno-chemical characterization of anti-TrkB Abs. Purified anti-TrkB Abs bound the native form of the TrkB receptor at the surface of PC12 cells as assessed by immunocytofluorescence in immunoprecipitation experiments, (a) Immunofluorescence experiments on PC12 cells treated with purified anti-TrkB Abs (I) or purified anti-CO Abs (II) or no primary Abs (III). The presence of rat Abs at the surface of the cells was revealed by anti-rat Abs coupled to alexa 288. Only cells treated by the anti-TrkB Abs showed red labeling at their surface indicating that anti-TrkB Abs generated by immunization against B1 peptide can interact with TrkB receptors at the surface of cells, (b) 100 µg of TrkB membrane preparations were incubated with 25 µg purified anti-TrkB Abs or 2.5 µg of commercially available anti-TrkB Abs as positive control. The mixture was immunoprecipitated with protein G. The samples were loaded on a 10% polyacrylamide gel and transferred onto nitrocellulose membranes. The presence of TrkB receptor was detected using commercially available anti-TrkB Abs. The anti-TrkB Abs generated by immunization against the B1 peptide immunoprecipitated the TrkB receptor indicating that these Abs interacted with the native form of the TrkB receptor
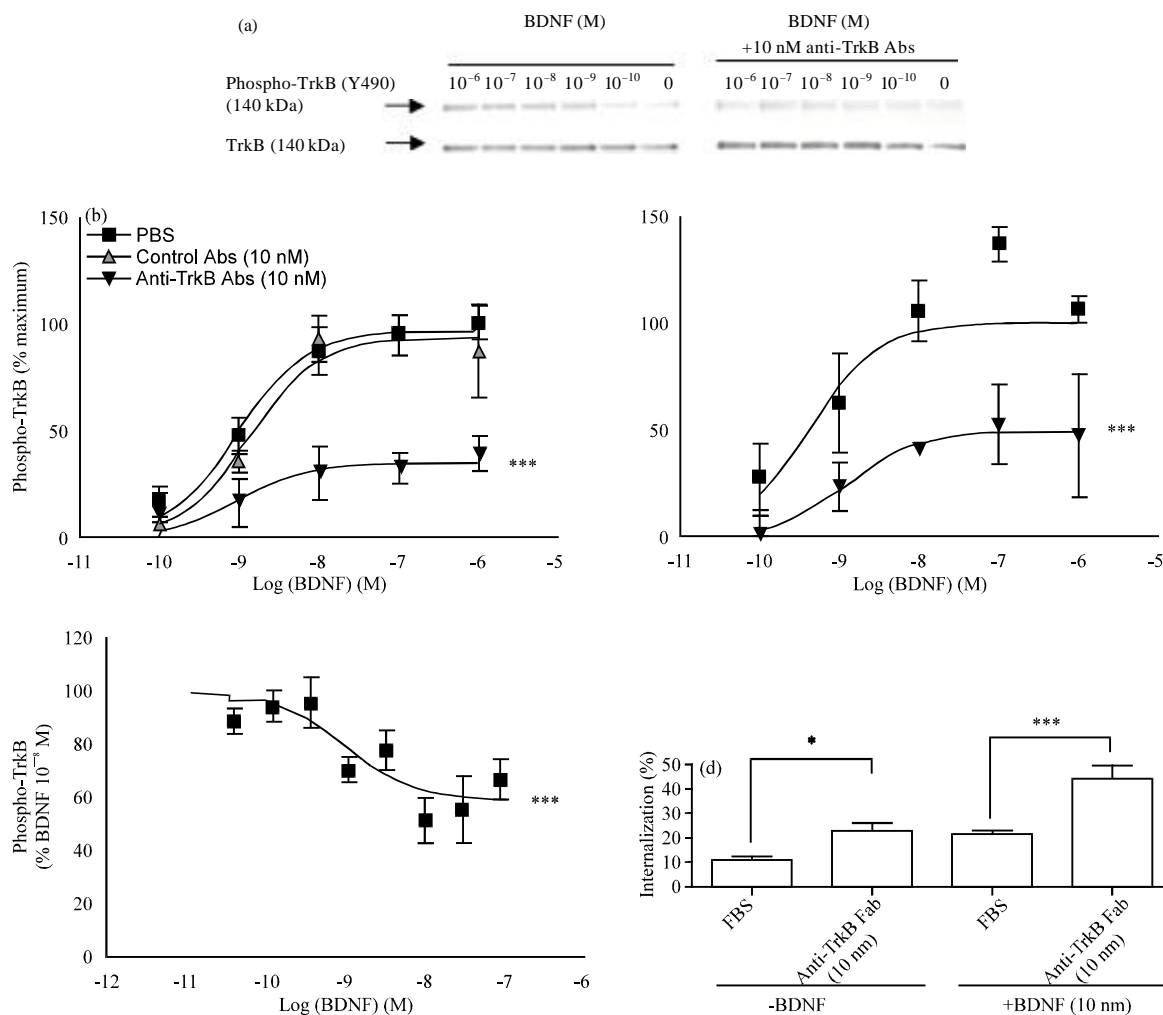
Fig. 3: Pharmacological characterization of anti-TrkB Abs upon BDNF stimulation, (a) Western blot of PC12-TrkB cell extracts treated with increasing concentrations of BDNF. The signal was revealed with anti-phospho-TrkB Abs and normalized for total anti-TrkB content, (b) Concentration-response curves obtained with purified anti-TrkB (n = 4) and control Abs (n = 4) on PC12 cells (left panel) or neurons prepared form ES cells (right panel). The presence of 10nM of rat anti-TrkB Abs decreased maximum efficacy while control Abs had no effect. Results are presented as Mean±SD; ***: $p < 0.001$, F-test. (c) Concentration-response curves obtained with purified anti-TrkB Abs in the presence of a fixed concentration of BDNF (10-8M) on PC12 cells. The presence of increasing concentrations of anti-TrkB Abs decreased the phosphorylation of TrkB in a dose-dependent manner. Results are presented as mean±SD; ***: $p < 0.001$, F-test. d. Internalization of TrkB studied by Western blot of PC12-TrkB cell extracts treated with or without BDNF in the presence or absence of anti-TrkB Abs (n=10). The presence of anti-TrkB A

with repeated measures, Fig. 6a). The effect was moderate and non-significant over the first 24 h (+15%) but became stronger (+28%) and significant ($p < 0.01$, two-way ANOVA with repeated measures) over the second 24 h period after injection (Fig. 6b, c). During the third 24 h the effect appeared to be still present (+19%) but was of borderline significance ($p < 0.08$) (Fig. 6d). No

side effects such as neurological disturbances were observed in these rats.

Selection of mAb D2: The mouse serum used for hybridoma production showed a pronounced response against the immunogenic peptide B1. Although the polyclonal response was high, only 5 clones were viable
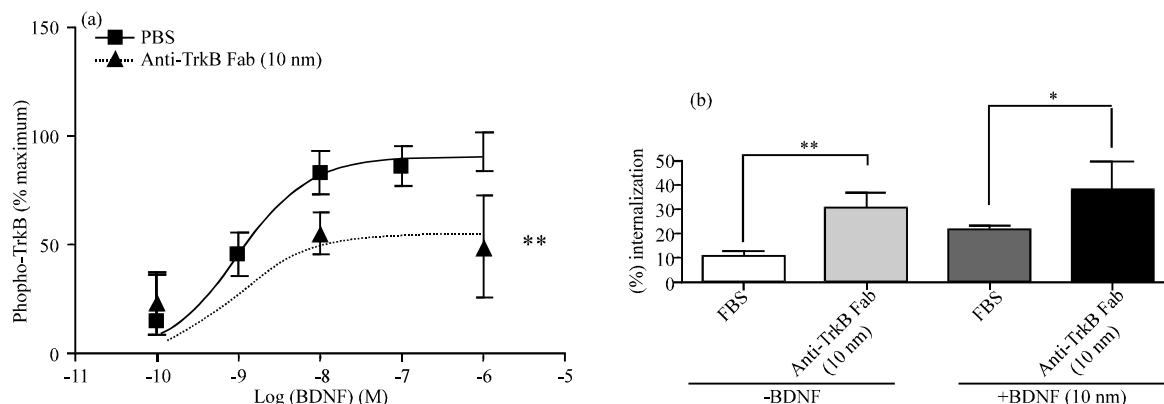
Fig. 4: Pharmacological characterization of anti-TrkB Fab upon BDNF stimulation, (a) Concentration-response curves obtained with purified anti-TrkB Abs and anti-TrkB Fabs in PC12 cells. The presence of 10nM of rat anti-TrkB Abs or Fabs decreased maximum efficacy. Results are expressed as percentage of maximum phosphorylation, **: $p < 0.01$, Student's t-test, (b) Internalization of TrkB studied by western blot of PC12-TrkB cell extracts treated with or without BDNF in the presence or absence of anti-TrkB Fab. The presence of anti-TrkB Fab increased the internalization under basal conditions and after stimulation by BDNF. Results are expressed as average percentage of internalization $\pm$SD; *, $p < 0.05$; **, $p < 0.01$, one-way ANOVA.
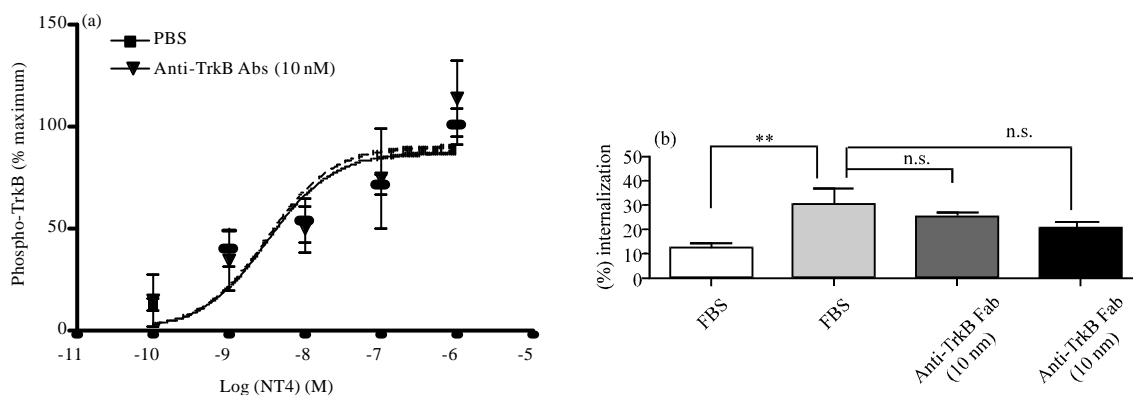

Fig. 5: Pharmacological characterization of anti-TrkB Abs upon NT4 stimulation, (a) Concentration-response curves obtained with purified anti-TrkB and control Abs on PC12 cells. The presence of 10 nM of rat anti-TrkB Abs did not influence the maximum efficacy of NT4 on TrkB phosphorylation. Results are presented as mean$\pm$SD, n.s.: non significant, F-Test. b. Internalization of TrkB studied by western blot of PC12-TrkB cell extract treated with 10 nM NT4 in presence of anti-TrkB Abs or anti-TrkB Fab. Neither the presence of anti-TrkB Abs nor of anti-TrkB Fabs influenced the internalization induced by NT4. Results are expressed as average percentage of Internalization$\pm$SD; **: $p < 0.01$, one-way ANOVA

until subcloning and amplification (3 $IgG_{,6}$ and 2 $IgG_{,6}$). Only 2 mAbs (D2 and 2C5) were able to interact with B1 peptide and TrkB receptor as assessed by ELISA. mAb D2 ($IgG_{2a,6}$) showed the strongest blockade of the TrkB activity in presence of BDNF.

Variable domain cloning and scFv expression: The scFv-encoding gene derived from the variable regions (V_H and V_L linked together via a short linker $(G_4S)_3$ of the mAb D2, with addition of a C-terminal six-His tag

encoding sequence), were inserted in frame with the PelB sequence into the pET22b expression vector. We confirmed that the cloned VL gene did not correspond to the aberrant kappa transcript of the SP20 hybridomas (Carroll *et al.*, 1998). The plasmid pET-scFv D2 was cloned into the Rosetta *Escherichia Coli* strain and the recombinant protein was expressed and exported to the bacterial periplasm by its leader sequence PelB (Lei *et al.*, 1987). The scFv D2 was purified and concentrated by IMAC chromatography.
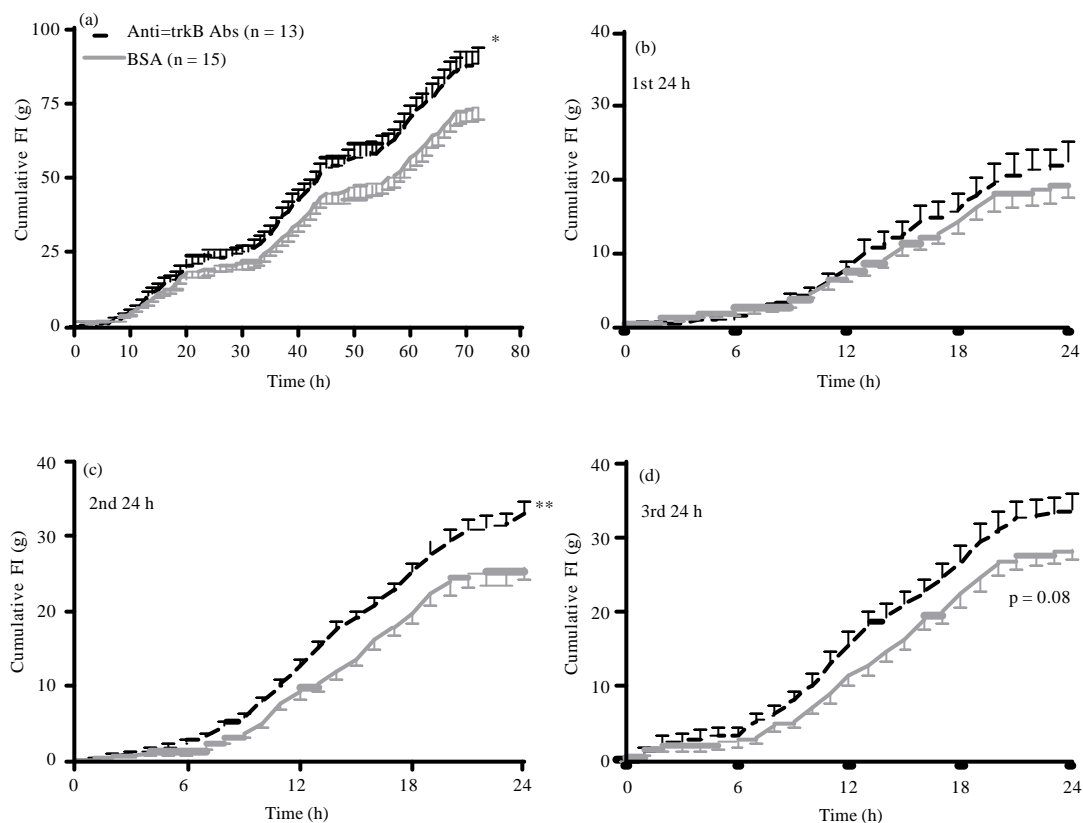
Fig. 6: Cumulative food intake of rats which received anti-TrkB Abs i.c.v., (a) 72-hour cumulative food intake (FI) in rats which received 1μg of anti-TrkB Abs (n = 13) or control Abs (n = 13) intracerebroventriculary (icv) into the third ventricle. The injection of anti-TrkB Abs increased FI compared to control Abs, (b) First 24 h period after icv application: a non-significant increase in food intake was observed in rats which received anti-TrkB Abs as compared with controls, (c) Second 24h period after icv application: rats which received anti-TrkB Abs had a significantly increased FI compared to the control group. d. Third 24h period after icv application: the increased food intake of rats which received anti-TrkB Abs as compared with controls is of borderline significance. Data presented as Mean±SEM. *: $p < 0.05$, **: $p < 0.01$, two-way ANOVA test

Pharmacological properties of an anti-TrkB mAb and its scFv derivative: The pharmacological activity of the anti-TrkB mAb was studied in PC12 cells expressing rTrkB. In these experiments, cells were treated with increasing concentrations of BDNF in the presence or absence of anti-TrkB mAb (mAb D2) or anti-TrkB scFv (scFv D2). In the presence of 30 nM of mAb D2 or 300nM of scFv D2, the maximum effect of BDNF on TrkB phosphorylation was significantly decreased ($p < 0.01$, F-test; Fig. 7a, b). Internalization of TrkB was studied in PC12 cell expressing rTrkB. Treatment with BDNF increased the internalization of TrkB (Fig. 7b). The presence of scFv D2 induced a 1.5-fold increase in the internalization of TrkB in the presence of BDNF (Fig. 7c).

Effects after passive immunization: Purified scFv D2 and BSA as control (1 μg) were injected into the third ventricle of rats. Rats which received scFv D2 showed a significant increase in food intake and a significant increase in body weight over 5 days as compared with control rats ($p < 0.05$, two-way ANOVA with repeated measures, Fig. 8a, b). No side effects such as neurological disturbances were observed in these rats.

DISCUSSION

In previous experiments we have shown that an immunological approach has several advantages for the characterization of receptors. Active immunization may produce a phenotype which could represent a useful
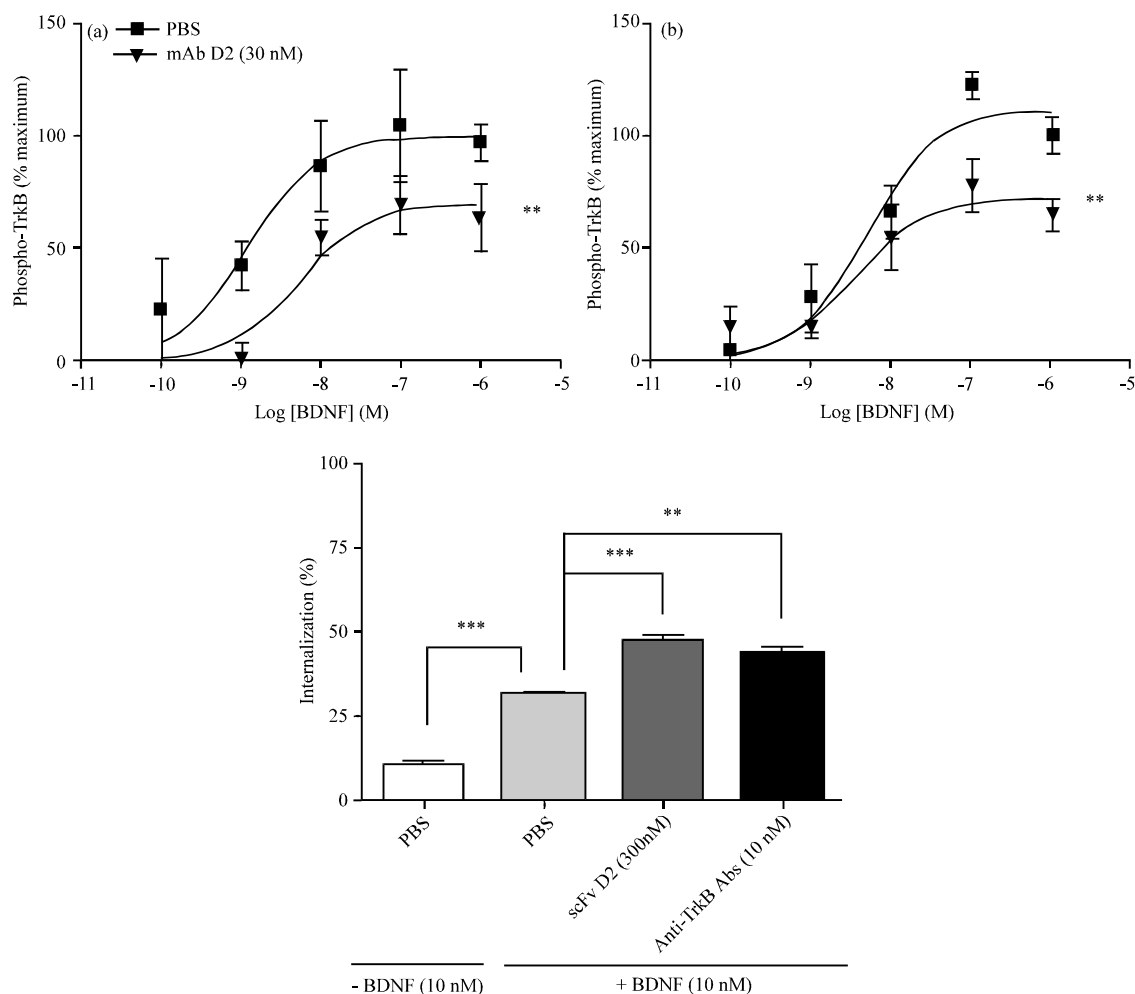
Fig. 7: Pharmacological characterization of anti-TrkB mAb D2 and scFv D2 upon BDNF stimulation. Concentration-response curves obtained with purified anti-TrkB mAb D2 (a) and anti-TrkB scFv D2 in PC12 cells (b), The presence of 30nM of rat anti-TrkB mAb D2 or 300 nM of scFv D2 decreased maximum efficacy. Results are expressed as percentage of maximum phosphorylation, **: $p < 0.01$, F-test. c. Internalization of TrkB studied by western blot of PC12-TrkB cell extracts treated with or without BDNF in the presence or absence of scFv D2 or anti-TrkB Abs. The presence of scFv D2 increased the internalization induced by BDNF. Results are expressed as average percentage of Internalization $\pm$ SD; **, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA

disease model. Polyclonal and monoclonal Abs can be used as pharmacological tools for the studies on receptor function *in vitro* and further investigations on its physiological and pathophysiological role *in vivo*. Finally mAbs and their derivatives generated in such experiments may provide a starting point for the development of novel therapeutic agents (Friedel *et al.*, 2005).

The aim of the present study was to apply such an immunological approach to the TrkB receptor which is part of the MC4R downstream pathway (Nicholson *et al.*, 2007). The selection of a suitable target peptide as an antigen was the prerequisite for a successful immunization. From the crystal structure of the D5

domain of TrkB receptor we selected a loop corresponding to the putative binding site of NT4 and BDNF (Banfield *et al.*, 2001). This loop is conserved between human rat and mouse TrkB isoforms. We hypothesized that Abs induced against this extracellular sequence may block the access of the neurotrophins and therefore prevent TrkB receptor activation. The peptide was injected without coupling to a carrier protein. This procedure had resulted in functionally active Abs against G-protein coupled receptors in previous experiments (Eftekhari *et al.*, 2001; Peter *et al.*, 2005, 2007, 2010).

Rats which developed anti-TrkB Abs showed a small but significant increase in body weight as compared to
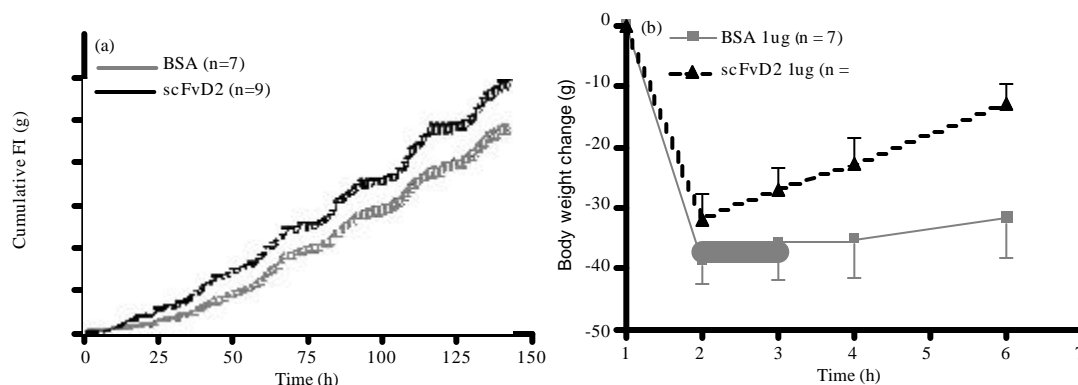
Fig. 8: Cumulative food intake of rats which received anti-TrkB scFv i.c.v., (a) 7-day cumulative food intake (FI) in rats which received 1 µg of scFv D2 (n=9) or BSA (n=7) icv into the third ventricle. The injection of scFv D2 increased FI compared to controls. b. Body weight change during 7 days after icv administration of scFv D2 or BSA as control. scFv D2 induced an increase in body weight as compared with rats which received BSA. Data presented as Mean±SEM. *: p<0.05, two-way ANOVA, Bonferroni post-hoc test

controls. This observation is consistent with several publications describing increased body weight in subjects with genetic TrkB (Xu *et al.*, 2003; Yeo *et al.*, 2004) or BDNF defects (Gray *et al.*, 2006). However, the presence of anti-TrkB Abs did not induce any disturbance of glucose metabolism. Neither glucose nor insulin tolerance tests showed any abnormalities and organ weights were unchanged. For an effect on a target in the central nervous system the penetration of the Abs into the brain, i.e. their passage across the blood-brain barrier, is a prerequisite. Although in the present studies we have not demonstrated the presence of Abs in the brain it is likely that they reached their central site of action. It has been reported that Abs can be demonstrated in the brain after peripheral administration (Banks *et al.*, 2002). In active immunization experiments Abs are circulating over periods of several weeks. It may be assumed that under these conditions a more favourable equilibrium between brain and plasma concentrations is achieved. Once Abs have gained access to the brain they appear to remain there for a prolonged period of time (Banks *et al.*, 2002).

When LPS was injected in rats that had been actively immunized against TrkB, the usual decrease in food intake was partially prevented. These results show that anti- TrkB Abs confer protection against LPS-induced anorexia. Taken into account that BDNF is released after MC4R stimulation, these observations extend our and other investigators' reports who have demonstrated that MC4R blockade has a protective effect in the LPS model (Marks *et al.*, 2001; Deboer and Marks, 2006; Hofbauer *et al.*, 2008) and suggest that TrkB plays a significant role in mediating the response to MC4R blockade.

When purified rat anti-TrkB Abs were administered intrcerebroventricularly (icv) into the third ventricle food intake increased by approximately 20% within 72 h. This is comparable to the increase in food intake induced by polyclonal anti-MC4R Abs observed in previous experiments (Peter *et al.*, 2007). After icv application of scFv D2 a comparable increase in food intake and body weight was seen. Lin *et al.*, (2008) described opposite effects on appetite after peripheral or central TrkB activation in non-human primates (Lin *et al.*, 2008). However, in our experiment performed in rats, the presence of circulating blocking anti-TrkB Abs or central administration of TrkB blockers (Abs or scFvD2) lead to the same effect i.e., a positive energy balance. In humans, a mutation of TrKB which is responsible for a decreased of the receptor lead to severe obesity and marked hyperphagia indicating that the system of energy balance regulation might be different in primates as compared with human (Yeo *et al.*, 2004).

Anti-TrkB Abs purified by immunoadsorption were used in a series of *in vitro* experiments in PC12 cells expressing the rat TrkB. Immunoprecipitation or inhibition ELISA demonstrated the specific binding of these Abs to TrkB. Their apparent affinity seemed to be lower for TrkB than for the peptide that had been used as an antigen. This is probably due to a lower accessibility of the sequence in the native receptor as compared with the free peptide. In PC12 cells expressing the rat TrkB receptor anti- TrkB Abs decreased the maximum efficacy of BDNF on TrkB phosphorylation. This effect was confirmed in neurons prepared from mouse ES cells and may be explained by allosteric modulation of the TrkB receptor. Such an effect has been described for leucocyte common antigen-related receptor which modulated

TrkB activity via interaction with the intracellular shc domain (Yang *et al.*, 2005, 2006).

Our internalization experiments showed that the presence of anti-TrkB Abs doubled the internalization rate in the absence and in the presence of BDNF. The decreased maximum phosphorylation efficacy of BDNF after anti-TrkB Abs may therefore be due to the Ab-triggered internalization of the receptor which limited its exposure to BDNF. By contrast, the internalization of TrkB was described to be initiated by binding of the neurotrophins which activates the receptor (Ehlers *et al.*, 1995; Grimes *et al.*, 1997). Indeed, complete mAbs generated against the TrkB receptor by immunization with the recombinant rat TrkB extracellular domain (Qian *et al.*, 2006) showed agonist properties. The bivalence of these Abs might have induced TrkB receptor dimerization and thereby activation. The obligatory requirement of bivalent ligands for receptor activation has been shown previously (Clary *et al.*, 1994). However, we observed that monovalent Fab fragments had the same blocking properties as the intact Abs and increased the internalization of TrkB to the same extent. Moreover, mAbD2 which targets the same peptide sequence as the polyclonal antibodies showed identical blockade of the TrkB receptor. Its monovalent derivative, scFvD2, also blocked the maximum efficacy of BDNF on TrkB phosphorylation and showed a comparable degree of internalization. We excluded a partial agonist effect by studying the effect of increasing concentration of anti-TrkB Abs in presence of fixed concentration of BDNF $(10^{-8}M)$ on TrkB phosphorylation.

The presence of anti-TrkB Abs induced a concentration dependent decrease of TrkB phophorylation (Fig. 2). These experiments have to be done in presence of BDNF in order to be above the detection threshold of phospho-TrkB receptor. Taken together, our observations indicate that the phenomenon of internalization is not necessarily coupled with activation of the receptor subsequent to dimerization.

After administration of NT4 our anti-TrkB Abs or Fabs neither influenced TrkB phosphorylation nor its internalization rate. These observations suggest that the residues 288-307 (1) are specifically responsible for the BDNF-induced internalization of TrkB and (2) are responsible for BDNF but not NT4 activation of the intracellular signaling cascade. This confirms a previous observation by Minichiello *et al.* (1998) who hypothesized that NT4 and BDNF may form different TrkB dimers that show distinct signaling properties. Furthermore it was demonstrated that the neurotrophin receptor p75[NTR] influenced the NT4-induced TrkB phosphorylation significantly more than that induced by BDNF (Bibel *et al.*, 1999).

In the present experiments, we applied an immunological approach to the characterization of the TrkB receptor. Rats were immunized against a sequence of an extracellular loop which is responsible for the specificity of the interaction of the TrkB receptor with its endogenous ligands BDNF and NT4 (Banfield *et al.*, 2001). Rats immunized with such a peptide developed specific Abs which were purified and evaluated in a series of *in vitro* experiments using PC12 cells expressing the rat TrkB receptor or neurons prepared from mouse Embryonic Stem (ES) cells. A monoclonal antibody (mAb) and its scFv derivative were produced against the same peptide antigen and their pharmacological profile was evaluated *in vitro* and *in vivo*. In contrast to other reports (Tsao *et al.*, 2008; Xu *et al.*, 2010), the Abs, mAb and scFv showed antagonist properties.

Here we report for the first time that immunization against the TrkB receptor in rats resulted in partial protection against LPS-induced anorexia. *In vitro* the anti-TrkB Abs showed non-competitive antagonism of TrkB activity and increased the TrkB internalization triggered by BDNF but not that induced by NT4. *In vivo* anti-TrkB Abs and scFvD2 increased food intake after central injection. Taken together, these observations are consistent with a role of the TrkB/BDNF system as a downstream mechanism of the MC4R (Nicholson *et al.*, 2007). Moreover, several studies underlined the possible contribution of BDNF/TrkB receptor in eating disorders (Monteleone *et al.*, 2004, 2006; Ribases *et al.*, 2004, 2005a, b; Friedel *et al.*, 2005; Monteleone and Maj, 2008). Blockade of the TrkB receptor may therefore be regarded as a new concept for the treatment of anorexia and cachexia. The mAbD2 and its scFvD2 derivative could provide a starting point for the development of specific and efficacious therapeutic agents.

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