Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 522 (2005) 30–37
www.elsevier.com/locate/ejphar

Central administration of small interfering RNAs in rats: A comparison with antisense oligonucleotides

Claudia Senn [a,1], Christoph Hangartner [b,1], Suzette Moes [a], Danilo Guerini [b], Karl G. Hofbauer [a,*]

[a] *Applied Pharmacology, Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH-4056 Basel, Switzerland*
[b] *Transplantation and Immunology, Novartis Pharma AG, CH-4002 Basel, Switzerland*

Received 11 April 2005; received in revised form 4 August 2005; accepted 8 August 2005
Available online 6 October 2005

Abstract

To date there are only few reports of the use of small interfering RNA (siRNA) in whole animals and most of these are restricted to systemic application of siRNAs targeting the liver. In our present studies we have investigated whether siRNAs can be used against a central target after intracerebroventricular (i.c.v.) application and compared their effects with those of antisense oligonucleotides. For this purpose we designed different siRNA and antisense oligonucleotide molecules against the rat hypothalamic melanocortin MC_4 receptor and selected the siRNA and antisense oligonucleotide with the highest efficacy in vitro. We observed that siRNA, encompassing the same gene sequence as antisense oligonucleotides, induced a stronger inhibition of melanocortin MC_4 receptor expression than antisense oligonucleotides. When fluorescence-labeled siRNA were applied i.c.v. in rats no label was detected in brain tissue in spite of the use of cell detergents to improve the delivery. In contrast to these findings the i.c.v. administered fluorescence-labeled antisense oligonucleotides reached the brain structures expressing melanocortin MC_4 receptor and were taken up by the cells in these areas. In summary it seems as if 'naked' antisense oligonucleotides have an advantage over 'naked' siRNA for experiments in vivo. The development of optimized vector systems seems to be a prerequisite before siRNA can be regarded as a suitable experimental tool for in vivo studies.

Keywords: Antisense oligonucleotide; Food intake; Melanocortin; Small interfering RNA; (Rat)

1. Introduction

Small interfering RNA (siRNA) has become a powerful tool for the investigation of gene function in cell culture in vitro (for review see Scherr et al., 2003). If siRNA showed similar effects in vivo it would be possible to generate "knock-down" rats, a model comparable to knock-out mice. The siRNA approach would have several advantages vis-à-vis related methods, e.g. the antisense oligonucleotide technology, including high efficacy and specificity (Ichim et al., 2004). Furthermore, since duplex siRNA is quite stable no chemical modifications are required to achieve a sufficient half-life in cell-culture media (Paroo and Corey, 2004).

To date there are only few reports of the use of siRNA in whole animals and most of these are restricted to systemic application of siRNAs targeting the liver (Paroo and Corey, 2004). In our present studies we have investigated whether siRNAs can be used against a central target after intracerebroventricular (i.c.v.) application. We used siRNA against the melanocortin MC_4 receptor, which is an important component in the regulation of energy balance (Fisher et al., 1999). A practical advantage of the melanocortin MC_4 receptor is that changes in food intake provide a simple biological read-out of its activity. The melanocortin MC_4 receptors are located in the hypothalamic arcuate and paraventricular nucleus, which are close to the third ventricle (Williams et al., 2000). They can therefore be easily accessed by i.c.v. administration via a chronically or acutely implanted cannula. In order to assess the potential of siRNA after central administration in vivo we compared their effects with those of antisense oligonucleotides against the melanocortin MC_4 receptor.

* Corresponding author. Tel.: +41 61 267 16 45; fax: +41 61 267 22 08.
E-mail address: karl.hofbauer@unibas.ch (K.G. Hofbauer).
[1] These authors contributed equally to this work.

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.08.021

2. Materials and methods

2.1. In vitro methods

2.1.1. Design of siRNAs

Three different double-stranded siRNAs (siRNA 1, siRNA 2, siRNA 3) targeting the melanocortin MC_4 receptor were synthesized by Qiagen-Xeragon (Basel, Switzerland). The targeting sequences were choosen according to Elbashir et al. (2001) using a 3′-end TT deoxynucleotide overhang.

siRNA1:Sense:5′-CAUUCUAGUGAUCGUGGCGdTdT-3′,
Antisense: 3′-dTdTGUAAGAUCACUAGCACCGC-5′.
siRNA 2: Sense: 5′-CGGGUCAGAAACCAUCGUCdTd-3′,
Antisense: 3′-dTdTGCCCAGUCUUUGGUAGCAG-5′.
siRNA 3: Sense: 5′-UCCAUACUGCGUGUGCUUCdTd-3′,
Antisense: 3′-dTdTAGGUAUGACGCACACGAAG-5′.

A negative control was obtained from the pre-designed siRNA database of Qiagen. siRNA 2 was also ordered from Qiagen with a 5′-fluorescein label for RNA diffusion analysis. For in vitro experiments high-performance purity grade siRNA, for in vivo experiments HPLC-purified siRNA was used. The siRNA was stored in 100 mM potassium acetate, 30 mM HEPES-KOH and 2 mM magnesium acetate, pH 7.4.

2.1.2. Design of antisense oligonucleotides

Two different single-stranded phosphothioate protected antisense oligonucleotides (ASO 1, ASO 2) targeting the melanocortin MC_4 receptor and their respective negative (non-silencing) control sequences (mismatch ASO 1, mismatch ASO 2) were synthesized by Novartis (Basel, Switzerland):

ASO 1: 5′-tggttTsCsTsGsAsCsCsCsgttcg-3′
ASO 2: 5′-tccgtGsTsCsCsGsTsAsCstgttt-3′
Mismatch ASO 1: 5′-tgggtGsCsTsTsAsCsCsCstt tcg-3′
Mismatch ASO 2: 5′-tccttGsGsCsCsTsTsAsCsggttt-3′.

For antisense oligonucleotides diffusion analysis a non-melanocortin MC_4 receptor specific fluorescence-labeled antisense oligonucleotide was used:

NCH 6549.1: 5′-Fs-ccttaCsCsTsGs CsTsAs Gsct ggc-3′.

The small letters indicate the 2′-methoxyethyl modified nucleotides, s indicates the phosphothioate linkage and F the fluorescein-5-isothiocyanate labeling.

2.1.3. Cell culture

HEK293 cells and their derivatives were maintained in monolayer cultures in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 50 μg/ml gentamicin. HEK293/$rMC_4/G_{\alpha 16}$, which upon addition of MC_4 agonists showed a robust intracellular Ca^{2+} release (Hangartner and Guerini, unpublished, supplement 1) were maintained in the same medium containing 500 μg/ml geneticin (G418), 250 μg/ml hygromycin B. Cells were cultured in a humidified incubator at 37 °C, in an atmosphere of 5% CO_2.

2.1.4. Transfection

HEK293 cells and derived cell clones were treated with the indicated concentration of antisense oligonucleotides per plate using liposomal transfection reagent Effectene (Qiagen, Basel, Switzerland) or DOTAP (Roche, Basel, Switzerland) according to the manufacturer's protocol. The cells were harvested 24 h after transfection for analysis of mRNA expression and 48 h after transfection for Fluorometric Imaging Plate Reader (FLIPR) assay. HEK293/$rMC_4/G_{\alpha 16}$ cells were transfected with 100 nM or 200 nM siRNA (Qiagen, Basel, Switzerland) and lipofectamine 2000 (Gibco, BRL) according to the manufacturer's protocol. The cells were harvested 24 h after transfection for analysis of mRNA expression and 48 h after transfection for FLIPR assay.

2.1.5. Fluorometric imaging plate reader (FLIPR) assay

Eighteen to twenty-four hours after transfection, HEK293/rMC_4 cells were seeded onto 384-well plates at a density of 12,500 cells per well in a volume of 25 μl and cultured for 18–24 further hours until used in the functional FLIPR assay. On the day of the experiment, one vial of FLIPR calcium assay reagent for FlexStation kit (R-8041, Molecular Devices, Bucher Biotec AG, Basel, Switzerland) dye was re-suspended in 10 ml of a FLIPR buffer consisting of Hank's balanced salt solution (HBSS), 20 mM HEPES, and 2.5 mM probenecid (pH 7.4). Cells were loaded with the calcium-sensitive dye by addition of an equal volume (25 μl) to each well of the 384-well plate and incubated for 1 h at 37 °C. The compound plate and cell plate were placed in the $FLIPR^2$ instrument (Molecular Devices). At the beginning of an experimental run, a signal test was performed to check the basal fluorescence signal from the dye-loaded cells and the uniformity of the signal across the plate. An aliquot (12.5 μl) of a 250 μM ATP solution was added to the existing 50 μl dye-loaded cells to prime the system and to check the response. The FLIPR was programmed to record baseline fluorescence for a duration of 20 s as 2-s intervals, followed by addition of 12.5 μl of 6× concentrated agonist solution (dilution from 10^{-7} to 10^{-10} M in 0.1% fatty acid free bovine serum albumin/FLIPR buffer). Fluorescence data were collected in real-time at 1 s intervals for the first 60 s and at 2 s intervals for additional 80 s. Peak fluorescence counts during the 20- to 40-s time points were used to determine agonist activity. FLIPR responses were measured as peak fluorescence intensity (F_{max}) minus basal fluorescence (F_{min}) (i.e. before addition of agonist). The data was exported from the FLIPR software into EXCEL spreadsheets for calculations.

2.1.6. RT-PCR

Tissue or cells were harvested for quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). The quantity was assessed by the RiboGreen RNA Quantitation Kit (Molecular Probes, Lucerne, Switzerland). One microgram from each RNA sample was reverse-transcribed to first

strand cDNA with the Omniscript Reverse Transcriptase kit (Qiagen) according to the manufacturer's protocol. In a total volume of 25 μl for a single reaction we used the TaqMan Universal PCR Master Mix (Applied Biosystems) and the melanocortin MC_4 receptor forward primer (5′-tgctggtgagcgtttcga-3′) in a final concentration of 300 nM, the melanocortin MC_4 receptor reverse primer (5′-cgtccgtgtccgtactgtttag-3′) in a final concentration of 900 nM and the TaqMan probe (5′-cgggtcagaaaccatcgtcatcacc-3′) in a final concentration of 150 nM. Real time PCR was performed under standard conditions in the ABI PRISM 7900HT. The rat melanocortin MC_4 receptor mRNA quantities were analysed in triplicate, normalised against ribosomal 18S RNA as a control and expressed in relation to a calibrator sample. As described by Livak and Schmittgen (2001), results are expressed as relative gene expression ±range ($2^{-\Delta\Delta Ct \pm S.D.}$) using the ΔΔCt method.

2.2. In vivo methods

2.2.1. Animals

Male Sprague–Dawley rats, delivered from Charles River (France) were used at a weight of 200 g. The rats were housed in individual plastic cages in a room with controlled temperature (21–22 °C) and a light cycle from 0600 to 1800 hours. The rats were acclimatized to the animal facilities for 7 days. Food (Nafag 3432, Provimi Kliba AG, Kaiseraugst, Switzerland) and tap water were given ad libitum.

2.2.2. I.c.v. surgery

Rats were anesthetized with 3%–4% isoflurane (Abbott, Baar, Switzerland) and placed in a stereotaxic apparatus (David Kopf Instrument, Tujunga, USA or TSE, Bad Homburg, Germany). A sterile 22-gauge, tubing length below pedestal 6mm, stainless steel single guide cannula (Plastics one Inc., Roanoke, USA) for acute studies or single connector-guide cannula for chronic studies was implanted for i.c.v. injection into the right lateral ventricle with the coordinates of 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 4.5 mm ventral to the surface of the skull. The cannula was fixed using Paladur dental cement (Heraeus Kulzer, Wehrheim, Germany). The position of the cannulas was verified one week after the surgery by applying 20 pmol/5 μl angiotensin II (Sigma, Buchs, Switzerland). Animals which drunk less than 5 ml within 15 min of injection were excluded from the experiment.

2.2.3. I.c.v. administration of fluorescence labeled-siRNA 2 for diffusion analysis

Fluorescence-labeled siRNA 2 was dissolved in siRNA suspension buffer (Qiagen) or in two specific detergents, namely the cell detergent DOTAP (Roche) or the detergent i-Fect (Neuromics Antibodies, Minnesota, USA) at the ratio of 1:4 (w:v). Fifty μg siRNA 2 dissolved in suspension buffer without detergent and 2 μg of siRNA2 dissolved in i-Fect or DOTAP were applied in a total volume of 5 μl. Three hours after injection brains were removed and postfixed overnight at 4 °C in 4% paraformaldehyde/PBS, dehydrated overnight in 20% sucrose and frozen in dry ice. Coronal sections were cut at 20 μm intervals and examined for fluorescence.

2.2.4. I.c.v. administration of siRNA 2 for food intake measurements

siRNA 2 and siRNA control were administered by two acute i.c.v. injections in rats at two consecutive days 1 h before onset of dark phase in a single dose of 25 μg/5 μl or 100 μg/5 μl in siRNA suspension buffer (Qiagen). Twenty-four hour food intake was measured on the two days of application and the following two days. At the end of the measurements animals were killed by CO_2, hypothalami were dissected and total RNA was prepared as described in the RT-PCR section for mRNA measurements.

2.2.5. I.c.v. administration of fluorescence-labeled antisense oligonucleotides for diffusion analysis

Fluorescence-labeled antisense oligonucleotides (NCH 6549.1) was injected i.c.v. in a dose of 25 μg/5 μl and 50 μg/5 μl sterile PBS. Three hours after administration, animals were killed by CO_2. Brains were removed and postfixed overnight at 4 °C in 4% paraformaldehyde/PBS, dehydrated overnight in 20% sucrose and frozen in dry ice. Coronal sections were cut at 20 μm intervals and examined for fluorescence. The pictures were analyzed with a quantification software to determine the percentage of labeled cells (GeneTool, SynGene, Biolabo Instruments SA, Châtel St. Denis, Switzerland). Cells were stained with Hoechst 33258 (nuclear staining; Molecular Probes, JURO Supply GmbH, Lucerne, Switzerland) to determine cell number.

2.2.6. I.c.v. administration of ASO 1 for food intake measurements

ASO 1 and mismatch ASO 1 were administered either by i.c.v. injections (2×25 μg/rat on day 0 and 2×50 μg/rat on day 1) or by continuous i.c.v. infusion (6.4 μg/h/rat) via osmotic minipumps (model 2001, Alzet, Charles River, France) over 7 days. Twenty-four hour food intake was measured daily from day 0 until day 4 in the acute experiment and during the 7 days in the chronic experiment. At the end of the measurements animals were killed by CO_2, hypothalami were dissected and total RNA was prepared as described in the RT-PCR section for mRNA measurements.

2.2.7. Statistics

Differences between food intake values were analysed by one-way Analysis of Variance (ANOVA) for each hour of measurement. Data were analysed for statistical significance using the SigmaStat software package (Chicago, USA). All data are expressed as means ± S.D.

3. Results

3.1. siRNA

3.1.1. Selection of active siRNA

Three independent siRNAs (siRNA 1–3) encompassing different regions of the rat melanocortin MC_4 receptor coding sequence were tested in a concentration of 100 nM for their ability to reduce melanocortin MC_4 receptor expression in the HEK293/rMC_4 cell line as measured by RT-PCR. Twenty-four hours after transfection siRNA 2 caused the strongest reduction of melanocortin MC_4 receptor mRNA levels to less than 15% of those seen in untreated controls. siRNA 1 exhibited moderate silencing ability, whereas siRNA 3 resulted in no decrease of melanocortin MC_4 receptor mRNA (Fig. 1A). Increasing the concentrations to 200 nM did not result in additional effect for any of the siRNAs (Fig. 1A). The functional siRNA

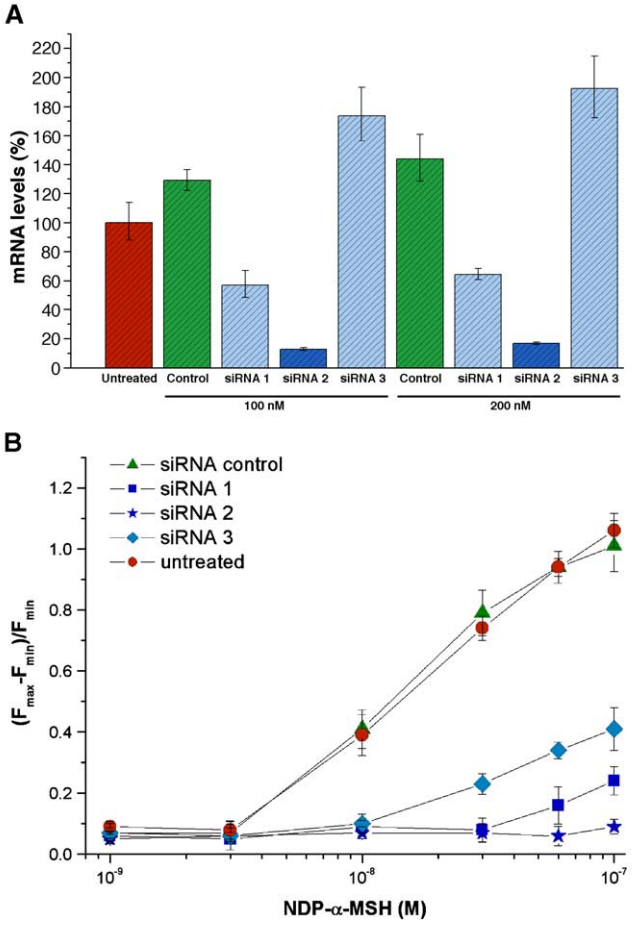

Fig. 1. (A) Down-regulation of rat melanocortin MC_4 receptor mRNA in MC_4 receptor expressing HEK293 cells after transfection with siRNAs measured by RT-PCR. mRNA levels are plotted in % of untreated HEK293/rMC_4/$G_{\alpha 16}$ cells. Melanocortin MC_4 receptor mRNA levels in wild type HEK293 cells were below the level of detection. Mean±Range of two independent transfections measured in triplicate. (B) Effect of siRNAs on the activity of rat melanocortin MC_4 receptor expressing HEK293 cells analyzed with FLIPR. Ca^{2+} release measured by fluorescence changes $((F_{max}-F_{min})/F_{min})$ is shown. Mean values±S.D. of two independent experiments measured in triplicate.

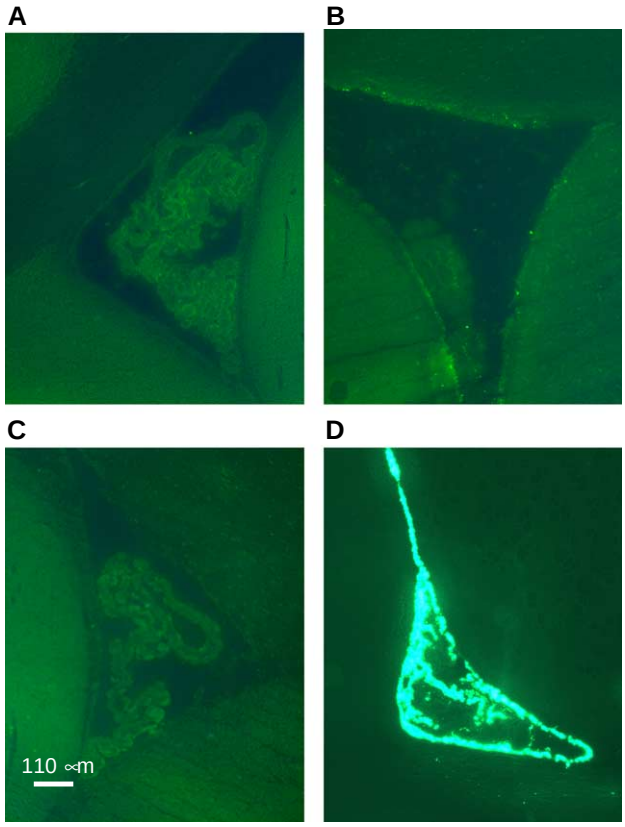

Fig. 2. The area around the lateral ventricle of the rat brain 3 h after i.c.v. injection. Fluorescence-labeled siRNA 2 were injected. A: 50 μg siRNA dissolved in siRNA suspension buffer without detergent, B: 2 μg siRNA: DOTAP (1:4 w/v), C: i-Fect only, D: 2 μg siRNA: i-Fect (1:4 w/v).

effects were tested by analyzing [Nle(4)-D-Phe(7)]-α-melanocyte-stimulating hormone (NDP-α-MSH) mediated intracellular Ca^{2+} release in HEK293 cells co-expressing rat melanocortin MC_4 receptor and $G_{\alpha 16}$ with the FLIPR. The strongest effect was observed with siRNA 2, which completely abolished agonist induced Ca^{2+} mobilization. siRNA 1 and siRNA 3 reduced the signal in FLIPR to 30% and 40% of control, respectively. siRNA control didn't show any reduction of intracellular Ca^{2+} release. The viability of the cells and their general capacity to release Ca^{2+} were not affected by the siRNA treatment since the addition of 10 μM ATP to treated and untreated cells elicited the same signal, suggesting that the siRNA had a specific effect on the melanocortin MC_4 receptor mediated signaling (data not shown). There was no difference between 100 nM (Fig. 1B) and 200 nM of siRNA for the transfection (data not shown).

3.1.2. Fluorescence-labeled siRNA 2 distribution and uptake in the brain

Fluorescence-labeled siRNA 2 was injected into the right lateral ventricle in a dose of 50 μg dissolved in suspension buffer or in a dose of 2 μg dissolved in the cell detergent i-Fect or DOTAP. The immunofluorescent microscopy images were analysed 30 min, 3 h, 12 h and 24 h after the i.c.v. injection. Representative for the result are the images obtained 3 h post application shown in Fig. 2. No fluorescence-labeled siRNA

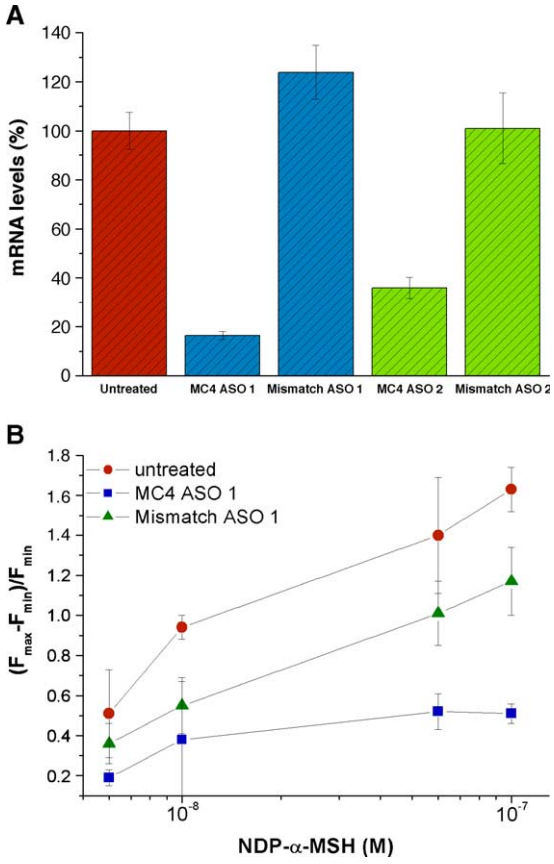

Fig. 3. (A) Down-regulation of rat melanocortin MC_4 receptor mRNA in MC_4 receptor expressing HEK293 cells after transfection with antisense oligonucleotides measured by RT-PCR. mRNA levels are plotted relative to those in untreated cells. Mean±Range of two independent transfections measured in triplicate. (B) Effect of ASO 1 on the activity of melanocortin MC_4 receptor expressing HEK293 cells analyzed with FLIPR. Ca^{2+} release measured by fluorescence changes is shown. Mean values±S.D. of two independent experiments measured in triplicate.

2 were detected in any brain region (e.g. around the third ventricle) including the area around the injection site regardless of the dose or the detergent used (Fig. 2A, B). With i-Fect detergent it was possible to detect fluorescence-labeled siRNA in the lateral ventricle 3 h post application (Fig. 2D). No fluorescence was detected after injection of the detergent i-Fect alone (Fig. 2C).

3.1.3. Effect of acute application of siRNA 2 on food intake

siRNA 2 and siRNA control were administered i.c.v. in rats on two consecutive days (25 or 100 μg/rat/day). Twenty-four hour food intake was measured over three days. Neither low (50 μg) nor high (200 μg) doses of siRNA 2 had an effect on food intake (food intake in rats which received 200 μg on day 4 was: siRNA 2 group: 28.8±6.5 g, mismatch siRNA 2 group: 30.9±5.8 g, siRNA suspension buffer group: 29.8±6.3 g, mean values±S.D., $n=5$). Melanocortin MC_4 receptor mRNA levels were not different between the siRNA treated rats and their controls (mRNA levels in % of untreated rats on day 4: siRNA 2 group: 110±5%, mismatch siRNA 2: 95±8%, siRNA suspension buffer group: 103±12%, mean values±S.D., $n=5$).

3.2. Antisense oligonucleotides

3.2.1. Selection of active antisense oligonucleotides

The HEK293/rMC_4 cell line was transfected (600 nM with DOTAP) with ASO 1 and 2 and melanocortin MC_4 receptor mRNA levels were analyzed by RT-PCR after 24 h. The mRNA level in the ASO 1 transfected cells was lowered to 17% of that found in the untreated controls (Fig. 3A). In contrast ASO 2 reduced mRNA level only to 35% of control. For functional analysis in HEK293/rMC_4 cells only the more effective ASO 1 was used. ASO 1 at 600 nM promoted a strong reduction of the NDP-α-MSH signal at different concentration of agonist (Fig. 3B), which was particularly pronounced at 10^{-7} M (up to 80% reduction compared to mock treated cells). An effect was also observed with the mismatch oligonucleotide, which indicated that after 48 h of treatment some unspecific effects might become apparent. The effect of the ASO 1 corresponds to a reduction of 60% when compared to the mismatched ASO. Experiments repeated at lower concentrations of ASO 1 confirmed the data presented in Fig. 3B (data not shown).

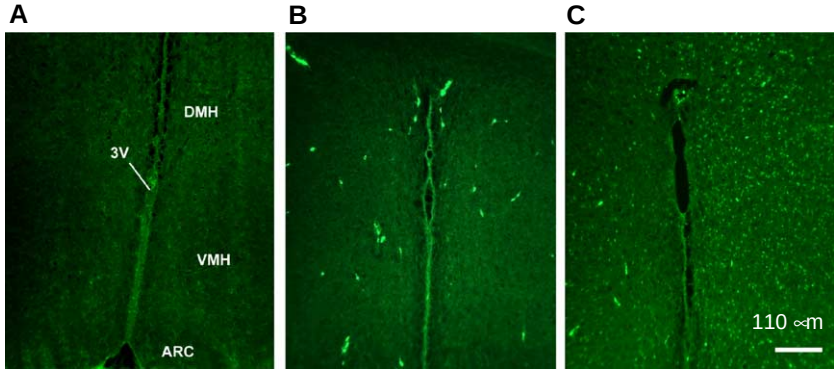

Fig. 4. Coronal sections of the third ventricle of rat brains 3 h after i.c.v. injection of fluorescence-labeled antisense oligonucleotides. A: negative control, B: 25 μg antisense oligonucleotides, C: 50 μg antisense oligonucleotides. 3V=third ventricle, DMH=dorsomedial hypothalamic nucleus, VMH=ventromedial hypothalamic nucleus, ARC=arcuate nucleus.

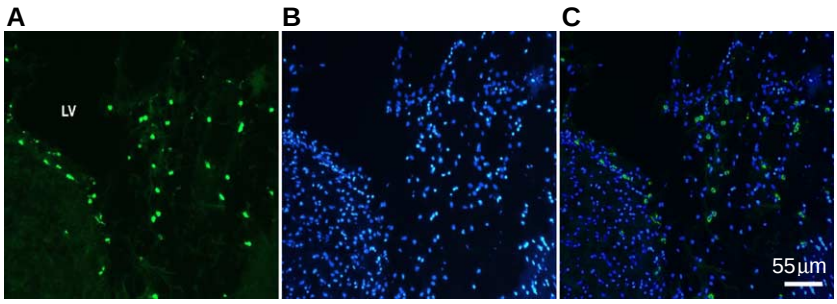

Fig. 5. Fluorescence-labeled antisense oligonucleotides uptake by neuronal cells 3 h after i.c.v. injection (25 μg). The epithelial lining of the lateral ventricle and cells of the choroid plexus are visible. Fluorescence microscope images of the same section were taken either with the filter for FITC staining (A) or for Hoechst nuclear staining (B). Picture C represents an overlay of pictures A and B for co-localization analysis. Coronal sections are shown of the lateral ventricle of the rat brain. LV=lateral ventricle.

Intracellular Ca^{2+} mobilization by ATP was not affected by the treatment (data not shown).

3.2.2. Fluorescence-labeled antisense oligonucleotides distribution and uptake in the brain

To study the distribution and the uptake of antisense oligonucleotides in the rat brain fluorescence-labeled antisense oligonucleotides were injected into the right lateral ventricle. Immunofluorescent microscopy images indicated that within 3 h of injection the distribution of the fluorescence had progressed to the thalamus and hypothalamus (Fig. 4). The fluorescence-labeled antisense oligonucleotide stained cells were detectable in those nuclei of the hypothalamus where melanocortin MC_4 receptor is expressed at high levels. I.c.v. injection of 50 μg fluorescence-labeled antisense oligonucleotides increased the amount of labeled cells in the proximity of the third ventricle compared to 25 μg. Increasing the amount of fluorescence-labeled antisense oligonucleotides injected to 100 μg had no additional effect (data not shown). No fluorescence-labeled antisense oligonucleotides were detected in control brains injected with vehicle. The co-localization of fluorescence-labeled antisense oligonucleotides with stained nuclei (Fig. 5) suggested cytoplasmic and nuclear uptake of fluorescence-labeled antisense oligonucleotides. The strongest labeling by was visible within the ependymal cell lining of the lateral ventricle and the cells of the choroid plexus. These observations indicate that 2′-methoxyethyl-modified antisense oligonucleotides rapidly penetrated into the brain parenchyma and was taken up into the cytosol and nuclei of neurons. Approximately 10% of all cells had taken up fluorescence-labeled antisense oligonucleotides in this experiment (Fig. 5).

3.2.3. Effect of acute and chronic application of ASO 1 on food intake

ASO 1 and mismatch ASO 1 were injected at a total dose of 150 μg within two days or infused at a dose of 6.4 μg/h/rat for seven days. During the four days of food intake measurements after the acute applications no increase of food intake was observed in the ASO 1 treated group in comparison to the mismatch ASO 1 group and the PBS group (24 h food intake on day 4: ASO 1 group: 30.8±1.4 g, mismatch ASO 1 group: 30.9±1.6 g, PBS group: 30.8±1.1 g, mean values±S.D., $n=8$). The infusion of ASO 1 had also no significant effect on food intake during the seven days of measurements (24 h food intake on day 7: ASO 1 group: 33.6±1.9 g, mismatch ASO 1 group: 32.0±2.8 g, PBS group: 35.2±1.2 g, mean values±S.D., $n=6$–7). At the end of the chronic infusion of ASO 1 hypothalami were dissected and melanocortin MC_4 receptor mRNA was measured. ASO 1 treated animals showed no decreased melanocortin MC_4 receptor mRNA levels as compared to their controls (mRNA levels in % relative to untreated animals on day 7: ASO 1 group: 79.8±10, mismatch ASO 1 group: 83.3±9%, PBS group: 80.7±13%, mean values±S.D., $n=6$–7).

4. Discussion

For our experiments we designed three different siRNA molecules against the hypothalamic rat melanocortin MC_4 receptor using the Qiagen siRNA design tool and developed an in vitro system to identify the siRNA with the highest efficacy. A marked down-regulation of melanocortin MC_4 receptor mRNA was observed with rat melanocortin MC_4 receptor siRNA 2 at a concentration of 100 nM and the functional responses (NDP-α-MSH promoted intracellular Ca^{2+} release) were reduced by more than 90%. While RNA down-regulation was seen after 24 h, functional effects were only evident after 48 h. This finding is not unexpected since down-regulation of mRNA usually precedes the effect on the corresponding protein by 12–24 h. Our results confirm that siRNA is a powerful tool for knocking-down genes in vitro. Three observations suggest that siRNA 2 was highly specific: the effect was concentration dependent; it was saturable; and no cellular toxicity was observed.

In a next step we applied fluorescence-labeled melanocortin MC_4 receptor siRNAs i.c.v. in rats to test their uptake and distribution in brain tissue in vivo. When saline was used as a vehicle, no label was detected even in brain regions adjacent to the cerebral ventricles. Since delivery systems, which form stable complexes with DNA or RNA have been shown to improve the cellular uptake and functional efficacy of siRNAs in vitro we applied two different non-toxic cationic lipid based reagents, DOTAP (Roche) and i-Fect (Neuromics Antibodies)

in further experiments in vivo. These vehicles were either used in the RNA-detergent ratio recommended by the manufacturer or with a higher proportion of RNA per unit volume. After injection with the i-Fect detergent fluorescence was detected both in the lateral ventricles and the third ventricle but at none of the time points measured (30 min, 3 h, 12 h, 24 h) was cell staining observed. Such a lack of neuronal uptake could also be an explanation for the observations by Isacson et al. (2003), who found that siRNA targeted to dopamine D1 receptors did not reduce dopamine D1 receptor messenger RNA levels or protein when applied via i.c.v. injection in rats. Furthermore it was recently shown by Thakker et al. (2004) that hypothalamus was resistant to the gene-silencing effect of siRNA in the first week of treatment with high amounts of siRNA (0.4 mg/d).

Positive results with synthetic RNAi have been obtained in vivo when high amounts of either short hairpin RNA (shRNA) or siRNA were administered via injections in the tail-vein of mice. Silencing was particularly pronounced in the liver where expression of specific genes was inhibited by approximately 80%. Although siRNAs primarily act in the cytoplasma, which should be easier to access by using nonviral methods than the nucleus, the most promising delivery method appears to be the expression of siRNAs via plasmid like shRNA or viral vectors (Lewis et al., 2002; McCaffrey et al., 2002; Xia et al., 2002). Despite such procedures it is generally difficult to achieve efficient uptake and long-term stability in relevant tissues in vivo (Dorsett and Tuschl, 2004).

For comparative studies we designed antisense oligonucleotides against the melanocortin MC_4 receptor and mismatch antisense oligonucleotides as controls. A set of 20 antisense oligonucleotides was synthesized and tested for binding to full-length melanocortin MC_4 receptor mRNA. The best sequences were further evaluated with the help of a luciferase-reporter system for their capacity to interfere with the translation of rmelanocortin MC_4 receptor cDNA in vitro (Husken et al., 2003). When the most promising candidates, which showed a good hybridization to the melanocortin MC_4 receptor mRNA, were tested in cells, the maximum down-regulation (>95%) was obtained with ASO 1 at a concentration of 600 nM. The corresponding effect at the functional level was approximately 80% inhibition compared to sham treatment and around 60% compared to the mismatch antisense oligonucleotides. Interestingly, ASO 1, the most efficacious antisense oligonucleotides, encompasses the same gene sequence as siRNA 2, the most efficacious siRNA, which suggests that this region of the melanocortin MC_4 receptor mRNA is highly accessible for DNA–RNA or RNA–RNA interaction. A comparison of our results in the functional assay shows that siRNA 2 induced a stronger inhibition than ASO 1 although it was used at a lower concentration.

I.c.v. administered fluorescence-labeled antisense oligonucleotides reached the brain structures expressing melanocortin MC_4 receptor (paraventricular nucleus, arcuate nucleus) within 3 h after application and a large amount of it was taken up by approximately 10% of the cells in these areas. Since antisense oligonucleotides were labeled with FITC at the 5′ end via a nuclease-resistant phosphorothioate linkage it can be assumed that this fluorescence was due to intact molecules and not free label. The pattern of intracellular distribution of antisense oligonucleotides was variable but in most of the cells both the cytoplasm and the nucleus were labeled. There were only few cells in which the fluorescence-labeled antisense oligonucleotides were visible in the cytoplasm. Sommer et al. (1993) demonstrated that FITC-labeled phosphorothioate antisense oligonucleotides are localized in both cytoplasm and nuclei of neurons whereas other groups found antisense oligonucleotides either mainly in the nuclei (Leonetti et al., 1991) or mainly in the cytoplasm (Caceres and Kosik, 1990). These differences might be explained by different ways of administration (microinjection into cultured cells versus i.c.v. injection), different labels (FITC and [^{32}P] versus biotin and digoxigenin label) or different backbone modifications (phosphorothioates versus phosphodiesters).

In further studies in vivo we investigated whether the extent of cellular uptake of antisense oligonucleotides was sufficient for a functional effect. In these experiments no significant changes in any of the measured parameters were detectable. Our negative results after acute and chronic i.c.v. administration of ASO 1 are at variance with observations by Obici et al. (2001) who showed that i.c.v. infusion of an antisense oligonucleotide targeting the melanocortin MC_4 receptor resulted in a consistent decrease (~50%) in hypothalamic melanocortin MC_4 receptor protein and in changes in glucose metabolism. However, when we used the same antisense oligonucleotides sequence in feeding experiment in rats we did not observe any changes in the expression of melanocortin MC_4 receptor mRNA in the hypothalamus or in food intake (unpublished observations).

In summary it seems as if ‘naked’ antisense oligonucleotides have an advantage over ‘naked’ siRNA for experiments in vivo. Even though we could not observe functional effects of antisense oligonucleotides after i.c.v. administration we demonstrated neuronal uptake with labeled antisense oligonucleotidess while labeled siRNA could not be detected in brain tissue. Chemical modifications may help to enhance the in vivo stability of siRNAs but the development of optimized vector systems seems to be a prerequisite before RNAi can be regarded as a suitable experimental tool for in vivo studies.

Acknowledgements

These studies were supported by a grant from Novartis Pharma AG, Basel, Switzerland. We thank Dr. F. Natt (Novartis Pharma AG) for generation of antisense oligonucleotides and for valuable discussion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2005.08.021.

References

Caceres, A., Kosik, K.S., 1990. Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. Nature 343, 461–463.

Dorsett, Y., Tuschl, T., 2004. siRNAs: applications in functional genomics and potential as therapeutics. Nat. Rev. Drug Discov. 3, 318–329.

Elbashir, S.M., Lendeckel, W., Tuschl, T., 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 15, 188–200.

Fisher, S.L., Yagaloff, K.A., Burn, P., 1999. Melanocortin and leptin signaling systems: central regulation of catabolic energy balance. Recept. Signal Transduct. Res. 19, 203–216.

Husken, D., Asselbergs, F., Kinzel, B., Natt, F., Weiler, J., Martin, P., Haner, R., Hall, J., 2003. mRNA fusion constructs serve in a general cell-based assay to profile oligonucleotide activity. Nucleic Acids Res. 31, e102.

Ichim, T.E., Li, M., Qian, H., Popov, I.A., Rycerz, K., Zheng, X., White, D., Zhong, R., Min, W.P., 2004. RNA interference: a potent tool for gene-specific therapeutics. Am. J. Transplant. 4, 1227–1236.

Isacson, R., Kull, B., Salmi, P., Wahlestedt, C., 2003. Lack of efficacy of 'naked' small interfering RNA applied directly to rat brain. Acta Physiol. Scand. 179, 173–177.

Leonetti, J.P., Mechti, N., Degols, G., Gagnor, C., Lebleu, B., 1991. Intracellular distribution of microinjected antisense oligonucleotides. Proc. Natl. Acad. Sci. U. S. A. 88, 2702–2706.

Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A., Herweijer, H., 2002. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat. Genet. 32, 107–108.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., Kay, M. A., 2002. RNA interference in adult mice. Nature 418, 38–39.

Obici, S., Feng, Z., Tan, J., Liu, L., Karkanias, G., Rossetti, L., 2001. Central melanocortin receptors regulate insulin action. J. Clin. Invest. 108, 1079–1085.

Paroo, Z., Corey, D.R., 2004. Challenges for RNAi in vivo. Trends Biotechnol. 22, 390–394.

Scherr, M., Morgan, M.A., Eder, M., 2003. Gene silencing mediated by small interfering RNAs in mammalian cells. Curr. Med. Chem. 10, 245–256.

Sommer, W., Bjelke, B., Ganten, D., Fuxe, K., 1993. Antisense oligonucleotide to c-*fos* induces ipsilateral rotational behaviour to d-amphetamine. Neuroreport 5, 277–280.

Thakker, D.R., Natt, F., Husken, D., Maier, R., Muller, M., van der Putten, H., Hoyer, D., Cryan, J.F., 2004. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. Proc. Natl. Acad. Sci. U. S. A. 101, 17270–17275.

Williams, G., Harrold, J.A., Cutler, D.J., 2000. The hypothalamus and the regulation of energy homeostasis: lifting the lid on a black box. Proc. Nutr. Soc. 59, 385–396.

Xia, H., Mao, Q., Paulson, H.L., Davidson, B.L., 2002. siRNA-mediated gene silencing in vitro and in vivo. Nat. Biotechnol. 20, 1006–1010.