

Pharmacological Blockade of G-Protein Coupled Receptors: Interventions to Alter Expression or Internalization

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Christoph Hangartner
aus Schaffhausen (Schweiz)

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Dissertationsleiter: Prof. Dr. Karl G. Hofbauer

Fakultätsverantwortlicher: Prof. Dr. Urs A. Meyer

Koreferent: Prof. Dr. Alex N. Eberle

Basel, den 16. November 2004

Prof. Dr. Hans-Jakob Wirz
Dekan

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CHAPTER A:

**Antisense Oligonucleotides and Small Interfering RNA as
Pharmacological Tools to Downregulate Melanocortin-4
Receptors *in Vitro* and *in Vivo***

SUMMARY

Obesity is the result of a long-standing imbalance between energy intake and energy expenditure and it has become a serious public health issue during the past decades. The control of food intake is complex, involving multiple interconnected pathways and signals. A large body of evidence supports a critical role of the melanocortin-4 receptor (MC4-R) in energy homeostasis as well as its involvement in other important physiological processes has prompted research efforts to investigate its pharmacology. Due to the lack of selective agonists or antagonists at the MC4-R our approach was to develop alternative tools for the blockade of the MC4-R *in vitro* and *in vivo*. Antisense oligonucleotides (ASO) and small interfering RNAs (siRNAs) were generated and analyzed. They down-regulated MC4-R mRNA levels by >90% *in vitro*. Due to the fact that the siRNA and the ASO encompassed the identical sequence, our results suggest that siRNA was much more effective than ASO. The siRNA down-regulated the functional activity of rMC4-R by >90% compared to 60-70% with ASO.

Distribution studies in rat brains indicate that labeled ASO after icv injection penetrated into the brain parenchyma. Around 8-10% of all neurons in the surrounding of the third ventricle had taken up the ASO into the cytosol and nuclei. However, after icv application no significant effects were detectable, neither in mRNA levels nor in food intake or bodyweight. Similar negative results were obtained by acute and chronic icv application of siRNA. In contrast to the ASO distribution study no staining in neurons with labeled siRNA were detectable. These results suggest that synthetic siRNA, when applied directly via icv injection to rat brain, is not capable of inducing RNA interference. Therefore, the further development of delivery systems is required to accomplish more efficient antisense *in vivo*.

Furthermore a polyclonal anti-ratMC4 antibody against the C-terminal portion of the receptor was generated and analyzed. The antibody proved to be useful to detect and measure in Western blots full length rMC4-R either in cells over-expressing the recombinant receptor or in rat hypothalami.

ABBREVIATIONS

AC	Adenylate cyclase
ACTH	Adrenocorticotropic hormone
AGRP	Agouti-related protein
ARC	Arcuate nucleus
ASO	Antisense oligonucleotides
BBB	Blood brain barrier
BMI	Body mass index
CCK	Cholecystokinin
CRF	Corticotropin releasing factor (= CRH)
CRH	Corticotropin releasing hormone
cAMP	Cyclic adenosin monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CNS	Central nervous system
DMH	Dorsomedial hypothalamic nucleus
DMV	Dorsomotor nucleus of the vagus
FACS	Flow Activated Cells Sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLIPR	Fluorescence image plate reader
GABA	Gamma aminobutyric acid
GI	Gastrointestinal
GLP-1	Glucagon-like peptide 1
GPCR	G-protein coupled receptor
GTP γ S,	Guanosine5 γ -3-O-(thio)triphosphate
HBSS	HEPES based salt solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(ethanesulfonic acid)
icv	Intracerebroventricular
IL	Interleukin
IPTG	Isopropyl β -D-thiogalactopyranoside

ip	Intraperitoneal
LHA	Lateral hypothalamic area
MCH	Melanin concentrating hormone
MC4-R	Melanocortin-4 receptor
MOE	2'-O-methoxyethyl
MOPS	3-(N-morpholino)propanesulfonic acid
α, β, γ -MSH	α, β, γ - Melanocyte stimulating hormone
NE	Noradrenaline
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
ORX	Orexin
PC1, 2	Prohormone convertase 1, 2
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
shRNA	Short hairpin RNA
Tris	Tris(hydroxymethyl)aminomethane
VMH	Ventromedial hypothalamic nucleus
5-HT	5-hydroxytryptamine (Serotonin)

1 INTRODUCTION

1.1 Aims and Outline of This Thesis

Obesity is the result of a long-standing imbalance between energy intake and energy expenditure and it has become a serious public health issue during the past decades. The control of food intake is complex, involving multiple interconnected pathways and signals. Numerous studies in the literature describe an important role of the melanocortin-4 receptor (MC4-R) in the regulation of food intake. Transgenic knockout mice, lacking MC4-R, show maturity-onset obesity, hyperphagia, hyperglycemia and increased linear growth (Huszar et al., 1997). Heterozygous MC4-R knockout mice show intermediate obesity, their average weight being between the homozygous MC4-R knockouts and wild-type mice, suggesting that the quantity of receptors is important for weight regulation (Huszar et al., 1997). Consequently, the MC4-R is an important drug target for the pharmaceutical industry. Generation and characterization of selective agonists and antagonists or inhibitors are a prerequisite for the evaluation of the role of MC4-R in the regulation of energy homeostasis *in vivo*. Until recently, no compounds with high affinity and selectivity for the melanocortin receptor subtypes were available (see chapter 1.10.4). To interpret pharmacological results, particularly from *in vivo* experiments, with confidence agonists or antagonists should have at least a 100-fold selectivity for the receptor of interest.

In this thesis two alternative approaches to block MC4-R signaling are described: Antisense oligonucleotides (ASO) and small interfering RNAs (siRNAs). One aim of this work was to establish ASO and siRNA against rat MC4-R as a pharmacological tools for *in vitro* and *in vivo* studies. Different *in vitro* methods were used to identify active sequences and the best ASO and siRNA were tested *in vivo*. In those experiments the distribution and uptake of labeled ASO in the brains of rats were analyzed. Furthermore, due to the inadequate performance of commercially available antibodies against rat MC4-R, a polyclonal antibody against the C-terminal portion of rat MC4 receptor was generated and analyzed.

1.2 Obesity

Obesity, defined as excess of fat mass for a given body size, results from an imbalance between energy intake and energy expenditure. In clinical practice, the grade of obesity is estimated by using a formula that combines weight and height, the so called body-mass index (BMI). The underlying assumption is that most variation in weight for persons of the same height is due to fat mass. Body mass index measured as body weight in kilograms over the square of the height in meters (kg/m^2) is a widely accepted measure of adiposity. A BMI value between 20 and 25 is considered normal, individuals with a BMI value between 25 and 30 are considered overweight and those with a BMI value over 30 obese ((WHO), 2000; (WHO), 2003). The prevalence of obesity in the United States is estimated at >30% of the population, the prevalence for overweight and obesity at >64% of the population (Flegal et al., 2002). Obesity is associated with a significantly higher probability of mortality, which can be attributed to increased risk of type II diabetes mellitus, hypertension and coronary heart disease and other diseases (Hall et al., 2002; Kopelman, 2000; Willett et al., 1999). Obesity predisposes to osteoarthritis and to respiratory problems including obstructive sleep apnoea. The associations between obesity and several cancers including breast, endometrial, prostate and colon cancer has been shown in several publications (Bianchini et al., 2002; Calle et al., 2003; Daling et al., 2001).

1.3 The Treatment of Obesity

It has been repeatedly proposed that treating obesity will reduce the risk for several obesity associated co-morbidities but obesity is often not considered as a disease. Avoidance of weight gain should become a major task in prevention of chronic disease in modern societies. Weight control can be achieved by a reduction in energy intake and by increasing the physical activity (energy expenditure). About half of the adult population of industrialized countries are insufficient active in their leisure time and more than a quarter are totally inactive (Bianchini et al., 2002).

Weight loss promoting therapies could theoretically be based on agents re-

ducing nutrient absorption, appetite suppressants, or thermogenic agents. In the past, once diet and exercise alone had failed, non-amphetamine anorectics, such as fenfluramine and dexfenfluramine had been used as appetite suppressants. These serotonergic agents were effective weight loss drugs, but they were withdrawn from the market because of cardiovascular and pulmonary side effects (Carek and Dickerson, 1999). On cessation of therapy with this drug, weight is rapidly regained (Van der Ploeg, 2000).

At the moment the most widely used anti-obesity drugs are sibutramine and orlistat. Sibutramine is a centrally acting drug with different mechanisms of action. It is a reuptake inhibitor of noradrenaline and serotonin and in addition, it may stimulate thermogenesis indirectly by activating the β_3 - system in brown adipose tissue. Side effects are increase in blood pressure, tachycardia, headache, insomnia (Poston and Foreyt, 2004; Weigle, 2003). Sibutramine is therefore contra-indicated in patients with a history of cardiovascular diseases. Orlistat is a lipase inhibitor inhibiting the digestion and consequently the absorption by the gut of dietary triglycerides (Ballinger and Peikin, 2002; Carek and Dickerson, 1999).

The maximal weight loss achievable with any dietary or pharmacological strategies appears to be around 5-10% of initial weight. Counter-regulatory mechanisms prevent further weight loss and make maintenance of the achieved weight loss difficult. There is some evidence that during evolution the appetite regulatory systems have evolved to conserve and store energy in times of plenty. There has been no evolutionary pressure to reduce intake to low levels once energy stores are full with the consequences that this system is biased strongly towards weight gain and storage of fat (Chakravarthy and Booth, 2004). The long-term regulation of adiposity involves both peripheral signals that relay information about adipose tissue mass to the central nervous system (CNS) and opposing circuits in the hypothalamus that control appetite and energy expenditure. To efficiently fight obesity, it will be necessary to intervene at key points within this regulatory network (Bray and Tartaglia, 2000; Schwartz MW et al., 2000).

1.4 Regulation of Energy Balance

The regulatory system for food intake and energy expenditure is coordinated by the central nervous system, which senses metabolic status from a wide range of humoral and neural signals. The hypothalamus is the key region in which the integration of a multiplicity of neuronal and humoral signals occur (Review: Schwartz MW et al., 2000).

During the last decade a number of different peptides and monoamines has been identified as the prominent players in food intake and energy expenditure. These agents can be classified into agents that increase food intake (orexigenic) and decrease energy expenditure when administered into the CNS and those that decrease feeding (anorexigenic) and increase energy expenditure (Table 1).

	Orexigenic	Anorexigenic
Neuropeptides,	Agouti-related peptide (AGRP)	α -melanocyte-stimulating hormone (α -MSH)
Hormones,	Neuropeptid Y (NPY)	Cocaine and amphetamine regulated transcript (CART)
Monamines	Melanin concentrating hormone (MCH)	Corticotropin-releasing hormone (CRH)
	Orexin	Serotonin (5-HT)
	Galanin	
	Noradrenaline	
Peripheral peptides	Ghrelin	Leptin
		Cholecystokinin (CCK)
		Glucagon-like peptide 1 (GLP-1)
		Bombesin
		Peptide YY (PYY)

Tab.1. Selected central and peripheral signals affecting food intake. (Schwartz MW et al., 2000)

These peptides and monamines can be divided into two broad categories: peripheral factors and central factors. The peripheral factors can be subdivided into long term regulation factors and short term regulation factors (Halford, 2001).

1.5 Peripheral Long Term Regulation Factors

The relative stability of weight in individuals indicates that energy balance may be controlled by a feedback regulation, which maintains energy stores constant. These signals are mediated by long term regulation factors which inform the brain about the state of depletion or repletion of energy reserves.

1.5.1 Leptin

Leptin, the product of the *ob* gene, is a 16 kDa protein (Reviews: Friedman and Halaas, 1998; Sahu, 2003) and is secreted by adipocytes (Zhang et al., 1994). It circulates at concentrations that are proportional to fat stores (cell number and size) (Considine et al., 1996). Leptin enters the hypothalamus across the modified blood–brain barrier of the arcuate nucleus-median eminence. In the arcuate nucleus it binds to the leptin-receptor, a member of the interleukin (IL)-6 receptor (IL6-R) family of class I cytokine receptors. Signaling through these receptors inhibits food intake and increases energy expenditure. *Ob/ob* mice, homozygous for a spontaneous mutation in the leptin gene, fail to produce functional leptin (Zhang et al., 1994). The *ob/ob* mice are hyperphagic and obese. Mutations in the leptin receptors in *db/db* mice and *fa/fa* rats result in the same phenotype. Administration of leptin to *ob/ob* and wildtype mice reduces food intake and body weight (Pellemounter et al., 1995). The leptin receptor (Ob-Rb, the long isoform) has been localized in various hypothalamic sites, which are known to regulate food intake and energy homeostasis (Mercer et al., 1996). In addition to its regulation of food intake leptin controls other important biological functions relevant for obesity. *Ob/ob* mice show some abnormalities also seen in starved animals including lower body temperature, decreased energy expenditure, decreased immune function (Farooqi et al., 2002) and infertility (Moschos et al., 2002).

1.6 Peripheral Short Term Regulation Factors

Most peripheral factors belong in this class. They are released in response to the chemical and physical presence of food in the gastrointestinal (GI) tract. The release of the gastrointestinal factors informs the brain that the stomach is full and the gut contains nutrients. These factors are short-lived and mediate a satiety signal.

1.6.1 Cholecystokinin (CCK)

Cholecystokinin (CCK) is produced by the intestinal endocrine cells in the GI tract but also in the brain. CCK is released both locally and into the plasma in response to the presence of nutrient digestion products and it was the first gut hormone shown to inhibit feeding (Review: Moran and Kinzig, 2004). Levels of CCK increase over 10-30 min after meal initiation and then gradually fall, although remaining elevated for as long as 3-5 h after eating. Peripheral CCK has a short-lived effect on feeding with a maximum inhibition 30 min after its first appearance in periphery (Moran, 2000). CCK is an important feedback signal for the control of meal size. CCK acts to coordinate the digestive process through both endocrine and paracrine/neurocrine actions by stimulating pancreatic and gallbladder secretions, inhibiting gastric emptying and modifying intestinal motility. CCK also plays a major role in satiation.

1.6.2 Glucagon-Like Peptide 1 (GLP-1)

Glucagon-like-peptide 1 (GLP-1) is secreted in the gut in response to nutrients (Review: Meier et al., 2002). Its major physiological role is a glucose-dependent stimulation of insulin secretion from pancreatic B-cells. GLP-1 reduces caloric intake and enhances satiety, most likely via specific receptors within the central nervous system, resulting in reduced weight gain in experimental animals (Turton et al., 1996).

1.6.3 Bombesin

The intestinal hormone Bombesin has an anorectic effect in rats as well as in humans (Review: Yamada et al., 2002). Bombesin administration enhances the termination of the test meal and prolonged inter-meal intervals, suggesting a strong effect on satiety (Thaw et al., 1998).

1.6.4 Ghrelin

Ghrelin is synthesized predominantly in the stomach and acts as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) or now called ghrelin receptor (Review: Inui et al., 2004). It has been shown that ghrelin in addition to stimulating growth hormone release, increases food intake when administered peripherally in humans (Wren et al., 2001a) as well as centrally in rats (Wren et al., 2001b). Ghrelin is thought to signal pre-meal hunger and stimulate meal initiation. Endogenous levels of ghrelin in man rise on fasting and fall rapidly after re-feeding with sharp surge occurring before each meal (Cummings et al., 2001). Ghrelin is transported across the blood-brain barrier. Whether the primary ligand for hypothalamic ghrelin receptor is peripheral ghrelin or ghrelin released by neurons projecting from the arcuate nucleus remains unknown. The orexigenic effect of ghrelin is mediated via an activation of AGRP/NPY neurons located in the medial portion of the arcuate nucleus (ARC)(Chen et al., 2004).

1.6.5 Peptide YY (PYY)

Peptide YY (PYY) is secreted by the endocrine L cells of the small and large bowel and it is released into the circulation after a meal (Review: Stanley et al., 2004). PYY is a member of the neuropeptide Y (NPY) family. The main circulating component of PYY is PYY₃₋₃₆. Central administration of PYY₃₋₃₆ increases food intake likely via Y₁/Y₅ receptor (Kanatani et al., 2000). In contrast, peripheral administration of PYY₃₋₃₆ have an inhibitory effect on food intake (Batterham et al., 2002) and this inhibiting PYY₃₋₃₆ effect is thought to be mediated via the Y₂ receptor on the NPY/AgRP neurones in the arcuate nucleus thereby disinhibiting POMC neurons to cause a reduction in food intake. Contradictory results concerning the effects in animals have been recently published (Tschöp et al., 2004). The significance of PYY₃₋₃₆ is still a matter of debate (Batterham et al., 2004).

1.7 Central Factors - Monoamines

Gamma aminobutyric acid (GABA) is one of the fast acting neurotransmitters that can increase or decrease food intake depending on where it is injected (Stanley et al., 1993). Other neurotransmitters that are involved in modulating feeding, include noradrenaline, serotonin, dopamine, and histamine (Review: Bray and Greenway, 1999).

1.7.1 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a short-acting widely distributed neurotransmitter (Review: Blundell and Halford, 1998). Serotonin is derived from the dietary amino acid tryptophan. It is known that agonists at the 5-HT receptors or drugs that inhibit the re-uptake of 5-HT reduce feeding. At the present time 14 different sub-types of 5-HT receptors have been identified and the most promising candidates for appetite regulation are the presynaptic 5-HT_{1A}, the postsynaptic 5-HT_{1B} and the 5-HT_{2C} receptors (Bickerdike, 2003; Blundell and Halford, 1998).

1.7.2 Noradrenaline

Noradrenaline (NE) can increase or decrease food intake depending upon the type of adrenergic receptors on which it acts in the brain. In experimental animal studies food intake was reduced by stimulation of α_1 adrenoreceptors, whereas stimulation of α_2 adrenoreceptors increased food intake (Leibowitz, 1970). Receptors can either be activated by agonist directly, by releasing noradrenaline or inhibiting its re-uptake in the vicinity of these receptors.

1.8 Central Factors – Neuropeptides

Numerous neuropeptides found in the hypothalamus affect feeding when injected centrally. Functional studies suggest that neuropeptide Y (NPY), agouti related protein (AGRP), Pro-opiomelanocortin (POMC), α -melanocyte-stimulating hormone (α -MSH), Cocaine- and amphetamine- regulated transcript (CART) and melanin-concentrating hormone (MCH) are the most prominent regulators of food intake and body weight (Review: Hillebrand et al., 2002). The melanocortin system (POMC, α -MSH, AGRP) will be dis-

cussed in more detail in a separate chapter (chapter 1.10) since this was an integral part of this work. Some other central factors like corticotropin releasing factor, galanin and orexin have secondary anorectic or orexigenic mode of action.

1.8.1 Neuropeptide Y (NPY)

NPY was first isolated from porcine brain more than 20 years ago (Tatemoto et al., 1982). It is a 36 amino acid peptide and a member of the pancreatic polypeptide family (Review: Pedrazzini et al., 2003). NPY is one of the most abundant peptides in the CNS. The highest concentration has been detected in the hypothalamus (Allen et al., 1983). NPY is a very potent feeding stimulator and it can increase food intake severalfold (Kalra et al., 1999). It is thought that NPY stimulates food intake via Y_1 or Y_5 receptors in the hypothalamus. Evidence of the involvement of the Y_1 and Y_5 receptors has been demonstrated by using specific Y_1 (Kanatani et al., 1999) and Y_5 antagonists (Criscione et al., 1998; Levens and Della-Zuana, 2003) or antisense oligonucleotides against the Y_5 receptor subtypes (Schaffhauser et al., 1997). There is still an ongoing debate about the involvement of Y_5 receptors as main mediator in the NPY action (Della-Zuana et al., 2004).

1.8.2 Cocaine- and Amphetamine- Regulated Transcript (CART)

Cocaine- and amphetamine- regulated transcript (CART) is a neuropeptide that decreases food intake (Hunter and Kuhar, 2003). CART mRNA is highly expressed in several parts of the hypothalamus and is co-localized with orexigenic as well as anorexigenic neuropeptides. Icv application leads to an inhibition of normal and NPY stimulated food intake (Kristensen et al., 1998). CART expression is upregulated after leptin administration, suggesting the mode of action is closely associated with the action of leptin and NPY (Edwards et al., 2000).

1.8.3 Melanin-Concentrating Hormone (MCH)

Melanin-concentrating hormone (MCH) is an orexigenic 19 amino acid peptide primarily expressed in neurons of the lateral hypothalamic area (LHA) (Review: Pissios and Maratos-Flier, 2003). Central administration of MCH induces hyperphagia (Qu et al., 1996) and chronic infusion into the lateral ventricle

lead to weight gain (Della-Zuana et al., 2002). MCH expression is increased after fasting in the LHA. MCH synthesis in the hypothalamus is elevated by both energy restriction and leptin deficiency (Qu et al., 1996), MCH-knockout mice are hypophagic and they show an increased metabolic rate, they have low leptin levels and they are lean (Shimada et al., 1998). MCH overexpression in the hypothalamus causes obesity (Ludwig et al., 2001). Leptin decreases MCH gene expression (Sahu, 1998a; Sahu, 1998b). Therefore it appears that MCH neurons function downstream of leptin signaling (Sahu, 1998b). MCH is therefore considered as a functional antagonist of the melanocortin system in the hypothalamus (Ludwig et al., 1998; Tritos et al., 1998).

1.8.4 Galanin

Galanin is a 29 amino acid peptide found in the gut and the brain (Review: Gundlach, 2002). Intracerebroventricular or intrahypothalamic injection of galanin stimulated feeding in satiated rats in a dose-dependent manner, in particular the intake of fat (Kyrkouli et al., 1990). Several galanin receptor antagonists have been shown to decrease the stimulatory effects of galanin (Koegler et al., 1999). Galanin appears to regulate both fat and glucose levels by its central and peripheral actions but the mode of action is still unknown. Galanin expression is indirectly influenced by leptin and insulin (Cone, 2000).

1.8.5 Corticotropin Releasing Factor (CRH)

Corticotropin releasing hormone (CRH) also called corticotropin releasing factor (CRF), a 41 amino acid peptide was found in a wide variety of mammalian species (Review: De Souza, 1995). CRH mRNA and protein are abundantly distributed in the central nervous system. The major sites of expression being the paraventricular nucleus of the hypothalamus, the cerebral cortex, the cerebellum and the amygdale-hippocampal complex, an area important for stress adaptation, learning and memory (Bittencourt and Sawchenko, 2000). It has been shown that intracerebroventricular administration of CRH suppresses appetite (Arase et al., 1988) and that the anorectic action of leptin may depend partially on the activation of hypothalamic CRH (Uehara et al., 1998). Leptin increases the expression of CRH (Schwartz et al., 1996). CRH

is a potent anorexigenic peptide likely acting downstream of leptin.

1.8.6 Orexins

Two novel orexigenic peptides have been found in the lateral hypothalamic area, named orexin-A and orexin-B (Sakurai et al., 1998) (Review: Ferguson and Samson, 2003). Administration of both orexins (also known as hypocretins) into the CNS stimulate food intake due to a delay in satiety (Rodgers et al., 2002). Orexins show a higher expression in fasted rodents so that the orexin system is sensitive to the nutritional status. Two orexin receptors have been identified. Leptin reduces orexin-A concentration and also blocks fasting induced changes in prepro-orexin mRNA and orexin receptor mRNA (Beck et al., 2001).

1.9 Brain Areas Involved in Food Intake Regulation

All the centrally acting factors for the regulation of food intake and body weight homeostasis are expressed in the hypothalamus. The hypothalamus consists of several subdomains (nuclei) involved to a different degree in food intake. These subdomains are the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the ventromedial hypothalamic nucleus (VMH), and the dorsomedial hypothalamic nucleus (DMH).

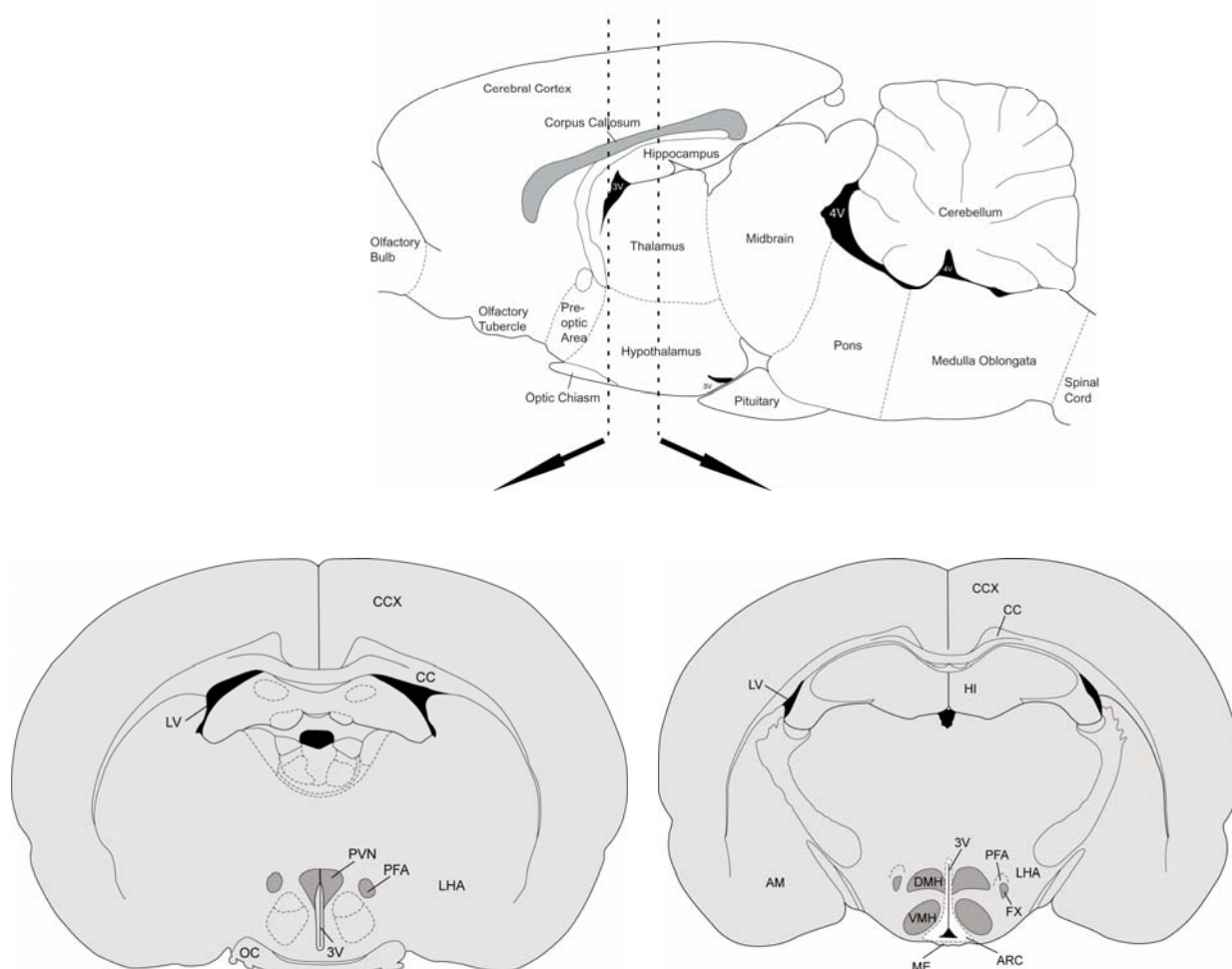


Fig. 1. Diagrams of rat brain, showing major hypothalamic regions implicated in adiposity signaling and regulation of food intake. The upper part of the panel shows a longitudinal view of a rat brain, with olfactory bulb at the anterior end on the left and the spinal cord on the right. Cross-sections of the brain (indicated by vertical dotted lines) are shown at the left and right of the lower part of the figure. First order neurons responding to adiposity signals are located in the arcuate nucleus (ARC) and project anteriorly to the paraventricular nucleus (PVN) as well as the perifornical area (PFA) adjacent to the fornix (FX) and the lateral hypothalamic area (LHA). Other regions implicated in regulating food intake include the ventromedial nucleus (VMH) and dorsomedial nucleus (DMH). Abbreviations of brain structures: AM, amygdala; CC, corpus callosum; CCX, cerebral cortex; HI, hippocampus; ME, median eminence; OC, optic chiasm; 3V, third ventricle. Modified after Schwartz (Schwartz et al., 2000).

1.9.1 Arcuate Nucleus (ARC)

ARC neurons are located at the base of the hypothalamus on either side of the third ventricle and are called 'first-order neurons' because of their 'first' contact with peripheral satiety factors like leptin, insulin or ghrelin. This is due to the fact that in the median eminence, which overlies the ARC, the blood brain barrier (BBB) is absent and ARC axons terminals are in direct contact with the bloodstream (Peruzzo et al., 2000). The ARC contains two distinct groups of neurons controlling energy balance (Fig. 2).

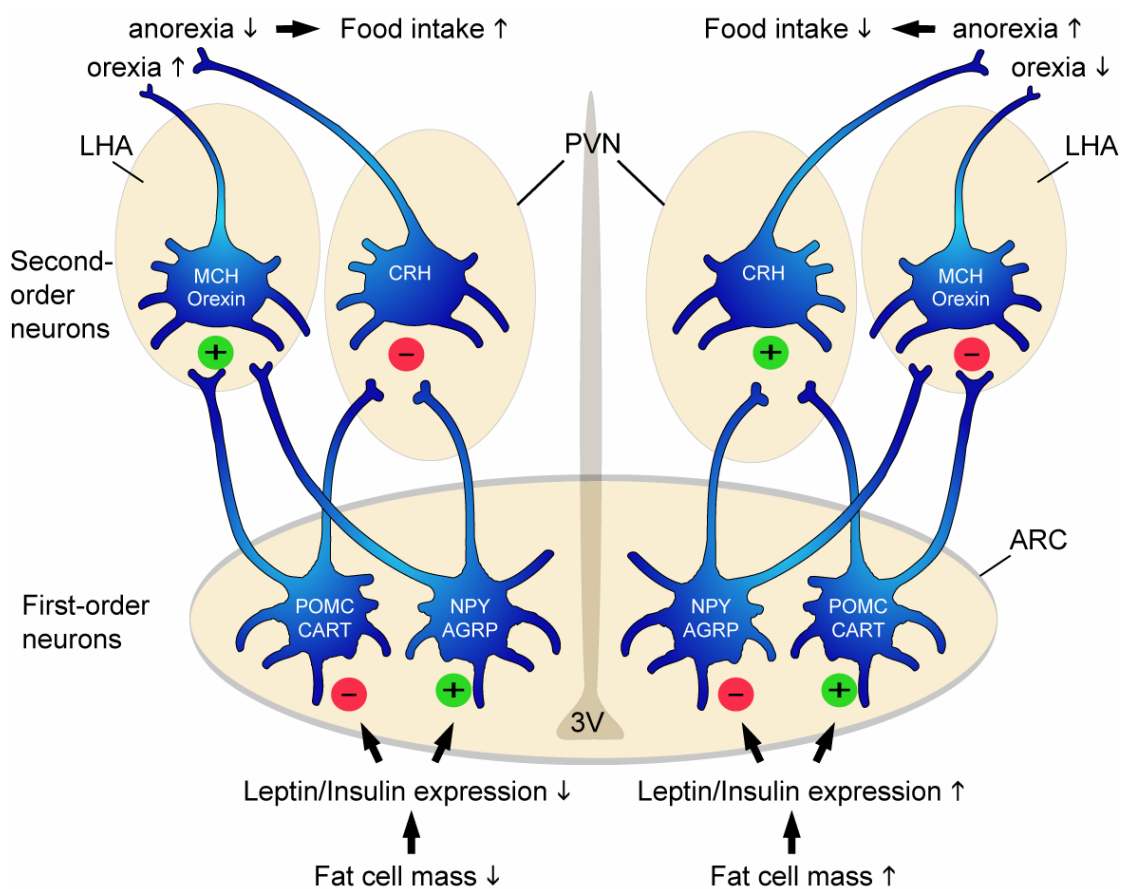


Fig. 2. The cascade of neuronal events that are triggered by increased (right side) or decreased (left side) levels of circulating leptin. Increased levels of leptin stimulate POMC/CART containing first-order neurons in the arcuate nucleus (ARC). That results in stimulation of anorectic CRH containing second-order neurons in the PVN and inhibition of orexigenic ORX/MCH containing neurons in the LHA. The result is a decrease in food intake.

Decreased levels of leptin result in disinhibition of NPY/AGRP containing neurons in the ARC. This results in inhibition of CRH neurons in the PVN and stimulation of ORX/MCH neurons in the LHA via antagonizing the MC4 receptor. The result is an increase in food intake. Modified after Schwartz (Schwartz et al., 2000).

One group expresses the orexigenic neuropeptides AGRP and NPY, the other expresses the anorectic neuropeptides POMC and CART. From the ARC, the neurons project to 'second-order neurons' in the PVN, VMH, DMH, and LHA (Schwartz et al., 2000). 'Second-order neurons' project amongst others to the nucleus of the solitary tract (NTS) in the brainstem and to the dorsomotor nucleus of the vagus (DMV). Leptin receptors are colocalized with NPY/AGRP and POMC/CART in the ARC (Cheung et al., 1997). The action of leptin on these neurons triggers the cascade of neuronal events, regulating the activity in other appetite controlling nuclei, including the LHA and PVN (Sahu, 2003).

1.9.2 The Lateral Hypothalamic Area (LHA)

The lateral hypothalamic area is vaguely defined and comprises a large, diffuse population of neurons. NPY, AGRP and α -MSH immunoreactive fibers innervate neurons in the LHA containing the orexigenic peptides MCH and Orexin (Elias et al., 1998). Therefore stimulation of the LHA can increase food intake. The LHA is suggested as a link between the hypothalamic feeding-center and higher functions, as it contains neurons innervating several regions of the cerebral cortex (Elmquist et al., 1998).

1.9.3 The Paraventricular Nucleus (PVN)

The PVN lies at the top of the third ventricle in the anterior hypothalamus (Fig. 1) and receives input from both NPY/AGRP and POMC/CART containing neurons (Elmquist et al., 1999) (Fig. 2). Fibers of orexin neurons stemming from the lateral hypothalamus have also been found. The nucleus is rich in terminals containing numerous food intake-modifying neurotransmitters, like NPY, α -MSH, serotonin and galanin. The PVN is also affected by leptin (Elmquist et al., 1998). This evidence implies that the PVN is the region where orexigenic/anorectic signals from the ARC converge and are integrated. Stimulation of the PVN neurons inhibits food intake by CRH containing neurons (Fig. 2) (Schwartz et al., 2000). Neurons in the PVN innervate regions in the medulla and spinal cord which are involved in autonomic and endocrine functions.

1.9.4 The Dorsomedial Hypothalamic Nucleus (DMH)

The DMH, located immediately dorsal to the VMH (Fig. 1), has extensive di-

rect connections with other hypothalamic nuclei such as the PVN and the lateral hypothalamus. The VMH and the lateral hypothalamus have no direct connections but connect indirectly through the DMH and the PVN. The PVN and the DMH may cooperate functionally as a unit, which is involved in initiating and maintaining food intake. The DMH contains high amounts of insulin as well as leptin receptors. Some ARC-NPY/AGRP neurones also terminate in the DMH (Kalra et al., 1999).

1.9.5 The Ventromedial Hypothalamic Nucleus (VMH)

The VMH, one of the largest nuclei of the hypothalamus, was long considered to be a “satiety center.” Stimulation of the VMH inhibits feeding, whereas a lesion in this region causes overeating and weight gain (Stellar, 1954). The VMH has direct connections with the lateral hypothalamus and the DMH. The precise role of the VMH remains unclear. However, there is compelling evidence that the VMH is an important integrator of glucose homeostasis (Routh, 2003). The VMH possesses receptors for virtually all neurotransmitters and peptides known to influence energy balance and glucose homeostasis.

1.10 Melanocortin System

The melanocortin system is one of the major pathways involved in the control of food intake/energy expenditure in man. This system includes a group of peptide hormones called melanocortins (MSH) and five G protein coupled receptors (GPCR), the melanocortin receptor (MCR), MC1-R to MC5-R. Additional components of the melanocortin system are proteins produced in the hypothalamus, which modulate the melanocortin system. Among them are the melanocortin receptor antagonists, Agouti and agouti-related protein (AGRP). Two recently discovered proteins, Syndecan (Reizes et al., 2001) and mahogany protein (Gunn and Barsh, 2000) are believed to be mediators of the melanocortin pathway. Further work is required to understand the role of the two latter compounds.

1.10.1 Pro-Opiomelanocortin and Melanocortins

The melanocortins (Review: Abdel-Malek, 2001) are a family of structurally related peptides which are derived through post-translational cleavage from the precursor pro-opiomelanocortin (POMC). The *POMC* gene is located at the locus p23.3 on human chromosome 2, and is composed of two coding exons and one upstream non-coding exon (Takahashi H, 1981). The 1200 base pair *POMC* transcript encodes a 267 amino acid pro-hormone with an N-terminal signal peptide of 26 residues (Fig. 3). After POMC is transported to secretory organelles it is cleaved by prohormone convertase 1 (PC1) to raise to pro-adrenocorticotrophic hormone (pro-ACTH) and β -lipotropin (Rouille et al., 1995). Pro-ACTH is cleaved again by PC1 producing the N-POC precursor and the mature ACTH peptide (Fig. 3). Cleavage of ACTH by PC2 results in the 13 amino acid peptide α -melanocyte-stimulating hormone (α -MSH) and the 20 amino acid peptide corticotropin-like intermediate lobe peptide (CLIP). α -MSH is further translationally modified by amidation of the C-terminus and acetylation of the N-terminus. The N-terminus of POMC is cleaved to give γ -MSH, and β -MSH is formed by cleavage of β -lipotropin. All melanocortins share a conserved tetrapeptide sequence, His-Phe-Arg-Trp, and α -MSH, β -MSH, and ACTH share a heptapeptide sequence, Met-Glu-His-Phe-Arg-Trp-Gly, which is important for the melanogenic effects of melanocortins.

Melanocortins are expressed throughout the brain as well as in peripheral tissues like testis, ovary, placenta, duodenum, liver, kidney, thymus, lymphocytes, macrophages and skin (DeBold et al., 1988; Tatro and Reichlin, 1987). In the brain, hybridization experiments have shown that POMC is expressed in the arcuate nucleus of the hypothalamus and the nucleus tractus solitarii of the caudal medulla (Young et al., 1998). Projections containing POMC derived peptides are found in many brain regions, such as nuclei within the hypothalamus (arcuate nucleus, paraventricular nucleus) (See chapter 1.9). A large amount of data has produced compelling evidence of the role of melanocortins in central processes, which are quite different from their peripheral effects (melanocyte and adrenal stimulation).

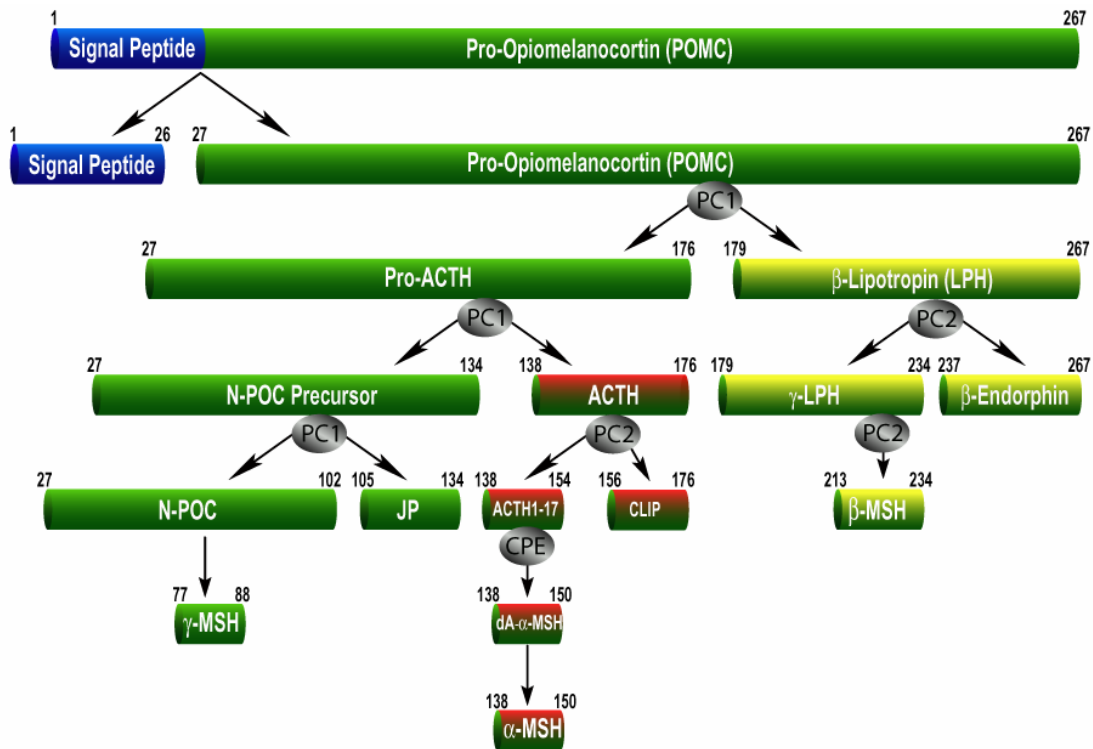


Fig. 3. The POMC processing pathway in the hypothalamus. Post-translational cleavage of pro-opiomelanocortin by prohormone convertases 1 (PC1) and 2 (PC2). PC1 and PC2 are endoproteases. Other factors may be involved in this process, for example carboxypeptidase E (CPE) removes C-terminal residues from the ACTH 1-17 peptide, to give des-acetyl α -MSH. Numbers indicate amino acid from the primary human POMC sequence (accession number P01189). Modified after Pritchard (Pritchard et al., 2002).

1.10.2 Effects of Melanocortins

α -MSH is the peptide derived from POMC that regulates energy homeostasis. This small peptide exerts a tonic inhibition on food intake by signaling through melanocortin-4 receptor (MC4-R). Studies using transgenic animals demonstrated the importance of melanocortins in the regulation system. POMC (Yaswen et al., 1999) and MC4-R knockout mice (Huszar et al., 1997) as well as mice which overexpress the antagonistic peptide, agouti (Yen et al., 1994) and AGRP (Ollmann et al., 1997), are obese and hyperphagic. Similar phenotypes are observed in humans with mutations in genes of the melanocortin system (O'Rahilly et al., 2004).

1.10.3 Endogenous Melanocortin Receptor Antagonists

The role of melanocortins in energy homeostasis was strongly supported by work with the 131 amino acid peptide termed agouti (Review: Dinulescu and Cone, 2000), which was found to be an antagonist of α -MSH at MC1-R, MC3-

R and MC4-R (Lu et al., 1994). This finding could explain the obese phenotype of the yellow agouti (A^y) mouse, a rodent model of obesity. The agouti mouse has a mutation within the promoter region of the agouti gene which leads to the ectopic expression of agouti. Agouti, expressed in the hypothalamus, antagonizes the α -MSH action on MC4-R leading to hyperphagia, reduced energy expenditure and ultimately obesity. Agouti expression is usually restricted to the hair follicle. Normally, its role is to block α -MSH action at MC1-R with the result that the cells switch from the synthesis of black pigment (eumelanin) to yellow pigment (phaeomelanin).

A gene that encoding a protein nearly identical in size and structure to the agouti was identified some years ago by searching an expressed sequence tag (EST) database (Ollmann et al., 1997). This protein was named agouti-related protein (AGRP) and is primarily expressed in the hypothalamus. AGRP is a competitive high-affinity antagonist of α -MSH at the MC3-R and MC4-R (Fong et al., 1997). *Ob/ob* mice and *db/db* mice (a leptin receptor deficient mutant) have an increased AGRP mRNA expression suggesting, that AGRP acts downstream of leptin (Mizuno and Mobbs, 1999; Shutter et al., 1997). Furthermore, overexpression of AGRP in transgenic mice results in obese phenotype similar to those in agouti mice and MC4-R knockout mice (Graham et al., 1997). Administration of AGRP, agouti or other antagonists increases food intake (Fan et al., 1997).

1.10.4 Synthetic Melanocortin Receptor Agonists and Antagonists

The most widely used endogenous ligands and synthetic peptides for studying melanocortin receptor function, α -MSH, MTII, AgRP, and SHU9119, show inadequate selectivity between receptor subtypes, particularly MC3-R and MC4-R (Table 2). The synthetic peptide, Ro27-3225, has been reported as an agonist with greater than 100-fold selectivity for MC4-R over MC3-R in functional assays. Ro27-3225 suppresses acute food intake in rats which was taken as evidence that the MC4 receptor subtype mediates these effects (Benoit et al., 2000). Similarly, peptide analogues with MC4-R antagonist activity (HS014; HS028) have been used in *in vivo* experiments to support the idea that the

MC4 receptor mediates the orexigenic effects of melanocortin receptor antagonists. Unfortunately, these compounds do not show greater than 75-fold selectivity for MC4-R over MC3-R to make this conclusion definitive. Recently, Bednarek *et al.* (Bednarek et al., 2001b) described a cyclic peptide (cyclic(1-6)suc-HFRWK-NH₂) which is a highly selective agonist for the MC4 receptor subtype. Another cyclic peptide, MBP10, is a low nanomolar affinity antagonist at MC4-R with greater than 100-fold selectivity for MC4 over MC3, MC5, and MC1 (Bednarek et al., 2001a). By virtue of their high affinity and selectivity, these peptides are the most selective reported *in vitro* for the evaluation of the contribution of the MC4 receptor subtype to body weight regulation.

Agonist	EC ₅₀ (nM)				Antagonist	K _i (nM)			
	MC1	MC3	MC4	MC5		MC1	MC3	MC4	MC5
α-MSH	9	40	29	97	AGRP(83-132)	>100	0.6	0.3	>40
ACTH	1	8	12	>100	SHU9119	0.2	1	0.1	1.5
γ-MSH	40	6	300	600	HS014	108	54	3	694
MTII	0.2	0.7	0.5	3	HS028	60	74	1	211
c(1-6)suc-HFRWK-NH₂	4	1000	1.5	>1000	MBP10	>1000	150	0.5	540
Ro27-3225	8	675	1	>1000					

Tab. 2. Affinity of melanocortin agonists and antagonists for melanocortin receptor subtypes (Foster et al., 2003).

1.11 Melanocortin Receptors

The melanocortin receptors are members of the superfamily of G-protein coupled receptors (GPCR) that are characterized by seven transmembrane domains (also named 7TM receptors). Mutagenesis studies have identified transmembrane domains one, two, three, six, and seven to be important for receptor binding (Schiøth et al., 1996b; Schiøth et al., 1997) (Fig. 4).

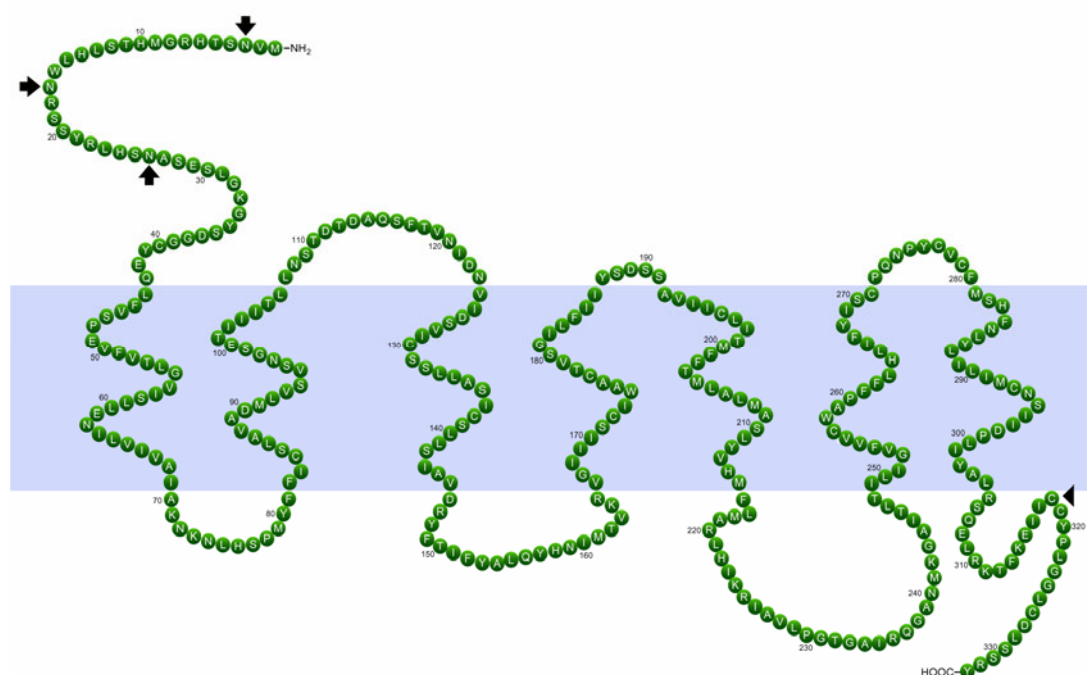


Fig. 4. Predicted domain structure of the human Melanocortin-4 receptor (Accession number: P 32245 UniProt/SwissProt). Upper part of the membrane (blue): extracellular region. Arrows: Potential N-glycosylation sites, arrowhead: Potential palmitoylation site.

Up to date five melanocortin receptors (MC1-R to MC5-R) have been cloned and have now been implicated in a number of human diseases. Melanocortin receptors are activated by one or more of the melanocortin peptides (ACTH, α -, β - or γ - MSH). Melanocortin binding to the receptor activates adenylate cyclase (AC) via the $G\alpha_s$ protein, leading to the increase of the levels of intracellular cyclic adenosine monophosphate (cAMP). The changes in intracellular cAMP modulate the physiological functions of the melanocortins. Depending on the cell type and the melanocortin receptor expression levels, signal transduction pathways other than via $G\alpha_s$ may be activated by melanocortin ligands. This includes the release of intracellular calcium ion levels via the inositol triphosphate pathway (Konda et al., 1994), the influx of extracellular calcium ions (Kojima et al., 1985), the activation of mitogen-activated protein

(MAP) kinase (Englaro et al., 1995), signaling by Janus kinase or signal transducer and activator of transcription (STAT) pathways (Buggy, 1998) and the protein kinase C (PKC) pathway (Kapas et al., 1995). The precise mechanisms by which these pathways are activated by melanocortins have not yet been elucidated. Each of the melanocortin receptors has a different tissue distribution and it has different binding affinities for the various melanocortins and their antagonists (Tab. 3). The five human melanocortin receptors share 40-60% amino acid similarity. None of the melanocortin receptor genes contain introns. Compared to other members of the GPCR superfamily the melanocortin receptors have shorter amino and carboxyl terminal ends. Several potential N-glycosylation sites are present in the N-terminal domain. There is evidence to suggest they are also regulated by phosphorylation since they have consensus recognition sites for protein kinase A and C. A conserved cysteine in their C-terminus may act as a site for fatty acid acylation anchoring the C-terminus to the plasma membrane (Fig. 4).

Receptor Homology (%)						Potency of agonist activation	Antagonist
	MC1	MC2	MC3	MC4	MC5		
MC1	100	42	50	53	47	α -MSH=ACTH> β -MSH>> γ -MSH	agouti
MC2		100	49	48	47	ACTH	agouti
MC3			100	64	61	α -MSH= β -MSH = γ -MSH= ACTH	AGRP, agouti
MC4				100	67	α -MSH=ACTH> β -MSH>> γ -MSH	AGRP, agouti
MC5					100	α -MSH>ACTH> β -MSH>> γ -MSH	(agouti)

Tab. 3. The homology among the human melanocortin receptors at the amino acid level and their relative potency of activation by various melanocortins and their natural antagonist (MacNeil et al., 2002).

1.11.1 Melanocortin-1 Receptor

The melanocortin-1 receptor (MC1-R) was first cloned from human melanocytes (Mountjoy et al., 1992). Human MC1-R is located on chromosome 16q24.3 and encodes a 317 amino acid protein. MC1-R equally binds to

ACTH and α -MSH (Chhajlani and Wikberg, 1992), with lower affinity to β -MSH and weakest to γ -MSH (Tab. 3). MC1-R shows the highest affinity for α -MSH among the five MC-Rs. The human and mouse MC1-R share 76% identity in amino acid sequence (Mountjoy, 1994). MC1-R is expressed abundantly in human and mouse melanoma cells (Xia et al., 1995) and at significantly lower levels in mouse (Donatien et al., 1992) and human melanocytes (Loir et al., 1999). Recently MC1-R has been found to be expressed in normal human skin glands and hair follicles as well as in neoplasms of the skin (Stander et al., 2002). The ubiquitous expression of MC1-R in human skin rules out MC1-R as a prognostic marker in skin tumours. α -MSH and agouti play an important role in regulating melanin production. α -MSH signals through MC1-R and $G\alpha_s$, resulting in the production of the dark pigment eumelanin (Suzuki et al., 1996a). Regulation of integumental pigmentation is the best known effect of α -MSH. This hormone is the physiological regulator of rapid color change in lower vertebrates, including fish, amphibians and reptiles (Sawyer et al., 1983). Agouti protein antagonizes the binding of α -MSH to MC1-R, allowing the production of the light pigment pheomelanin. MC1-R signaling in the mouse is a major determinant of coat colour and agonism or antagonism regulates the type of pigment produced.

The human MC1-R is highly polymorphic and many allelic variants of the gene have been identified in Northern European populations (Smith et al., 1998) and in Australia, but not in African populations (Sturm et al., 1998). Some of these variants are associated with the inheritance of red hair phenotype (the red-yellow pigment pheomelanin), poor tanning ability and an increased risk of melanoma (Palmer et al., 2000).

MC1-R is also expressed in human anti-inflammatory cells including neutrophils, endothelial cells, glioma cells and astrocytes, macrophages and monocytes and fibroblasts (Neumann et al., 2001). α -MSH have anti-inflammatory and immunomodulatory effects mainly due to its capacity to alter the function of antigen-presenting, inhibition of cytokine production and decline of adhesion molecule expression (Review: Catania et al., 2004).

1.11.2 Melanocortin-2 Receptor

The human melanocortin-2 receptor (MC2-R) gene encodes a 297 amino acid protein and it is located on chromosome 18p11.2. ACTH is the only melanocortin with binding affinity for MC2-R (Tab. 3) (Schiøth et al., 1996a). It increases the cAMP level via the G-protein $G\alpha_s$. The main role of MC2-R is to induce glucocorticoid production by the adrenal glands in response to ACTH released from the pituitary glands (Xia and Wikberg, 1996). MC2-R is expressed in the zona fasciculata and zona glomerulosa of the adrenal cortex (Mountjoy et al., 1992). In both mouse and human adrenocortical cell lines the addition of ACTH or cAMP increases MC2-R mRNA (Mountjoy et al., 1994a). The pituitary and hypothalamus do not appear to express MC2-R (Xia and Wikberg, 1996). Missense mutations in the coding region of MC2-R have been identified which lead to glucocorticoid deficiency syndromes (Tsigos, 1999). MC2-R is also expressed in murine adipocytes where ACTH binds to it, possibly regulating adipocyte function (Boston and Cone, 1996). MC2-R is not expressed in human adipocytes and there is no evidence suggesting that human adipose tissue is responsive to the lipolytic functions of ACTH or α -MSH (Chhajlani, 1996).

1.11.3 Melanocortin-3 Receptor

The human melanocortin-3 receptor (MC3-R) gene encodes a 361 amino acid protein and it is located on chromosome 20q13.2-13.3. MC3-R is expressed in brain, placenta and gut but not in melanoma cells or adrenal gland (Gantz et al., 1993a). The highest levels of MC3-R expression in the brain occur in the VMH, ARC, preoptic nucleus, LHA and posterior hypothalamic area (Roselli-Reh fuss et al., 1993). MC3-R binds α -MSH, β -MSH and γ -MSH with similar affinities and it is the only MC-R activated by γ -MSH (Tab. 2) (Gantz et al., 1993a). POMC neurons of the ARC were found to express mRNA for MC3-R (Jegou et al., 2000). Melanocortin binding to MC3-R is antagonised by agouti-related protein (AGRP) in the hypothalamus (Ollmann et al., 1997). MC3-R knockout mice are hypophagic, have an increased fat mass and reduced lean mass (Chen et al., 2000). Remarkably, MC3-R knockout mice are hypophagic but do not have alterations in body weight and do not develop diabetes. The

observation that MC3-R knockout mice have increased adiposity supports a role for MC3-R in energy homeostasis. The mechanism is still unknown. Furthermore it has been demonstrated that MC3-R activation modulates host inflammatory response (Getting et al., 2003). There are speculations for a cardiovascular effect but heart rate and blood pressure are not affected by γ -MSH signaling via MC3-R (Humphreys, 2004).

1.11.4 Melanocortin-4 Receptor

The human melanocortin-4 receptor (MC4-R) gene encodes a 332 amino acid protein and it is localized on chromosome 18q21.3. The rat gene is 95%, and the mouse 99%, identical to the human gene, which suggests a high conservation in mammals (Tab. 4). MC4-R was found to be expressed predominantly in the brain (Gantz et al., 1993b). Areas of the brain showing significant expression include olfactory cortex, hippocampus, amygdala, corpus striatum, nucleus accumbens, hypothalamus, nucleus tractus solitarius and the dorsal horn of the spinal cord (Kishi et al., 2003; Mountjoy et al., 1994b). No expression was detected in the adrenal cortex, melanocytes and placenta. The highest level of expression was found in the paraventricular nucleus (PVN) in the hypothalamus (Kishi et al., 2003). ACTH and α -MSH increase the cAMP levels in COS-1 cells, transfected with MC4-R, which suggests that this receptor signals via $G\alpha_s$ (Gantz et al., 1993b). MC4-R binds to ACTH and α -MSH with equal affinities and to β -MSH with lower affinity (Tab. 3).

The distribution of MC4-R is consistent with its involvement in autonomic and neuroendocrine functions. Many lines of evidence indicate that MC4-R is a strong candidate for appetite regulation and energy expenditure. Knockout mice, lacking MC4-R, show maturity-onset of obesity, hyperphagia, hyperinsulinemia (gender specific), hyperglycemia and increased linear growth (Huszar et al., 1997). Heterozygous MC4-R knockout mice show intermediate obesity, their average weight being between the homozygous MC4-R knockouts and wild-type mice, suggesting that receptor number is important for weight regulation (Huszar et al., 1997). Further evidences for the role of MC4-R came from works with AGRP which is expressed in the hypothalamus of humans

and rodents and acts as an antagonist on MC4-R. The interplay of α -MSH and AGRP switches MC4-R from the active to the inactive state. Agouti protein as AGRP antagonizes MC4-R in rodents and possibly in humans (Ollmann et al., 1997). 3-4% of cases with severe obesity showed pathogenic mutations in MC4-R (Hinney et al., 1999; O'Rahilly et al., 2004; Yeo et al., 1998).

	1	10	20	30	40	50																																																						
mouse	M	N	S	T	H	H	G	M	Y	T	S	L	H	L	W	N	R	S	S	Y	G	L	H	S	N	A	S	E	S	L	G	K	G	H	P	D	G	G	C	Y	E	Q	L	F	V	S	P	E	V	F	V	T	L	G	V	I	S	L		
rat	M	N	S	T	H	H	G	M	Y	T	S	L	H	L	W	N	R	S	S	H	G	L	H	G	N	A	S	E	S	L	G	K	G	H	S	D	G	G	C	Y	E	Q	L	F	V	S	P	E	V	F	V	T	L	G	V	I	S	L		
human	M	V	N	S	T	H	R	G	M	H	T	S	L	H	L	W	N	R	S	S	Y	R	L	H	S	N	A	S	E	S	L	G	K	G	Y	S	D	G	G	C	Y	E	Q	L	F	V	S	P	E	V	F	V	T	L	G	V	I	S	L	
	60	70	80	90	100	110																																																						
mouse	L	E	N	I	L	V	I	V	A	I	A	K	N	K	N	L	H	S	P	M	Y	F	F	I	C	S	L	A	V	A	D	M	L	V	S	V	S	N	G	S	E	T	I	V	I	T	L	L	N	S	T	D	T	D	A	Q	S	F	T	V
rat	L	E	N	I	L	V	I	V	A	I	A	K	N	K	N	L	H	S	P	M	Y	F	F	I	C	S	L	A	V	A	D	M	L	V	S	V	S	N	G	S	E	T	I	V	I	T	L	L	N	S	T	D	T	D	A	Q	S	F	T	V
human	L	E	N	I	L	V	I	V	A	I	A	K	N	K	N	L	H	S	P	M	Y	F	F	I	C	S	L	A	V	A	D	M	L	V	S	V	S	N	G	S	E	T	I	I	I	T	L	L	N	S	T	D	T	D	A	Q	S	F	T	V
	120	130	140	150	160	170																																																						
mouse	N	I	D	N	V	I	D	S	V	I	C	S	S	L	L	A	S	I	C	S	L	L	S	I	A	V	D	R	Y	F	T	I	F	Y	A	L	Q	Y	H	N	I	M	T	V	R	R	V	G	I	I	S	C	I	W	A	A	C	T	V	
rat	N	I	D	N	V	I	D	S	V	I	C	S	S	L	L	A	S	I	C	S	L	L	S	I	A	V	D	R	Y	F	T	I	F	Y	A	L	Q	Y	H	N	I	M	T	V	R	R	V	G	I	I	S	C	I	W	A	A	C	T	V	
human	N	I	D	N	V	I	D	S	V	I	C	S	S	L	L	A	S	I	C	S	L	L	S	I	A	V	D	R	Y	F	T	I	F	Y	A	L	Q	Y	H	N	I	M	T	V	K	R	V	G	I	I	S	C	I	W	A	A	C	T	V	
	180	190	200	210	220	230																																																						
mouse	S	G	V	L	F	I	I	Y	S	D	S	S	A	V	I	I	C	L	I	S	M	F	F	T	M	L	V	L	M	A	S	L	Y	V	H	M	F	L	M	A	R	L	H	I	K	R	I	A	V	L	P	G	T	G	T	I	R	Q	G	T
rat	S	G	V	L	F	I	I	Y	S	D	S	S	A	V	I	I	C	L	I	T	M	F	F	T	M	L	V	L	M	A	S	L	Y	V	H	M	F	L	M	A	R	L	H	I	K	R	I	A	V	L	P	G	T	G	T	I	R	Q	G	A
human	S	G	I	L	F	I	I	Y	S	D	S	S	A	V	I	I	C	L	I	T	M	F	F	T	M	L	A	L	M	A	S	L	Y	V	H	M	F	L	M	A	R	L	H	I	K	R	I	A	V	L	P	G	T	G	A	I	R	Q	G	A
	240	250	260	270	280	290																																																						
mouse	N	M	K	G	A	I	T	L	T	I	L	I	G	V	F	V	C	W	A	P	F	F	L	H	L	L	F	Y	I	S	C	P	Q	N	P	Y	C	V	C	F	M	S	H	F	N	L	Y	L	I	L	I	M	C	N	A	V	I	D	P	
rat	N	M	K	G	A	I	T	L	T	I	L	I	G	V	F	V	C	W	A	P	F	F	L	H	L	L	F	Y	I	S	C	P	Q	N	P	Y	C	V	C	F	M	S	H	F	N	L	Y	L	I	L	I	M	C	N	A	V	I	D	P	
human	N	M	K	G	A	I	T	L	T	I	L	I	G	V	F	V	C	W	A	P	F	F	L	H	L	I	F	Y	I	S	C	P	Q	N	P	Y	C	V	C	F	M	S	H	F	N	L	Y	L	I	L	I	M	C	N	S	I	I	D	P	
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mouse	L	I	Y	A	L	R	S	Q	E	L	R	K	T	F	K	E	I	I	C	F	Y	P	L	G	G	I	C	E	L	S	S	R	Y																											
rat	L	I	Y	A	L	R	S	Q	E	L	R	K	T	F	K	E	I	I	C	F	Y	P	L	G	G	I	C	E	L	P	G	R	Y																											
human	L	I	Y	A	L	R	S	Q	E	L	R	K	T	F	K	E	I	I	C	Y	P	L	G	G	L	C	D	L	S	S	R	Y																												

Tab. 4. Deduced amino acid sequences of the melanocortin-4 receptor gene sequence of mouse, rat and human. The amino acids marked in red, are different at least in two species. The GenBank accession numbers for the melanocortin-4 receptor are AF201662 (mouse) RNU67863 (rat), L08603 (human).

Taken together, a large amount of data demonstrates that MC4-R activation decreases food intake and increases energy expenditure. Disruption of MC4-R signaling results in overweight. Recent results indicate that beside food intake, MC4-R modulates also sexual behaviour and influences penile erection. (Van der Ploeg et al., 2002; Wessells et al., 1998).

Further evidence for MC4-R regulation of body weight can be deduced from mice with cachexia. Cachexia is a syndrome where loss of lean body mass is

observed in individuals with chronic diseases such as cancer and AIDS. These individuals have a decrease in appetite and an increase in energy expenditure leading to a dramatic body weight loss. Malignant tumours induce cachexia in mice, which can be used to examine the role of melanocortin signaling in this disorder (Marks et al., 2001). Blockade of MC4-R signaling inhibited the cachexic phenotype in these mice. Administration of AGRP or other MC4-R antagonist as well as MC4-R knockout prevented the weight loss, the reduction of food intake and the loss of activity which are normally observed in animals with tumors.

1.11.5 Melanocortin-5 Receptor

The human melanocortin-5 receptor (MC5-R) gene encodes a 325 amino acid protein and it is located on chromosome 18p11.2 (Fathi et al., 1995). MC5-R is the most widely expressed melanocortin receptor. MC5-R binds to all of the melanocortins except γ -MSH (Abdel-Malek, 2001). It is still not known if there is an endogenous antagonist of MC5-R as neither agouti protein (Lu et al., 1994) nor AGRP (Fong et al., 1997) binds MC5-R under physiological conditions in mice. Tissue distribution in humans includes adrenal glands, adipose tissue, kidney, leukocytes, lung, lymph nodes, mammary glands, ovary, testis and uterus (Chhajlani, 1996). MC5-R expression has not been found in placenta, heart or thyroid. In the brain the receptor is expressed in the hypothalamus, cortex, cerebellum, hippocampus, substantia nigra and the pituitary (Chhajlani et al., 1993). Although MC5-R has a wide tissue distribution, its function is unclear. Presence of MC5-R in B- and T-lymphocytes suggests a function in immune regulation (Buggy, 1998). Since MC5-R is expressed in brain, adipose tissue and skeletal muscle, it might have a role in energy homeostasis pathways. A polymorphism study of MC5-R in a Quebec population revealed four polymorphisms and significant association with obesity phenotypes (Chagnon et al., 1997). The MC5-R knockout mouse has impaired exocrine gland function as seen by a reduced sebum secretion. This leads to a severe defect in water repulsion of the fur while the decreased production of sebaceous lipids may result in defective thermoregulation (Chen et al., 1997). Activation of MC5-R by ACTH or α -MSH may regulate exocrine gland function.

1.12 Modulation of the Melanocortin System

The melanocortin system responds to physiological inputs related to the nutritional status. These peripheral signals are largely mediated by leptin. Additionally, it interacts at various levels with other appetite-regulating factors, in particular AGRP and NPY.

1.12.1 Leptin and the Melanocortin System

POMC neurons in the arcuate nucleus express leptin receptors (Cheung et al., 1997). Fasting reduces leptin levels and decreases POMC mRNA levels in the hypothalamus (Schwartz et al., 1997). Injection of leptin to fasted animals increases POMC mRNA expression. Additional evidence for the effects of leptin on the melanocortin system comes from *ob/ob* and *db/db* mice. These mice have low amounts of POMC mRNA. Leptin injection in *ob/ob* mice has been shown to restore POMC mRNA to normal level (Mizuno et al., 1998; Thornton et al., 1997). Further hints that the melanocortin system is located downstream of leptin signaling come from experiments with MC4-R antagonists. Pretreatment with SHU-9119, a synthetic MC4-R antagonist, prevented both the leptin-induced reduction of food intake and the increase in c-Fos expression in the PVN, a indicator for MC4-R activation (Seeley et al., 1997).

1.12.2 NPY and the Melanocortin System

The activation of the NPY/AGRP neurons probably stimulates feeding through a dual effect: activation of NPY receptors and antagonism of MC4-R by AGRP. Additionally, activity of MC4-R neurons could be antagonized by NPY, because these neurons also express the NPY-Y₁ receptor.

1.13 Antisense Technology

Antisense oligonucleotides (ASO) are a tool for modulating gene expression. ASO have shown pharmacological activity in several animal models and clinical trials in humans are currently underway. Up to now only one treatment with ASO has been approved for use in men by the Food and Drug Administration (FDA) (Pirollo et al., 2003). The antisense technology involves the introduction of antisense oligonucleotides (ASO) complementary to mRNA into cells (Reviews: Dias and Stein, 2002; Myers and Dean, 2000). The inhibition of gene expression is believed to be highly specific and it is dependent on the formation of a complex by complementary Watson-Crick base pairing between antisense ASO and the target mRNA (hybridization). For this hybridization to occur the targeted mRNA sequence must be accessible to the oligo-nucleotide and the oligonucleotide must be taken up by the cell, tissue or organ in a quantity sufficient to cause a biological response. The ASO might cause inhibition of the translation of a specific gene by preventing transcription of DNA, by blocking export of RNA, by interfering with RNA splicing, by reducing RNA stability or by preventing RNA translation. The first inhibition of gene expressions using antisense strategy was demonstrated 25 years ago (Zamecnik and Stephenson, 1978).

1.13.1 Mechanism of Action

Once introduced into cells ASO are hybridized with target mRNA in the cytoplasm and inhibit protein expression by a variety of mechanisms (Fig. 5). ASO might sterically interfere with ribosome binding to mRNA and thereby prevent translation (Boiziau et al., 1991). Evidence for steric interference comes from studies, in which ASO specific for the 5' cap of mRNA have been found to be effective in inhibiting protein synthesis. In the 5' cap region binding of a number of initiation factors is required for ribosome assembly, unwinding of DNA and ribosome translocation along the mRNA (Kozak, 1986).

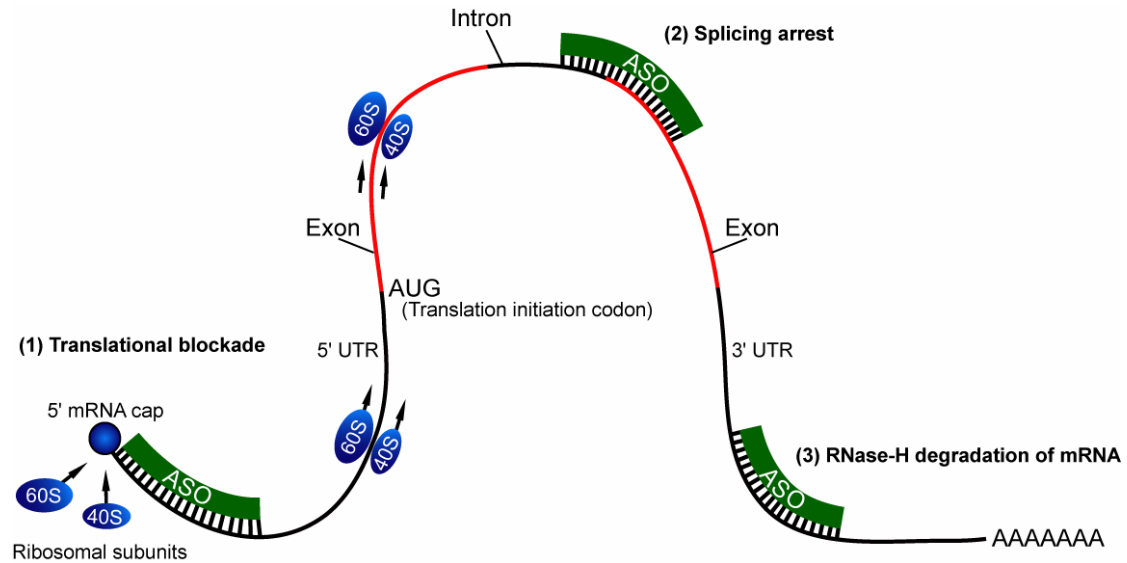


Fig. 5. Mechanisms by which antisense oligonucleotides disrupt protein synthesis: (1) steric blockade of ribosomal subunit attachment to mRNA at the 5' cap site; (2) interference of ASO with mRNA splicing through binding to splice donor or splice acceptor sites; and (3) RNase-H-mediated degradation of ASO-mRNA complex. Degradation can occur in the mRNA wherever an antisense molecule binds with sufficient affinity, including the 5' and 3' UTRs, at the translation initiation codon, and in exons or introns. Modified after Myers (Myers and Dean, 2000).

The major mechanism by which ASO inhibits gene expression has been proposed to be the cleavage of mRNA by the ribonuclease H (RNase H) that specifically recognizes DNA-RNA duplexes (Dash et al., 1987). ASO may inhibit splicing in the nucleus (Daum et al., 1992), thus preventing the process of pre-mRNA formation, or it may block transport of the mRNA to the cytoplasm. In conclusion ASO could cause reduction of the levels of specific mRNA thereby preventing translation of the corresponding protein. If RNase H mediated degradation takes place, or ASO could simply cause a reduction of protein level, without a reduction in mRNA levels, ASO interfere with translation by steric interference.

1.13.2 Design and Stability

Antisense molecules are mostly 15–20 bases long, a length sufficient to hybridize to a unique sequence in the human genome (Branch, 1998). To avoid any homology with other mRNA, antisense sequences are routinely checked against all sequences available in the GenBank™. Unfortunately, identifying the best sequence for antisense is still largely an empirical exercise. Not all

areas of a mRNA molecule are equally amenable to be used for the design of ASO. The likely reason lay in the mRNA secondary structure, proteins bound to the mRNA or accessibility of hybridized mRNA to RNase H. One strategy, which has been quite successful, is based on the prediction of the secondary structure of the mRNA. Regions which have low levels of secondary structure are the most likely candidate for ASO. Such predictions can be easily verified by *in vitro* hybridization to synthetic RNA (Milner et al., 1997). The best candidates have then to be evaluated in cell-culture based assays (Husken et al., 2003). Despite these pre-selecting criteria the success rate for generating effective antisense oligos varies greatly from target to target and is generally 10-20%. Therefore, a minimum of 15-20 different oligonucleotide sequences for each mRNA target should be evaluated (Myers and Dean, 2000).

For *in vitro* or *in vivo* use, antisense oligonucleotides are chemically modified to prevent their degradation by nucleases. Many chemical modifications have been applied (Urban and Noe, 2003). Phosphorothioates are one of the most frequent classes of ASO. One of the oxygens in the phosphate backbone in such molecules is replaced by a sulfur atom. This modification results in an increased protection against cleavage by both exo- and endonucleases.

1.13.3 Toxicity

ASO are well tolerated when infused continuously or injected repeatedly over several days (Agrawal et al., 1995). The continuous infusion of phosphorothioate-ASO into the cerebrospinal fluid for at least one week caused no obvious systemic or neurological CNS toxicity (Whitesell et al., 1993). There is now a large body of data suggesting that while at high doses ASO might induce adverse effects, there are doses that are safe but still provide a sufficient therapeutic effect to justify clinical studies (Marquis and Grindel, 2000).

1.13.4 Delivery and Uptake

While the mechanisms involved in the cellular ASO uptake are still not well understood, there is a great variation in the efficiency of this process between different cell types. Receptor mediated endocytosis seems to mediate ASO uptake, followed by the release of ASO from endocytotic vesicles into the cytoplasm. (Review: Shi and Hoekstra, 2004). Although cells have the capacity

to take up naked DNA, this process is extremely inefficient. Therefore more efficient delivery methods for ASO have been developed: microinjection, electroporation, membrane permeabilization with chemical agents and all the vector-facilitated delivery methods (liposomes, polymers). Liposomes and polymers efficiently complex ASO and enhance the adsorption of complexed ASO to the plasma membrane. For every cell type and tissue the best delivery method has to be established. Delivery and uptake of ASO *in vivo* have been reviewed recently (Wang et al., 2003).

1.14 RNA Interference

RNA interference (RNAi) is currently the most widely studied method to knock out a gene. (Reviews: Downward, 2004; Hannon, 2002). The first use of RNAi was reported in plants, *Caenorhabditis elegans* and *Drosophila melanogaster*. RNAi is a natural phenomenon by which the formation of double-stranded RNA (dsRNA) tags a specific mRNA for degradation (Mechanism see Fig. 6). Introducing foreign dsRNA into cells can mediate a RNAi response. Long dsRNA are cleaved intracellularly into short 21-25 nucleotide small interfering RNAs (siRNA) by a ribonuclease known as Dicer. These short RNAs assemble with the protein components of the RNA-induced silencing complex (RISC). The activated RNA-RISC complex binds to the mRNA by base pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved and subsequently degraded. The mRNA degradation leads to decreased protein formation from the corresponding gene and therefore establishes long lasting gene silencing. Researchers have already shown that siRNAs might be functional *in vitro* and *in vivo* in mammals by using synthetic siRNAs or siRNA expression vectors. siRNA is becoming a powerful tool to knock down gene expression, but one big hurdle is still remaining: the efficient delivery of the siRNAs to the target tissue or cell type *in vivo*.

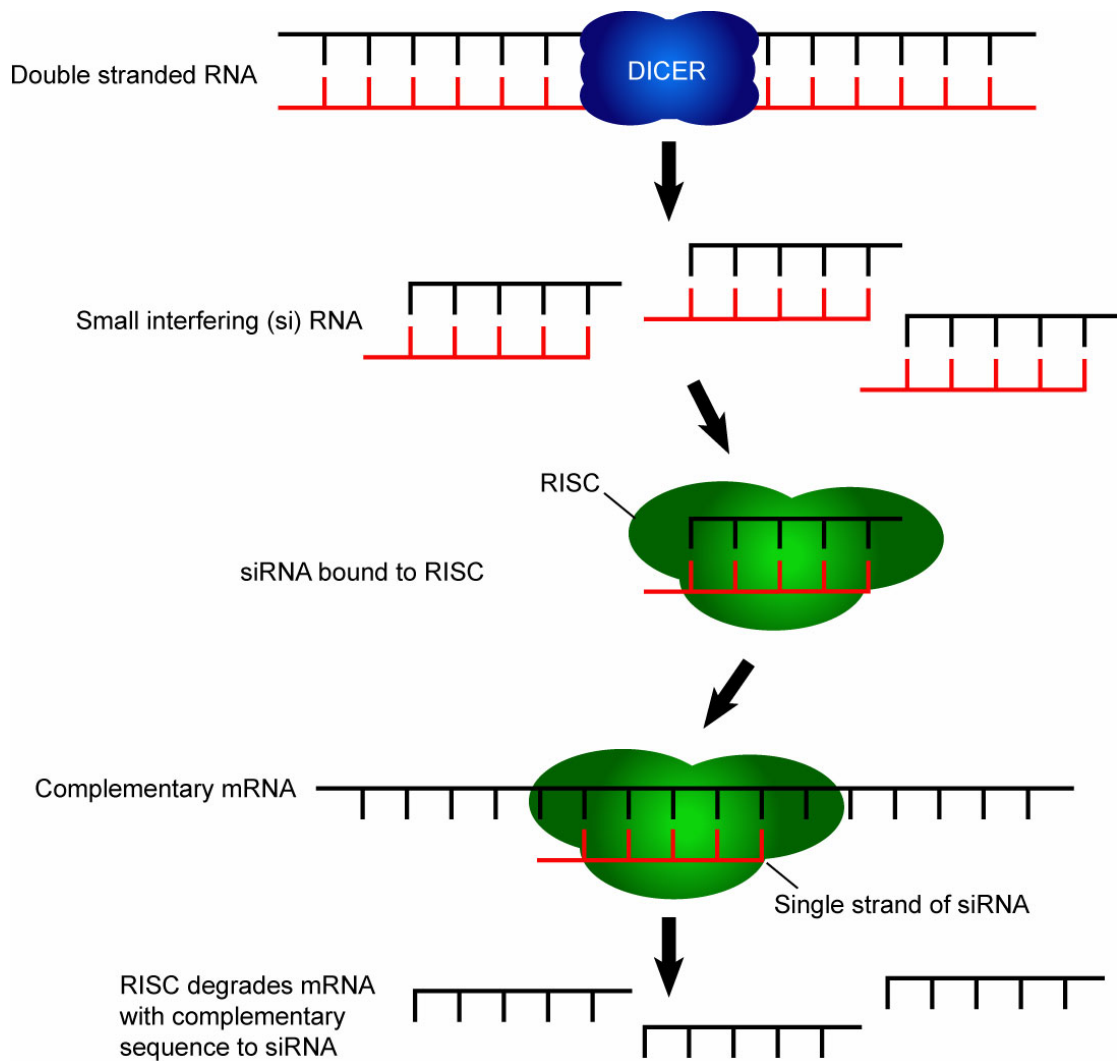


Fig. 6. Mechanisms of RNA interference. The appearance of foreign double stranded (ds) RNA within a cell -for example, as a result of viral infection - triggers an RNA interference response. The cellular enzyme dicer binds to the dsRNA and cuts it into short pieces of 21-25 nucleotide pairs in length known as siRNAs. These bind to the cellular enzyme complex RISC (RNA induced silencing complex) that uses one strand of the siRNA to recognize complementary single stranded RNA molecules. RISC degrades the mRNA, thus silencing expression of the viral gene.

2 MATERIAL AND METHODS

2.1 Material/Compounds

2.1.1 Vectors

Name	Application	Source/Supplier
pcDNA 3.1/myc-His A	expression of recombinant myc- His tagged proteins in eukaryotic cell lines	Invitrogen, Basel, Switzerland
pCR II-Topo	Cloning vector for PCR fragments	Invitrogen, Basel, Switzerland
pRSETB	expression of recombinant 6His tagged proteins in <i>E. coli</i>	Invitrogen, Basel, Switzerland
pIRES (G α 16)	expression of recombinant G α 16	Carmen Barske (Novartis, NIBR, Basel Switzerland).

2.1.2 Cell Lines

Name	Application	Source/Supplier
HEK293	Transient and stable transfection, expression of recombinant proteins	ATTC, LGC Promochem, Molsheim, France
HEK293/G α 16	Coupling of G s to PLC pathway	Klaus Seuwen (Novartis, NIBR, Basel Switzerland).
HELA	Transient and stable transfection, expression of recombinant proteins	ATTC, LGC Promochem, Molsheim, France

2.1.3 *E. coli* Strains

Name	Application	Source/Supplier
Top 10	Cloning/Sequencing	Invitrogen, Basel, Switzerland
BI21 (DE3)	Protein expression	Invitrogen, Basel, Switzerland
JM109	Transformation/Sequencing	Promega, Wallisellen, Switzerland

2.1.4 Antibodies

Name	Application	Source/Supplier
PentaHis-MAb (34660)	Western blot (1:500)	Qiagen, Basel, Switzerland
Anti Rabbit IgG-HRP (#7074)	Western blot (1:1000)	Cell signaling, Bioconcept, Allschwil, Switzerland
Anti mouse IgG-HRP (#7072)	Western blot (1:1000)	Cell signaling, Bioconcept, Allschwil, Switzerland
Alexa Fluor [®] 488 goat anti rabbit IgG (A-11008)	FACS, Immunocytochemistry (1:200)	Molecular Probes, JURO Supply GmbH, Lucerne, Switzerland

2.1.5 siRNA

Name	DNA Target Sequence	Sense Antisense
siRNA control	AAT TCT CCG AAC GTG TCA CGT	r(UUC UCC GAA CGU GUC ACG U) d(TT)
		r(ACG UGA CAC GUU CGG AGA A) d(TT)
siRNA 1	AAC ATT CTA GTG ATC GTG GCG	r(CAU UCU AGU GAU CGU GGC G) d(TT)
		r(CGC CAC GAU CAC UAG AAU G) d(TT)
siRNA 2	AAC GGG TCA GAA ACC ATC GTC	r(CGG GUC AGA AAC CAU CGU C) d(TT)
		r(GAC GAU GGU UUC UGA CCC G) d(TT)
siRNA 3	AAT CCA TAC TGC GTG TGC TTC	r(UCC AUA CUG CGU GUG CUU C) d(TT)
		r(GAA GCA CAC GCA GUA UGG A) d(TT)

All siRNA were synthesized by Qiagen-Xeragon (Basel, Switzerland) in HPP purity. The siRNA were stored in 100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4

2.1.6 Antisense Oligonucleotides (ASO)

For the following studies, two different rMC4 phosphothioate protected antisense oligonucleotides (ASO) and their respective control sequences were used. rMC4 ASO 1 (MW 6438) target the rMC4 start codon mRNA on closed regions and were initially described by Obici (Obici et al., 2001).

Name	DNA Target Sequence	Product
rMC4 ASO 1	TGC AGG AAG ATG AAC TC	GsAG TTC ATC TTC CTG CsA
Mismatch ASO 1	GGA CTT AGT ACA GGA AC	CCT GAA TCA TGT CCT TG
NCH 6549.1	GCC AGC TAG CAG GTA AGG	Fs-cct taCs CsTsGs CsTsAs Gsct ggc
rMC4 ASO 2	CGA ACG GGT CAG AAA CCA	tgg ttTs CsTsGs AsCsCs Csgt tcg
Mismatch ASO 2	CGA AAG GGT AAG CAC CCA	tgg gtGs CsTsTs AsCsCs Cstt tcg
rMC4 ASO 3	TCC GTG TCC GTA CTG TTT	tcc gtGs TsCsCs GsTsAs Cstg ttt
Mismatch ASO 3	TCC TTG GCC TTA CGG TTT	tcc ttGs GsCsCs TsTsAs Csgg ttt
		small lettering : MOE-modified s : phosphorothioate linkage F: FITC

Antisense rMC4 (rMC4 ASO 2, 3), Mismatch rMC4 (mismatch ASO 2, 3) and NCH 6549.1 were synthesized by Novartis (Basel, Switzerland). rMC4 ASO 1, mismatch ASO 1 were synthesized by Biognostik (Göttingen, Germany) in HPP Purity.

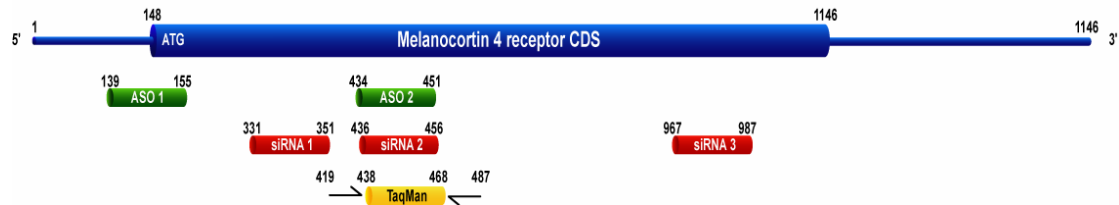


Fig. 7. Localization of ASO and siRNA constructs on the MC4-R sequence (RNU67863) Green: ASO constructs, red: siRNA constructs, Yellow: TaqMan probe and primers, CDS: coding sequence

2.1.7 Chemicals

30% Acrylamide/Bis solution (37.5:1)	Gibco BRL Life Technologies, Basel, CH
Agar, imMedia Amp Blue	Invitrogen, Basel, CH
Agarose SeaKem (electrophoresis grade)	BMA, Bioconcept, Allschwil, CH
Ammonium persulfate	BioRad, Basel, CH
Ampicillin	Fluka, Buchs, CH
Bacto tryptone	DIFCO, BD, Basel, CH
Bacto yeast extract	DIFCO, BD, Basel, CH
β -mercaptoethanol	Fluka, Buchs, CH
DMEM, Medium	Gibco Invitrogen, Basel, CH
DMSO	Fluka, Buchs, CH
dNTPs (100mM each)	Pharmacia, Dübendorf, CH
DTT	BioRad, Basel, CH
EDTA	Sigma, Buchs, CH
Ethidium bromide	Merck, Darmstadt, D
FBS	Gibco Invitrogen, Basel, CH
Geneticin (G418)	Gibco Invitrogen, Basel, CH
Gentamicin	Gibco Invitrogen, Basel, CH
Hygromycin B	Gibco Invitrogen, Basel, CH

IPTG	Roche, Basel, CH
L-Glutamine 200mM	Gibco Invitrogen, Basel, CH
NaCl	Fluka, Buchs, CH
NaF	Fluka, Buchs, CH
NaHCO ₃	Merck, Darmstadt, D
NDP- α -MSH	Bachem, Bubendorf, CH
Ni-NTA	Qiagen, Basel, CH
Non fat dry milk	BioRad, Basel, CH
Nonidet P-40 detergent	Fluka, Buchs, CH
PBS	Gibco Invitrogen, Basel, CH
Phenylmethylsulphonylfluoride (PMSF)	Fluka, Buchs, CH
Probenecid	Sigma, Buchs, CH
SDS	BioRad, Basel, CH
Sodium deoxycholate	Sigma, Buchs, CH
Sodium orthovanadate	Sigma, Buchs, CH
Sucrose	Sigma, Buchs, CH
Paraformaldehyd	Sigma, Buchs, CH
TEMED	BioRad, Basel, CH
Tris base	Sigma, Buchs, CH
Triton X-100	Sigma, Buchs, CH
Tween 20	BioRad, Basel, CH
Urea	Merck, Darmstadt, D

2.2 *In Vitro* Methods

2.2.1 General Cloning/Transformation/DNA Purification

DNA constructs were carried out according to standard cloning protocols (Ausubel et al., 1994; Sambrook et al., 1989). Restriction enzymes (Roche, Basel, Switzerland) were used as described in the protocols of the manufacturer. The Topo cloning method (Invitrogen) was carried out with the pCR II-TOPO-vector according to the manufacturer's protocol. Briefly, the method takes advantage of the template-independent activity of *Taq* DNA polymerase adding a single adenosine nucleotide to the 3' end of double stranded DNA.

This PCR product can be inserted into the thymidine-tailed vector pCR II-TOPO-vector without any further treatment. PCR products were purified by gel extraction according to manufacturer's protocol (QIAquick gel extraction Kit, QIAGEN, Basel, Switzerland) and the concentration of eluted DNA was measured with a GeneQuant II (Amersham, Otelfingen, Switzerland). Top 10 bacteria were transformed according to the manufacturer's protocol.

2.2.2 Analysis of Transformed Clones

Liquid cultures of *E. coli* clones pre-selected on agar plates were incubated overnight. The Qiagen Miniprep System (Qiagen, Basel, Switzerland) was used to obtain pure plasmid DNA for subsequent approaches (protein expression, DNA sequencing) according to the manufacturer's protocol. Plasmid DNA was digested with appropriate restriction enzymes to analyze the insertion of PCR fragment. All restriction digests were performed as recommended by the manufacturer, using the provided 10x reaction buffer. Separation of the DNA fragments was done by agarose gel electrophoresis.

2.2.3 Cloning of C- term rMC4-R Construct

The C-term sequence (36 amino acids) of the rMC4-R was obtained by PCR amplification (63°C, 30 cycles) on the pcDNA3.1ratMC4 vector using Amplitaq Gold polymerase (Roche, Basel, Switzerland) and the following oligos: 5' **CCCATATG-ACAGATCTCATCGACCCTCTCATTTATG** (blue: Nde I, red: Bgl II restriction enzyme site) and 5' **CAAGCTTGGATCC**TTAATACCTGCCAGG TAAC (blue: Bam HI, red: Hind III restriction enzyme site). The PCR fragment was purified in an agarose gel and then cloned into pCR II-Topo (Invitrogen). After sequencing, the correct construct was digested with Bgl II/Hind III and cloned into pRSETB (Invitrogen) cut with Bam HI/Hind III (Fig. 8).

2.2.4 Protein Expression and Purification of Recombinant C- term rMC4

Expression and purification of the protein constructs were done following the manufacturer's protocol (QIAexpressionist Qiagen). Briefly, *E. coli* cells, transformed with one of the expression vectors, were inoculated into 25 ml L-Broth (10 g Bacto-Trypton, 5 g yeast extract, 10 g NaCl in 1 L H₂O) using 100 mg/L ampicillin and incubated overnight. Fresh L-Broth with antibiotics was inocu-

lated with the overnight culture (20 ml overnight culture / 1 L fresh medium) the following day. The cells were grown at 37°C to $OD_{600} \text{ nm} = 0.5-0.8$, when the protein production was induced by addition of IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation after four hours of shaking. The pelleted cells were resuspended in lysis buffer (100 mM NaH_2PO_4 , 10 mM Tris-Cl, 6 M GuHCl, pH 8.0). The protein solution was centrifuged at 4300 rpm for 10 min at 4°C to pellet the cellular debris. The supernatants containing solubilized fusion proteins were mixed with appropriate amounts of Ni-NTA-Superflow (Qiagen, Basel, Switzerland) and incubated for 1 h at room temperature on a rotating wheel. The lysate-Ni-NTA-Superflow mix was loaded onto a column and washed twice with wash buffer (100 mM NaH_2PO_4 , 10 mM Tris-Cl, 8 M Urea, pH 6.3). Purification was performed with a pH step gradient. The recombinant protein was eluted with elution buffer (100 mM NaH_2PO_4 , 10 mM Tris-Cl, 8 M Urea, pH 4.5). 20 μl of each protein fraction were analysed by sodium dodecyl sulphate-polyacrylamide gel electro-phoresis (SDS-PAGE) to evaluate the purity of the protein. Protein bands were visualized by staining the gel in Coomassie blue solution. Pure protein fractions were pooled. The proteins were stored at -70°C until required.

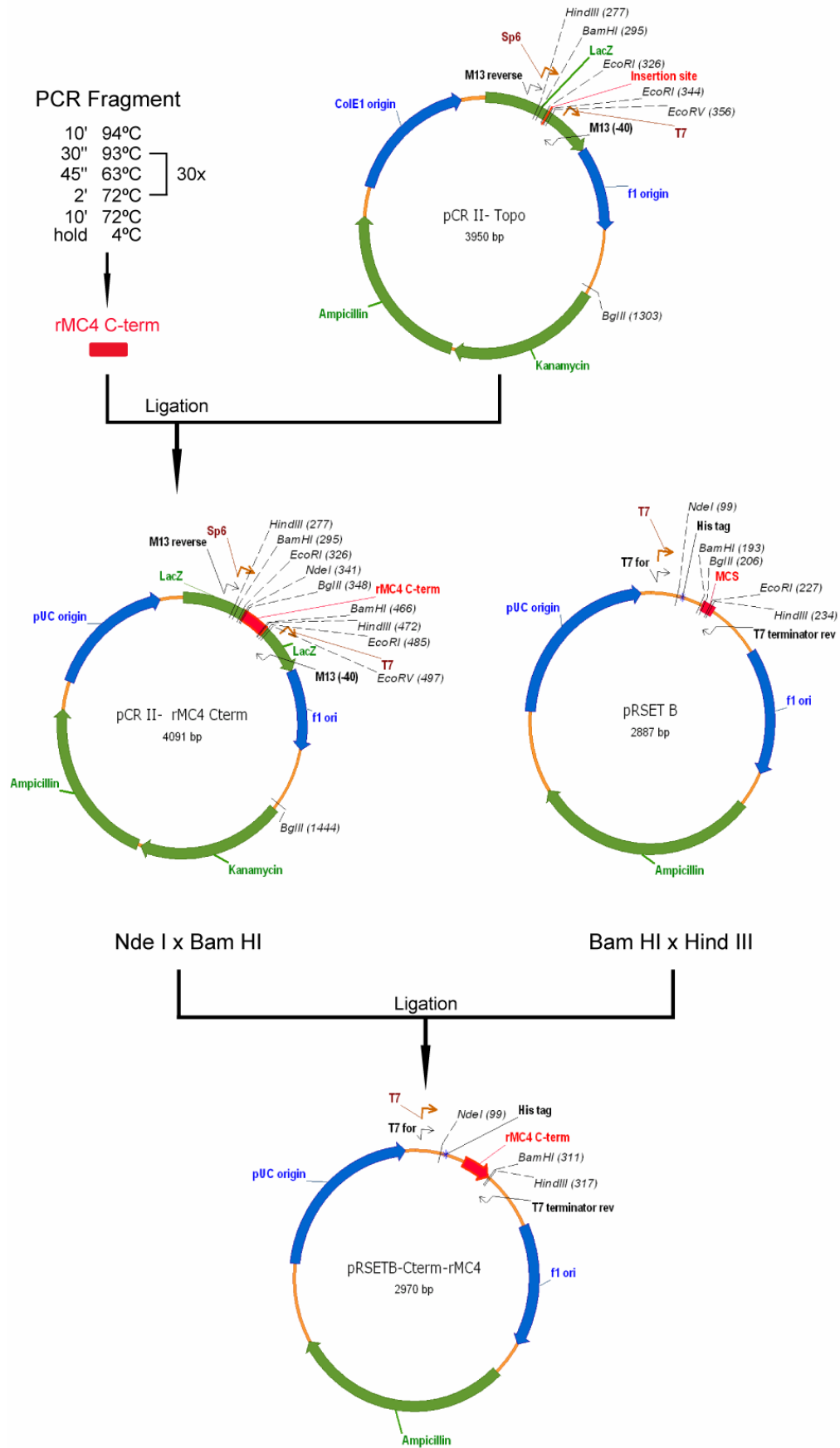


Fig. 8. Cloning scheme of the rat MC4 receptor C-terminal part for antigen production.

2.2.5 Antibody Production and Immunoaffinity Purification

The peptide was linked with the help of the Imject Maleimide activated Immunogen Conjugation Kit (Pierce, Socochim, Lausanne, Switzerland) to a carrier molecule (KLH: keyhole limpet hemocyanin) to increase its immunogenicity. Immunization of two rabbits was done at Davids Biotechnologie (Regensburg, Germany).

The rabbit sera were tested in an ELISA and Westernblot and the sera were stored at $-70\text{ }^{\circ}\text{C}$ until required.

The serum of one rabbit was immunopurified according to the following method:

The recombinant peptide solution (see 2.2.4) was dialysed against coupling buffer (0.1 M NaHCO_3 , 0.5 M NaCl pH 8.3) to remove urea. 1g of CNBr-activated Sepharose 4 B (Amersham Biosciences, Otelfingen, Switzerland) was washed and rehydrated with 200 ml of 1 mM HCl to preserve the activity of the reactive groups which hydrolyze at high pH. 1g resulted in 3.5 ml of activated gel suspension. The gel was then washed with coupling buffer (0.1 M NaHCO_3 , 0.5 M NaCl , pH 8.3 (5 ml per gram dry gel)). This material should be used without delay since reactive groups on the gel hydrolyze at the coupling pH 8.3. 0.75 ml of gel was mixed with 1.2 mg of the dialyzed peptide in a final volume of 3 ml coupling buffer for 2 h on a rotating wheel at room temperature. After sedimentation of the gel the supernatant was saved because it could still contain antigen. To eliminate uncoated active groups the gel was incubated with 0.2 M glycine pH 8.0 for 2 h at room temperature. The gel was then washed with 5 volumes of 0.1 M NaAcetate / 0.5 M NaCl pH 4.0 followed with one volume of coupling buffer to remove unbound protein.

The antigen-coupled sepharose was rinsed with 10 mM Tris-HCl pH 7.4 and the gel let sediment slowly. The sedimented gel was washed with 4 ml 10 mM Tris-HCl pH 7.4, followed with 6 ml of 100 mM glycine (HCl) pH 2.5. The column was neutralized with a solution of 10 mM Tris-HCl pH 8.8 and washed with 5 ml of 100 mM Triethylamine (prepared freshly: 0.7 ml (100%) solution in 50 ml H_2O , pH to 11.5 with HCl , $M_w = 100.19\text{ g/moles}$; $d = 0.75$). Then the gel

was washed again with 4 ml of 10 mM Tris-HCl pH 7.4 until eluted solution was neutral. In between 20 ml of 1:1 in PBS diluted antibody serum was centrifuged at 9500 g (SS-34, 9000 rpm) at 4°C for 10 min. The clear supernatant was loaded onto the pre-washed column. The flow-through was collected and passed a 2nd time through the column. The column was washed with 8 ml of 10 mM Tris-HCl pH 7.4, followed by 8 ml of 10 mM Tris-HCl, 0.5 M NaCl, pH 7.4.

The bound antibodies were eluted with 5 x 1 ml of 100 mM glycine-HCl pH 2.5 and the fractions were collected directly into tubes containing 100 µl 1 M Tris-HCl pH 8.0. The column was washed with 4 ml of 10 mM Tris-HCl pH 8.8 until the flow-through solution was neutral.

Additionally bound antibodies were eluted with 5 x 1 ml of 100 mM Triethylamine pH 11.5 and the fractions were collected directly into tubes containing 100 µl 1 M 10 mM Tris-HCl pH 8.0. The column was re-equilibrated with 10 mM Tris-HCl pH 7.4 and stored in 10 mM Tris-HCl, 0.01 M NaN₃ pH 7.4.

2.2.6 Cell Culture

HEK293 cells were maintained in monolayer cultures in DMEM media, supplemented with 10% fetal bovine serum, 50 µg/ml gentamicin, and if needed 500 µg/ml geneticin (G418), 250 µg/ml hygromycin B in a humidified incubator at 37°C, in an atmosphere of 5% CO₂.

2.2.7 Generation of Stable Cells Expressing rMC4-R

The complete coding sequence of the rMC4-R was obtained by PCR amplification on rat genomic DNA using following oligos: 5' **AT-GAACTCCACCCACCA** and 5' **CTTAATACCTGCCAGGTAAC** (blue: the start codon; red: stop codon). This strategy was possible since the coding sequence of rMC4-R is encompassed in one exon. The cDNA was cloned in a pcDNA 3.1 vector and sequenced. A nucleotide difference from the published sequence (nt 765 T to C of the sequence under the GenBank™ accession number U67863) was observed which did not result in a change in the amino acid sequence. A vector (pIRES) containing the human G_{α16} was a gift of

Carmen Barske (Novartis, NIBR, Basel, Switzerland). The pcDNA3.1 rat MC4 vector was used to transfect Hek293 wild type or $G_{\alpha 16}$ -HEK293 cells (gift of Klaus Seuwen (Novartis, NIBR, Basel, Switzerland)). Transient and stable transfections were performed with the Lipofectamine Plus reagent (Gibco Invitrogen, Basel, Switzerland) according to the procedure provided by the manufacturer.

Stable clones expressing rMC4-R were selected in the presence of 400 $\mu\text{g}/\text{ml}$ of G418 or in the presence of 400 $\mu\text{g}/\text{ml}$ G418, 250 $\mu\text{g}/\text{ml}$ hygromycin for the clone co-expressing rMC4-R and $G_{\alpha 16}$. Co-expression of rMC4-R with $G_{\alpha 16}$ allows measuring MC4-R mediated signaling in the FLIPR.

2.2.8 RNA Extraction from Cultured Cells

Cells of a 35 mm \varnothing were lysed in 200 μl of RLT buffer (RNeasy Mini Kit, Qiagen, Basel, Switzerland) following the manufacturer's protocol. The lysates were centrifuged through a Qias shredder column, mixed with an equal volume of 70% ethanol and transferred to the RNeasy column. The DNA digestion step was performed on the column for 12 min at room temperature. The total RNA was eluted either with 30 μl (for 5×10^5 starting cells) or with 50 μl (for 5×10^6 starting cells) using RNase-free water and immediately stored at -70°C .

2.2.9 RNA Extraction from Rat Tissue

After ASO or siRNA treatment, rats were killed by decapitation after CO_2 anaesthesia. Hypothalami were dissected, weighed, divided in two portions and immediately stored in the RNA/later RNA Stabilization Reagent (Qiagen, Basel, Switzerland) until RNA extraction with the RNeasy Mini Kit (Qiagen, Basel, Switzerland).

RNA extraction was performed according to the protocol provided by Qiagen. Hypothalami (160 mg in 1000 μl RLT buffer, Qiagen) were placed in FastRNA Tube (Bio101, Qbiogene, Basel, Switzerland) and homogenized with the Fast-Prep FP120 instrument on level 6 for 45 s (Bio101, Qbiogene, Basel, Switzerland). The lysate was centrifuged for 3 min at maximum speed and an equivalent of 30 mg tissue (190 μl) was transferred to an Eppendorf tube (Vaudeaux-

Eppendorf, Schönenbuch, Switzerland). 410 µl of buffer RLT were added, mixed with an equal volume of 70% ethanol and transferred to the RNeasy column. The DNA digestion step was performed on the column for 12 min at room temperature using the RNase-free DNase set (Qiagen, Basel, Switzerland). After the washing steps, the total RNA was eluted with 35 µl using RNase-free water and immediately stored at -70 °C.

2.2.10 RNA Quantification

The quality of the RNA was assessed by Agilent 2100 Bioanalyzer (Agilent, Basel, Switzerland) which showed intact 28S and 18S rRNA species.

The concentration of RNA was determined by the RiboGreen RNA Quantitation Kit (Molecular Probes, JURO Supply GmbH, Lucerne, Switzerland)

2.2.11 cDNA Synthesis and RT-PCR

1 µg from each RNA sample was reversely transcribed to first strand cDNA by using 100 ng (final concentration 1 µM) random hexamer primer (pd(N)₆, Amersham Pharmacia Biotech Inc.), 2 µl dNTP-Mix (5mM each nucleotide), RNase Inhibitors (4 U RNasin, Promega), 2 µl 10x Master Mix (Omniscript Reverse Transcriptase Kit, Qiagen) and 4 U Omniscript reverse transcriptase (Qiagen) in 20 µl at 37°C for 60 minutes followed by 30 min at 42°C.

cDNA equivalent to 20 ng total RNA were used for the TaqMan PCR. PCR primers and fluorogenic probes were designed using Primer Express software (Applied Biosystems, Rotkreuz, Switzerland). The primers were synthesized by Microsynth (Balgach, Switzerland) and purified by high-performance liquid chromatography (HPLC). The fluorogenic probes contained a reporter dye (FAM, 6-carboxy-fluorescein) covalently linked at the 5' end and a quencher dye (TAMRA, 6-carboxy-tetramethyl-rhodamine) covalently attached at the 3' end.

The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (huGAPDH, Applied Biosystems No. 4310884E; rodent GAPDH, No. 4308313) or the 18S ribosomal RNA (rRNA, Applied Biosystems No. 4308329) were used as endogenous reference for the amount of total RNA in the sample. Real time PCR was performed in the ABI PRISM 7700 or

7900HT. Standard thermal cycling conditions are 10 min at 95°C for the activation of AmpliTaq Gold followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C (annealing and extension phase). Forward, reverse primers and TaqMan probe concentrations are listed in the table below. The 2x TaqMan Universal PCR Master Mix (Applied Biosystems, No 4324018) contained dATP, dCTP, dGTP, dUTP, AmpliTaq Gold in optimized concentrations. The total volume was 50 µl for duplex reactions and 25 µl for single reactions.

Used primer sequences and concentration:

Gene	5' Forward primer 3'	Final conc.	5' Reverse primer 3'	Final conc.	5' Probe 3'	Final conc.
rMC4R	tgctggtagcggttcga	300 nM	cgcccggtgcccactgttttag	900 nM	cgggtcagaaaccatcgcatcacc	150 nM

The relative quantification was done by using either the standard curve method or the comparative C_T method as described in the user bulletin #2 (PE Applied Biosystems).

2.2.12 Transfection of ASO and siRNA

The day before transfection, Hek293 cells and derived cell clones were plated on poly-D-lysine coated plates (0.1 mg/ml poly-D-Lysine, BD Bioscience, Basel, Switzerland) or poly-D-lysine pre-coated plates: 4 x 10⁵ cells were used for a 35 Ø mm wells. The cells were cultured in their normal growth medium containing serum and antibiotics.

HEK293/rMC4/G_{α16} cells were treated with the indicated concentration of antisense oligonucleotides (ASO, Novartis, Basel, Switzerland) per plate using Effectene (Qiagen, Basel, Switzerland) or DOTAP (Roche, Basel, Switzerland) according to the manufacturer's protocol. The cells were harvested 24h after transfection for analysis of mRNA expression and 48h after transfection for FLIPR assay.

HEK293/rMC4/G_{α16} cells were transfected with 100 nM or 200 nM siRNA (Qiagen, Basel, Switzerland) lipofectamine 2000 (Gibco, BRL) according to the manufacturer's protocol.

In the case of siRNA, Lipofectamin 2000 (Invitrogen) reagent was used. For a

35 mm Ø plate 20 µl of a 5.5 µM siRNA stock were diluted into 240 µl of OPTI-MEM I and incubated for 10 min at room temperature. 12.6 µl of Lipofectamin 2000 (LF2000) reagent were diluted into 420 µl OPTI-MEM I (GibcoBRL, Basel, Switzerland) medium and incubated for 5 min at room temperature. The diluted siRNA were combined with the diluted LF2000 reagent at room temperature for 20 min. The DNA-LF2000 reagent complexes (680 µl) were directly added to the cell, which were overlaid with 400 µl of OPTI-MEM.

The cells were incubated at 37°C in a CO₂ incubator for 4 h. After the addition of 960 µl DMEM with 20% FBS the cells were incubated for total of 24 h.

2.2.13 Fluorometric Imaging Plate Reader (FLIPR®) Assay

Eighteen to twenty-four hours after transfection, HEK293/rMC4 cells were replated onto a 384-well plate at a density of 12'500 cells per well in a volume of 25 µl and cultured additional for 18–24 h.

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 reconstituted in 10 ml of Hank's balanced salt solution (HBSS), 20 mM HEPES and 2.5 mM probenecid, pH 7.4 (FLIPR buffer). Cells were loaded with the calcium-sensitive dye in 25 µl/well for 1 h at 37°C. The compound plate and cell plate were placed in the FLIPR2 (Molecular Devices). The basal fluorescence signal from the dye-loaded cells was collected for 20 sec prior to the addition of the agonist. 10 µM ATP were added to the dye-loaded cells to check for the integrity of the cellular response. The addition of the agonist solution (dilutions from 10⁻⁷ to 10⁻¹⁰ M in 0.1% fatty acid free BSA/FLIPR buffer) was performed 20-30 min after the addition of ATP, after the cells had recovered and re-established the basal level of internal Ca²⁺. Experiments where the pre-testing with ATP was omitted, gave essentially the same results. Fluorescence data were collected at 1 s intervals for the first 60 s and at 2 s intervals for an additional 80 s. Peak fluorescence counts during the 20- to 40-s time points were used to determine the MC4-R activity.

FLIPR responses were measured as peak fluorescence related to basal fluorescence with the following equation: $(F_{\max} - F_{\min}) / F_{\min}$ where F_{\max} is the peak height after the addition of agonist and F_{\min} corresponds to the fluorescent basal counts before the addition of the agonist.

2.2.14 Total Membrane Preparation (Freeze & Thaw)

Cells are rinsed once in and scraped in (for a Ø 35 mm plate = 6 well) 300 µl PBS or TBS (10 mM TrisHCl, pH 7.5 and 140 mM NaCl) and centrifuged at 350 g. The supernatant is carefully discarded, the cell pellet is resuspended in 200 µl hypotonic buffer (10 mM Tris.HCl pH 7.8-8.0) and allowed to swell on ice for 10-15 min (cell density of $1-10 \times 10^6$ cells/ml). After addition of 1/25 anti-protease-mix-ready (Boehringer) the cells are homogenized by freezing and thawing (three times at -70°C , until frozen; then 3 times at 37°C until melted). The homogenate is centrifuged for 15 min at $14'000$ g at 4°C (Eppendorf centrifuge). The supernatant is discarded and the pellet is resuspended (with the help of a Hamilton syringe) in 100-200 µl of 5 mM Tris.HCl pH 8.0, 10% sucrose. The membranes are stored at -70°C .

2.2.15 Total Protein Extraction

Total protein extraction was performed by using two different lysis solutions. After washing with cold PBS, cells were scraped into RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40 detergent, 1 mM PMSF, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM NaF, and 0.25% sodium deoxycholate and proteinase inhibitors (Complete mini, Roche, Basel, Switzerland) and incubated on ice for 30-60 min. Alternatively the cells were lysed in 10 mM Tris.HCl pH 8.8, 0.5% Triton X100, 150 mM NaCl and protease inhibitors mix (Complete mini, Roche, Basel, Switzerland).

2.2.16 Cytosol and Membrane Fractionation

Cells were harvested on ice; all procedures were performed at 4°C . Cells were washed twice with ice-cold PBS, scraped into PBS and centrifuged at 2000 rpm for 5 min. The cells (pellet) are resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.8) and were allowed to swell on ice for 15 min (density of cells around 4×10^6 cells/ml). After the addition of 1/100 protease inhibitor mix (Roche, Basel, Switzerland) and 0.5 mM DTT the cells were homogenized

with 30 strokes in a tight-fitting glass Wheaton Dounce homogenizer (Fisher Scientific AG, Wohlen, Switzerland). The homogenate was diluted two times with 10 mM Tris-HCl, pH 7.8, 20% sucrose, 300 mM KCl. The homogenate was centrifuged at 750 g (RCF) for 10 min at 4°C. Supernatants (collected as postnuclear cell lysates) were centrifuged at 100'000 g (43'000 rpm TLA 100.3, Beckman TL-100) for 30-60 min, and the resulting supernatant was used as the cytosolic fraction. The pellet was resuspended (with the help of a Hamilton syringe) in Tris-HCl, pH 8.0, 10% sucrose. The membranes were divided in two fractions and stored at -70°C.

2.2.17 Protein Measurement

The protein concentration was determined by using the BCA Protein Assay Kit (Pierce, Perbio Science Switzerland S.A., Lausanne, Switzerland).

2.2.18 Western Blot Analysis

Samples were denatured at 95°C for 5 min in Lämmli sample buffer and a total of 20 – 30 µg of protein were loaded on SDS-PAGE (Criterion XT Bis-Tris gels, BioRad, Reinach, Switzerland) in XT MOPS or XT MES buffer. The proteins were transferred onto PVDF membrane (BioRad). For detection of tagged proteins, different antibodies were used. For signal detection, the LumiGLO detection kit (Cell signaling, Bioconcept, Allschwil, Switzerland) was used according to the manufacturer instructions. The membrane was exposed to Hyperfilm ECL chemiluminescence film (Amersham Biosciences, Otelfingen, Switzerland).

2.3 *In Vivo* Methods

2.3.1 Animals

Male Sprague-Dawley rats (SD), delivered from Charles River (France) were used at a weight of 200 g. Rats were housed individually in plastic cages at $21.5 \pm 0.5^\circ\text{C}$ on a 12 h light/ 12 h dark cycle (lights off at 6 p.m). They were acclimatized to the animal facilities at least for 7 days prior to the experiment. Food (Nafag 3432, Provimi Kliba AG, Kaiseraugst, Switzerland) and water were given ad libitum.

2.3.2 Icv Surgery

Rats were anesthetized with 3-4 vol% 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 icv 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 then fixed using Paladur dental cement (Heraeus Kulzer, Wehrheim, Germany) The positions of the cannula were verified one week after the surgery by applying 20 pmol/5 μ l angiotensin II (Sigma, Buchs, Switzerland). Animals which drunk less than 5 ml in 15 min were excluded from the experiment.

2.3.3 ASO Diffusion Analysis

Fluorescence-labelled MC4-R ASO (NCH 6549.1, Novartis Pharma AG, Basel, Switzerland) were icv injected in a dose of 25 μ g/5 μ l (dissolved in sterile saline). Three hours after administration, animals were killed by decapitation. Brains were dissected out, postfixed 4h at 4°C in 10% paraformaldehyde/PBS, dehydrated overnight in 20% sucrose and frozen in dry ice. Coronal sections were cut at 20 μ m intervals with a cryostat (Microm HM560, Volketswil, Switzerland) and examined for fluorescence in a Zeiss Axioskop 2 plus microscope (Zeiss, Feldbach, Switzerland). 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 show the cells number.

2.3.4 Acute and Chronic icv Administration of ASO

ASO and mismatch ASO were administered either by icv injection or by chronic icv infusion via subcutaneously implanted osmotic minipumps (model 2001, Alzet, Charles River, France) over 7 days.

2.3.5 Statistics

Results are expressed as means \pm SEM (standard error of the mean) or as means \pm S.D (standard deviation). Comparisons between experimental groups were done with one way ANOVA for repeated measurements. Probability values smaller than 0.05 were considered significant.

3 RESULTS

3.1 Generation of Stable Cell Lines Co-Expressing $G_{\alpha 16}$ and the Rat MC4 Receptor

In order to evaluate the functional effect of ASO and siRNA, stable cell lines expressing the rMC4-R were generated. Since it has been reported that co-expression of the promiscuous G-protein like $G_{\alpha 16}$ allows $G_{\alpha s}$ coupled receptors as MC4-R (Offermanns and Simon, 1995) to promote mobilization of intracellular Ca^{2+} , attempts were made to generate cell lines co-expressing rMC4-R and $G_{\alpha 16}$. The Ca^{2+} mobilization and therewith the signaling through the MC4-R could be analyzed by Fluorometric Imaging Plate Reader (FLIPR[®]). In preliminary experiments, it could be shown that in cells transiently co-transfected with rMC4-R and $G_{\alpha 16}$, the MC4-R agonist NDP- α -MSH promoted a robust, concentration dependent Ca^{2+} signal (Fig. 9). No effects were observed in cell transfected with rat MC4-R or $G_{\alpha 16}$ alone. The same was observed for stable clone expressing both rat MC4-R and $G_{\alpha 16}$ (data not shown).

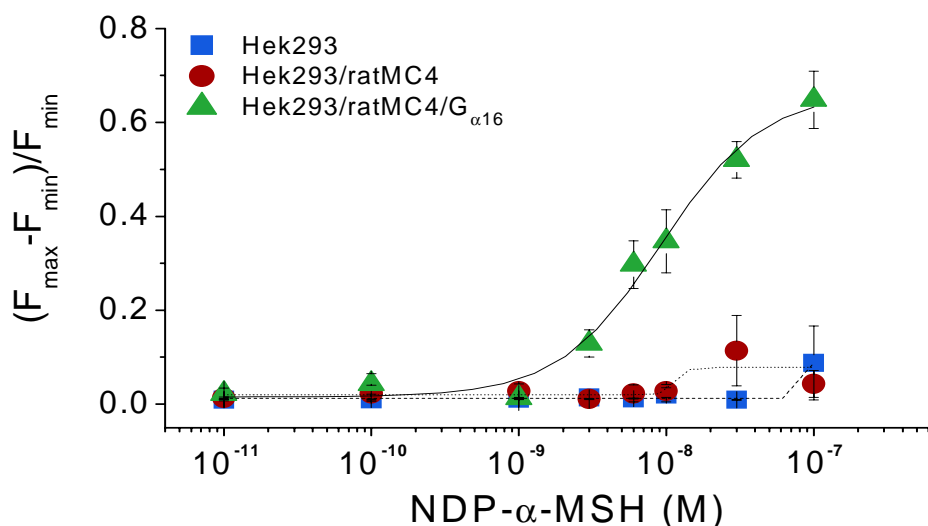


Fig. 9. Transient Ca^{2+} release of $G_{\alpha 16}$ coupled rMC4-R after agonist stimulation. Ca^{2+} release was analyzed by FLIPR[®] as described in the methods section. Ca^{2+} release (fluorescence) is dependent on the NDP- α -MSH concentration and on the construct used for transfection. Mean values \pm S.D. of at least two independent experiments.

3.2 Selection of Antisense Oligonucleotides

A whole set of about 20 antisense oligonucleotides were synthesized in the functional genomics department (Lab F. Natt, NIBR, Novartis, Basel). These ASO were tested for binding to full-length MC4-R mRNA and the best candidates were evaluated for their capacity to interfere with the translation of rMC4-R cDNA *in vitro* with the help of a GFP-reporter system. The HEK293rMC4 cell line was transfected (600 nM with DOTAP) with the two pre-selected ASO (named rMC4 ASO 2 and 3) and after 24 h rMC4-R mRNA levels were analyzed by real-time RT-PCR. ASO 2 (Fig. 10) showed the best down-regulation: after treatment with this ASO the mRNA level was lowered to 17% of that found in the untreated controls. ASO 3 was also able to down-regulate mRNA but only to 35% of control.

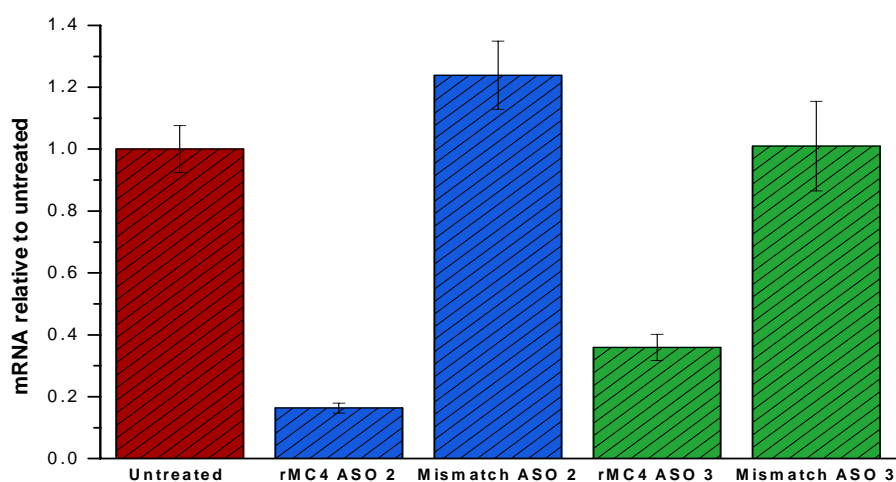


Fig. 10. Down-regulation of rat MC4-R mRNA in HEK293 by ASO. HEK293-rMC4 cells were transfected (DOTAP) with 600 nM rMC4 ASO 2 or 3 and the Mismatch ASO 2 or 3. After 24 h RNA was extracted, reverse transcribed and analyzed by Real-time RT-PCR. mRNA levels are relative to untreated cells. Mean \pm S.D. of two independent transfections (measured in triplicate) are shown.

To optimize the ASO effect, different transfection conditions and transfection agents were evaluated. The best results were obtained 36 h after transfection with Effectene (data not shown). The optimal ASO concentration for the Hek293rMC4 cells was also determined (Fig. 11) with Effectene. The down-regulation of the rMC4-R specific mRNA by ASO 2 was concentration de-

pendent and at 600 nM less than 5% of the amount of rMC4-R mRNA present in untreated cells was detectable. Higher concentrations only marginally promoted further down-regulation. The corresponding mismatch ASO had no effect on the rMC4-R mRNA levels. No toxicity as measured by the viability of the cells was observed with any of the ASO used.

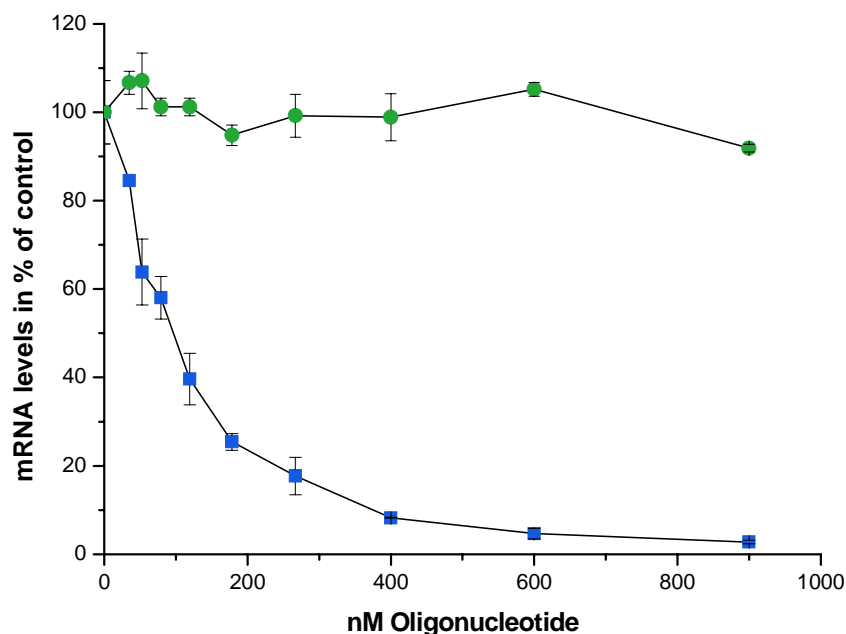


Fig. 11. Down-regulation of rat MC4-R mRNA in HEK293 after transfection with ASO revealed by TaqMan Analysis. HEK293-rMC4 cells were transfected (Effectene) with rMC4 ASO 2 (blue squares) or Mismatch ASO 2 (green circles). After 36 h RNA was extracted and analyzed with Real-time RT-PCR. mRNA levels in % of control (untreated cells) \pm S.D. of two independent transfections (measured in triplicate) are shown.

To demonstrate that ASO 2 resulted in down-regulation of the protein, functional assays based on rMC4-R dependent intracellular Ca^{2+} release were performed. In HEK293rMC4 cells 600 nM ASO 2 promoted a strong reduction of the NDP- α -MSH signal at different concentration of agonist (Fig. 12), which was particularly pronounced at 10^{-7} M (up to 80% reduction compared to mock treated cells). An effect was observed with the mismatch oligonucleotide, which indicated that after 48h of treatment some unspecific effects might become apparent. ASO 2 effects were becoming weaker after 72 h (not shown), probably because the ASO concentration in the cells were becoming

limiting. Therefore weak to no effects were observed after 72h transfections in the FLIPR® (data not shown).

The effect of the ASO 2 would correspond to a reduction of around 60% of the

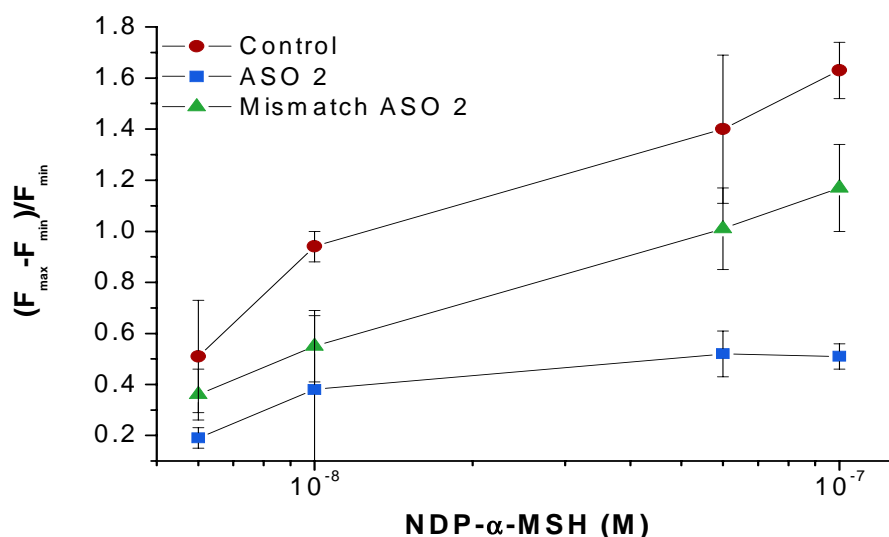


Fig. 12. The effect of the transfection with rMC4 ASO 2 on the activity of rMC4 receptor expressing HEK293 cells. HEK293 cells expressing rMC4 receptor were transfected with the rMC4 ASO 2 or the mismatch ASO 2 (both at 600 nM). 48 hours later the agonist induced transient Ca²⁺ release was analyzed with FLIPR® as described in the methods section. The concentrations of NDP- α -MSH were 6 × 10⁻⁹, 10⁻⁸, 6 × 10⁻⁸, 10⁻⁷ M. Ca²⁺ release as measured by fluorescence changes are shown. Mean values \pm S.D. of two independent experiments.

signal if compared to the mismatched ASO. Experiments repeated at lower concentrations of ASO 2 confirmed the data presented in Fig. 12. Intracellular Ca²⁺ mobilization by ATP was not affected by the treatment (not shown).

3.3 ASO Distribution and Uptake in the Brain

The next step was to analyze the delivery of ASO *in vivo*. To study the uptake and the distribution of ASO in the rat brain fluorescein-labeled ASO was injected into the left lateral ventricle. Immunofluorescent microscopy images (Fig. 13) were analyzed (see 2.3.3) and they indicated a dose and time dependent uptake of fluorescein-labeled ASO in the rat brain after a single icv injection. ASO exhibited rapid uptake by ventricular ependyma and cells in the immediate vicinity of the injection site. Neurons along the injection cannula showed labeling (Fig. 13, 0.5 h, arrow). Fluorescent staining of neurons over a 3–5 mm distance from the injection site was observed by 2 hours, while more

diffused staining is detectable by 24 hours. Remarkably, the labeled cells are often located in the proximity of blood vessels or the ventricles (Fig. 13, 2 h, arrow). No fluorescein labeled ASO was detected in control brains injected with PBS vehicle.

After 3 hours the distribution of the fluorescence had progressed to the thalamus and hypothalamus (Fig. 14). The ASO stained cells were detectable in the paraventricular hypothalamic nucleus (PVN), the region where rMC4-R is predicted to be expressed at high levels (Fig. 14). Icv injection of 50 μg fluorescent ASO increased the amount of labeled cells compared to 25 μg in the proximity of the third ventricle after 3 hours (Fig. 14). Increasing the amount of ASO injected to 100 μg had no additional effect (data not shown).

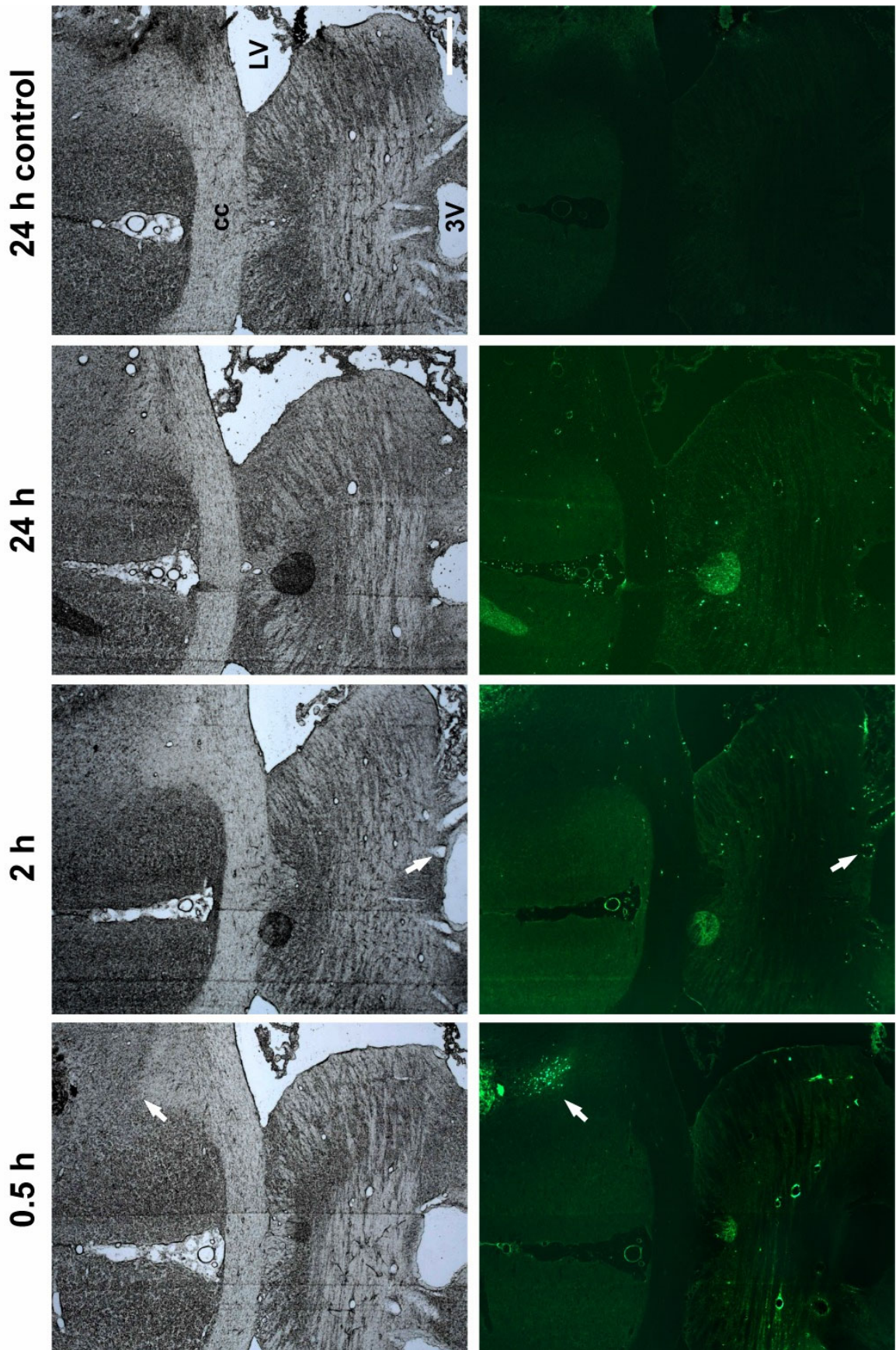


Fig. 13. Distribution and kinetics of fluorescein labeled oligo-nucleotides at various times after icv injection into the rat brain. Legend see next page.

Fig. 13. . **Distribution and kinetics** of fluorescein labeled oligo-nucleotides at various times after icv injection into the rat brain. Arrows indicated regions where many neurons showed intensive fluorescence.

Distribution and cellular uptake of fluorescein-labeled ASO (NCH 6549.1) in the rat brain. The ASO (25 μg) were icv injected into the left lateral ventricle (LV). Coronal section of lateral ventricle of the rat brain, Bregma -0.8 mm at different time points (30 min, 2 h, 24 h). Upper panels: Light microscopy. Lower panels: Fluorescence microscopy. Negative control: icv injection of PBS vehicle. Bar = 440 μm

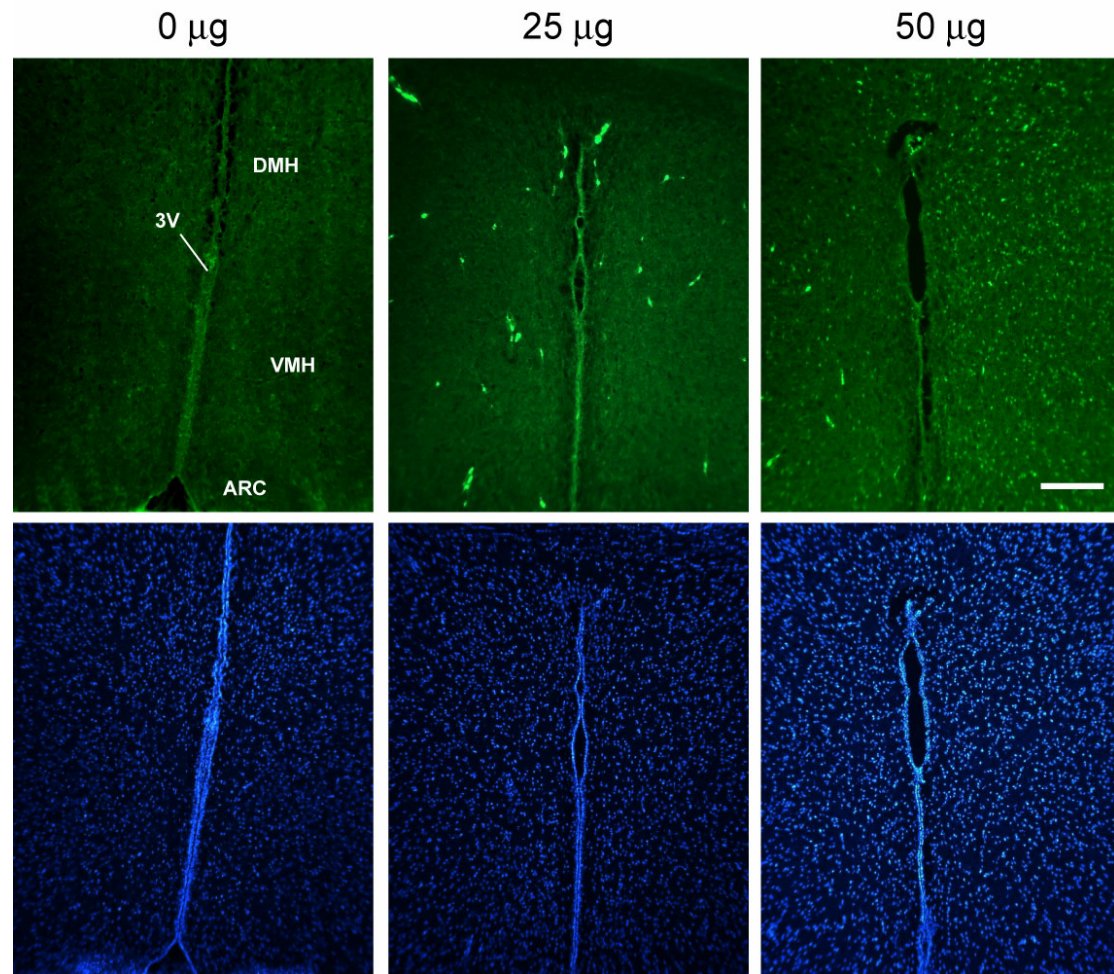


Fig. 14. Concentration dependent effect of ASO injection. Coronal section of the third ventricle (3V) of the rat brain, Bregma -3.0 mm, 3 hours after injection with different amount of labeled ASO (0 μg , 25 μg , 50 μg). After preparation of the brain sections slides were stained with Hoechst 33258 for nuclear staining and analyzed with fluorescence microscopy. Dorsomedial nucleus (DMH), ventromedial nucleus (VMH), arcuate nucleus (ARC).

Upper panels: A specific filter for FITC staining was used to detect the labeled ASO.

Lower panels: The same image as in the upper panel but with a specific filter for nuclear staining with Hoechst 33258 was used. Negative controls (0 μg) are PBS injected rat brains. Bar = 110 μm

The colocalization of ASO labeled cells with stained nuclei (Fig. 15) suggested cytoplasmic and nuclear uptake of ASO. The strongest labeling by the fluorescent ASO was visible within the ependymal cell lining of the lateral ventricle and cells of the choroid plexus. These observations indicate that MOE-modified ASO rapidly penetrated into the brain parenchyma and are taken up into the cytosol and nuclei of neurons. It was estimated that around 8-10% of all cells (see 2.3.3) have taken up labeled ASO in this picture.

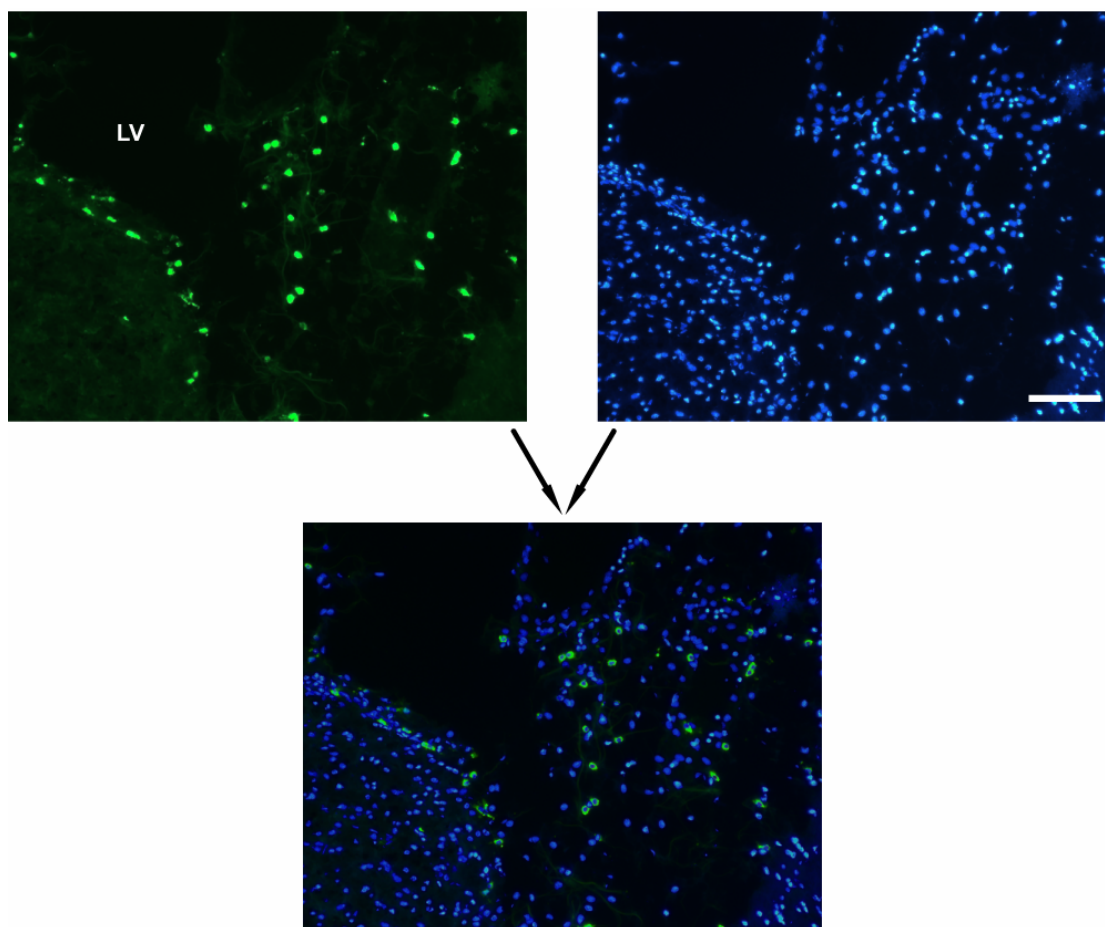


Fig. 15. Evaluating the efficiency of the ASO taken up by neuronal cells. Coronal section of the lateral ventricle (LV) of the rat brain, Bregma -1.2 mm, 3 h after injection of labeled ASO (25 μ g). Epithelial lining of the lateral ventricle and cells of the choroid plexus are shown. Fluorescence microscope images of the same section taken with the filter for FITC (left upper panel) and for Hoechst nuclear staining (right upper panel) as indicated in Fig. 14.

Lower panel: Overlay of the upper panels for co-localization analysis. Bar = 55 μ m

3.4 *In Vivo* Experiments with ASO

Obici et al. (Obici et al., 2001) had published data showing ASO mediated down-regulation of MC4-R. The ASO (ASO 1) used by Obici et al. was prepared (Biognostik) and tested *in vivo* to reproduce their results.

3.4.1 Acute Application of ASO 1

MC4-R ASO 1 and mismatch controls were administered icv in rats at two consecutive days: two times (in the morning and afternoon) 25 µg/rat/injection on day one and two times (in the morning and afternoon) 50 µg/rat/injection on day two. Food intake was measured over four days at 12 h intervals. ASO 1 showed no significant effect on food intake, neither on the two days where it was applied nor on the consecutive two days (Fig. 16).

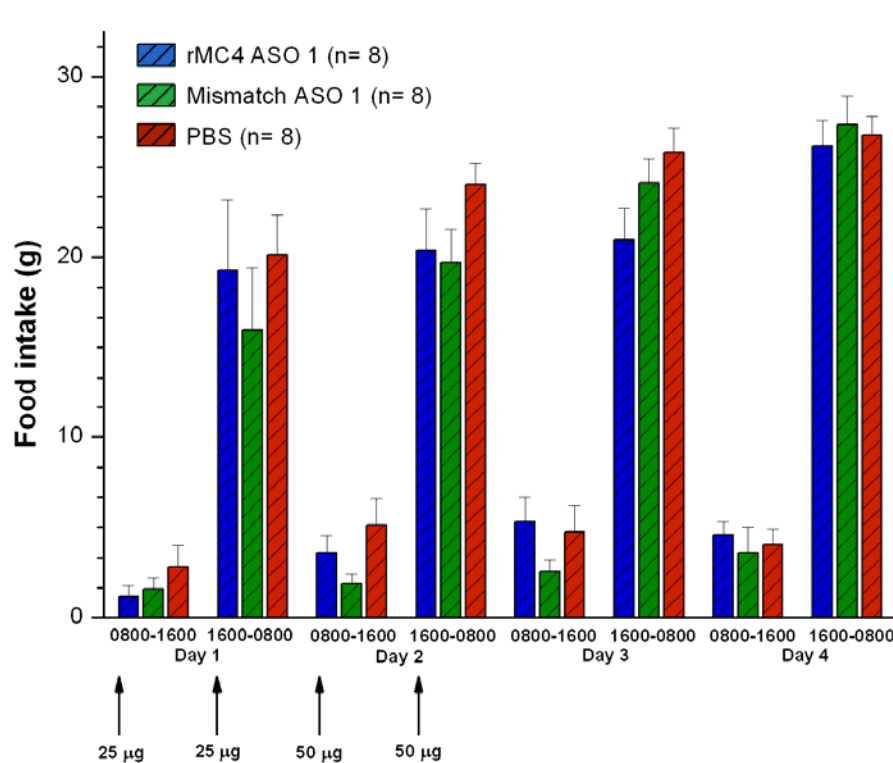


Fig. 16. Food intake after icv injection of ASO 1. SD rats were treated with rMC4 ASO 1, Mismatch ASO 1 and PBS (control). Concentrations are given on the x-axis. Arrows: Application time points: 1./3. application 1 h after onset of light phase. 2./4. application 1 h before onset of dark phase. Blue bars: rMC4 ASO 1; Green bars: Mismatch ASO 1; Red bars: Control 5 µl PBS. Mean values +SEM. n = 8.

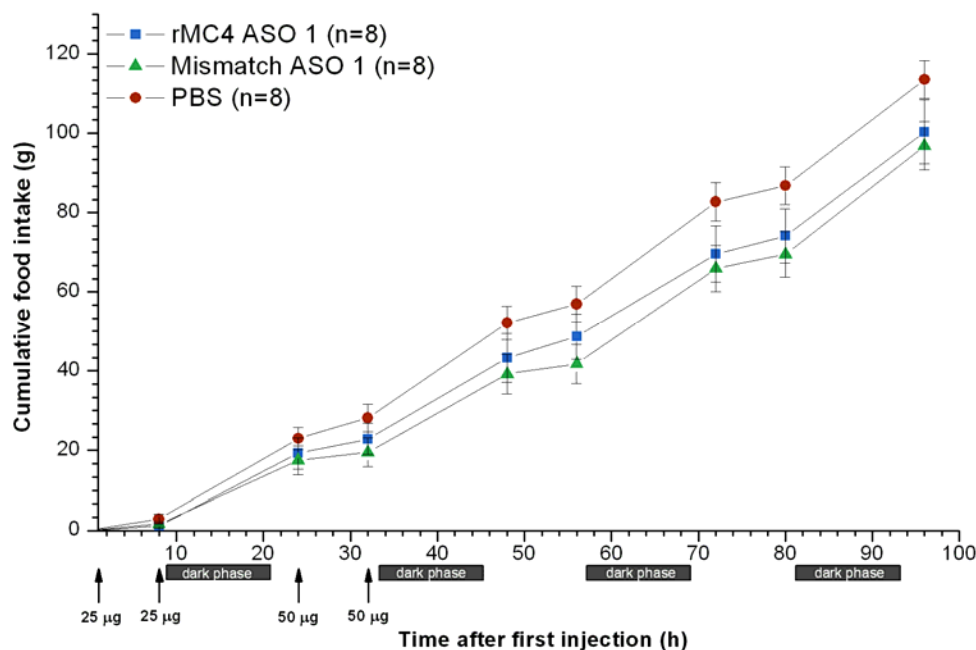


Fig. 17. Cumulative food intake after icv injection of ASO 1. SD rats were treated with rMC4 ASO 1, Mismatch ASO 1 and PBS (control). Arrows: Application time points: 1./3. application 1 h after onset of light phase. 2./4. application 1 h before onset of dark phase. Blue square: rMC4 ASO 1; Green triangle: Mismatch ASO 1; Red circle: Control 5 μ l PBS. Mean values \pm SEM. n = 8.

When the cumulative food intake in the same experiment as represented in Fig. 16 was evaluated, application of ASO 1 and the mismatch ASO1 resulted in a decrease of food intake (Fig. 17). Nevertheless, this difference did not result in a change in body weight (Fig. 18).

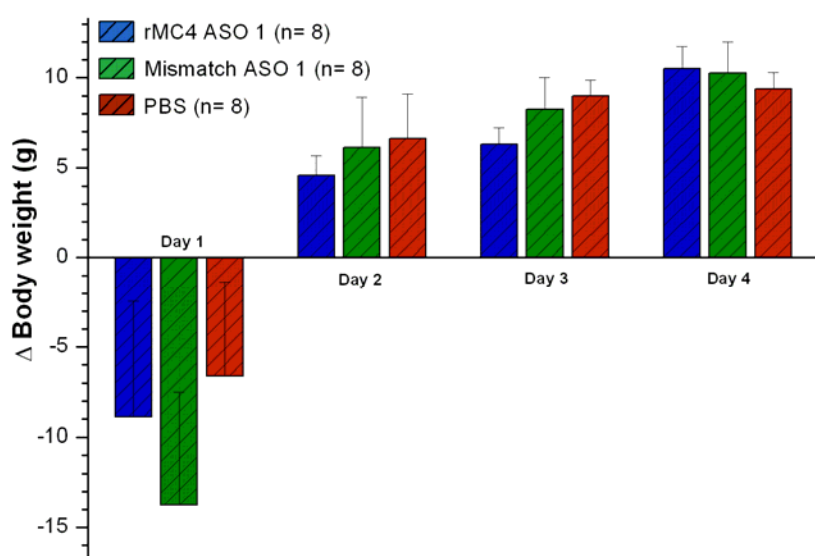


Fig. 18. Body weight change after application of ASO 1. Rats were treated with the rMC4 ASO 1, Mismatch ASO 1 and PBS control. Day 1: Day of first application; Blue bars: rMC4 ASO 1; Green bars: Mismatch ASO 1; Red bars: Control 5 μ l PBS. Mean values \pm SEM. n = 8.

3.4.2 Chronic Application of ASO 1

ASO 1 was applied via Alzet minipumps (1 nmol/h/rat) for seven days as described in Obici et al. (Obici et al., 2001). The total amount of ASO delivered icv per 24 h was 154 μ g. Over this period of ASO 1 infusion no significant increase in food intake (Fig. 19) and cumulative food intake (Fig. 22) was observed in comparison to mismatch and PBS controls. Water intake (Fig. 20) and body weight/body weight gain (Fig. 21) also did not differ between the controls and the treated animals. The high water intake (Fig. 20) values of ASO 1 treated rats on day 4 and 7 were due to leaks of two water bottles. The body weight gain (Fig. 21) on day 1 with the PBS treated animals was reduced in comparison to the ASO and mismatch treatment, but this observation could not be reproduced in a second experiment.

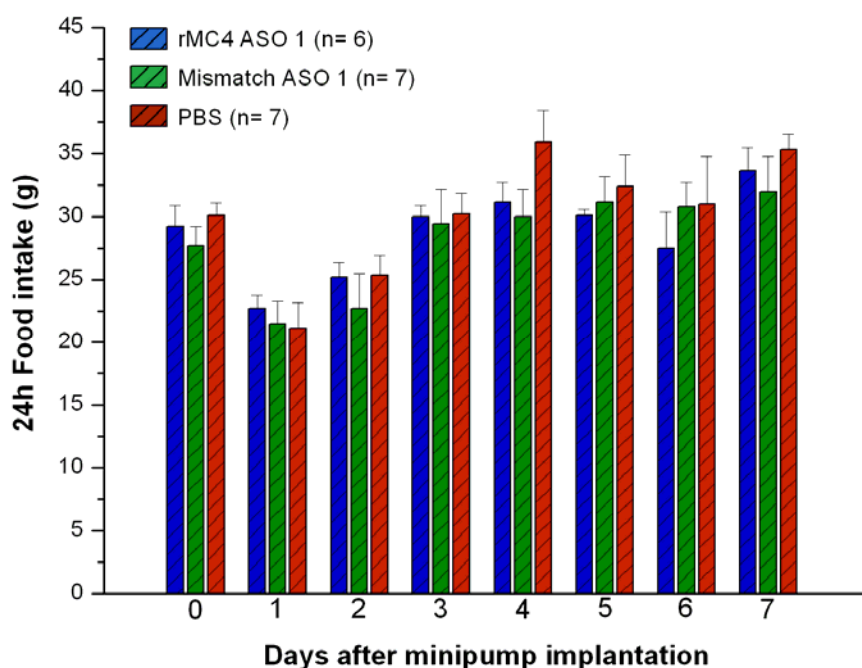


Fig. 19. 24 h food intake after minipump application of ASO 1. On day 0 minipumps were implanted. Rats were infused into the lateral ventricle with rMC4 ASO 1, mismatch ASO 1 and PBS (control) via Alzet osmotic minipump (6.4 μ g/h) for 7 days. Blue bars: rMC4 ASO 1, (n = 6); Green bars: Mismatch ASO 1, (n = 7); Red bars: PBS control, (n = 7). Mean values +SEM.

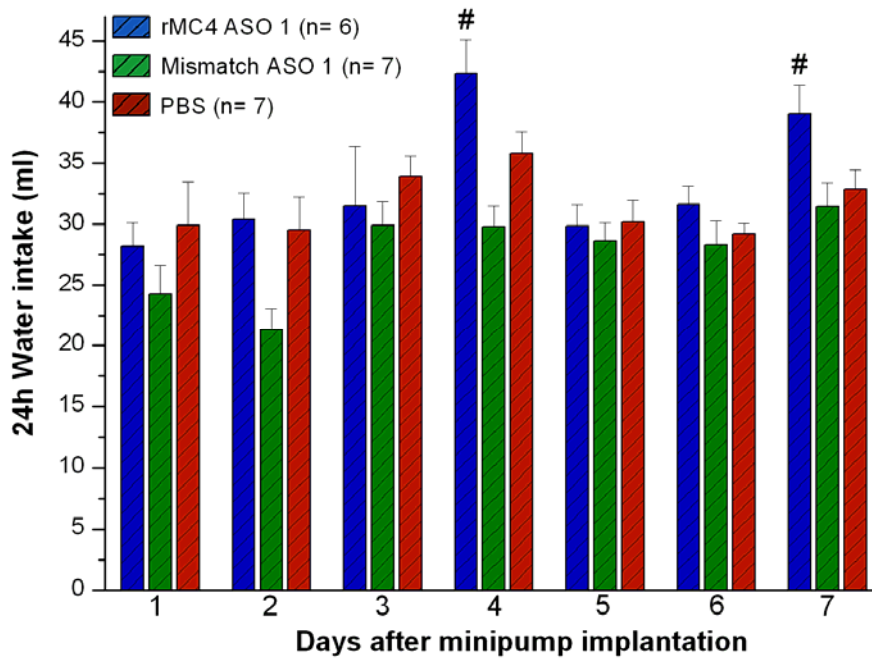


Fig. 20. 24 h water intake after minipump application of ASO 1. On day 0 minipumps were implanted. Rats were infused into the lateral ventricle with rMC4 ASO 1, mismatch ASO 1 and PBS (control) via Alzet osmotic minipump (6.4 $\mu\text{g}/\text{h}$) for 7 days. Blue bars: rMC4 ASO 1, (n = 6); Green bars: Mismatch ASO 1, (n = 7); Red bars: PBS control, (n = 7). # leaky water bottles. Mean values \pm S.D.

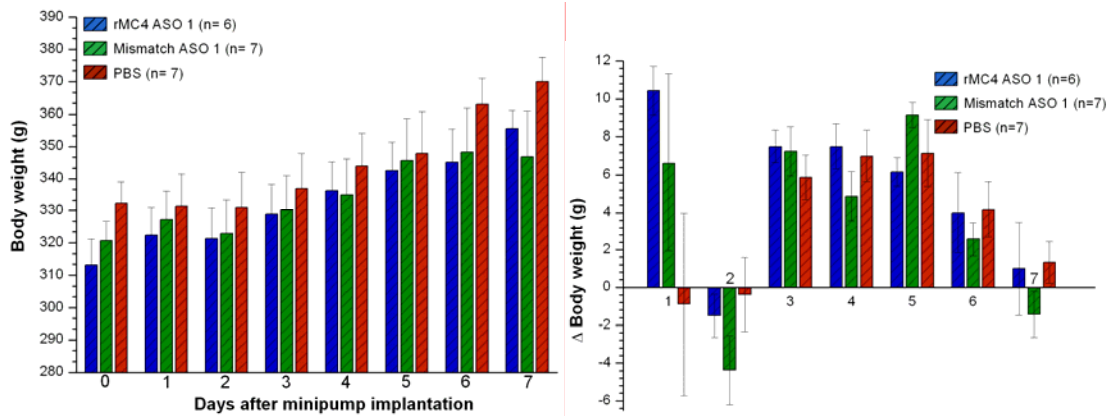


Fig. 21. Body weight after minipump application of ASO 1. On day 0 minipumps were implanted. Rats were infused into the lateral ventricle with rMC4 ASO 1, mismatch ASO 1 and PBS (control) via Alzet osmotic minipump (6.4 $\mu\text{g}/\text{h}$) for 7 days. Left panel: Body weight; right panel: Body weight gain; Blue bars: rMC4 ASO 1, (n = 6); Green bars: Mismatch ASO 1, (n = 7); Red bars: PBS control, (n = 7). Mean values \pm S.D.

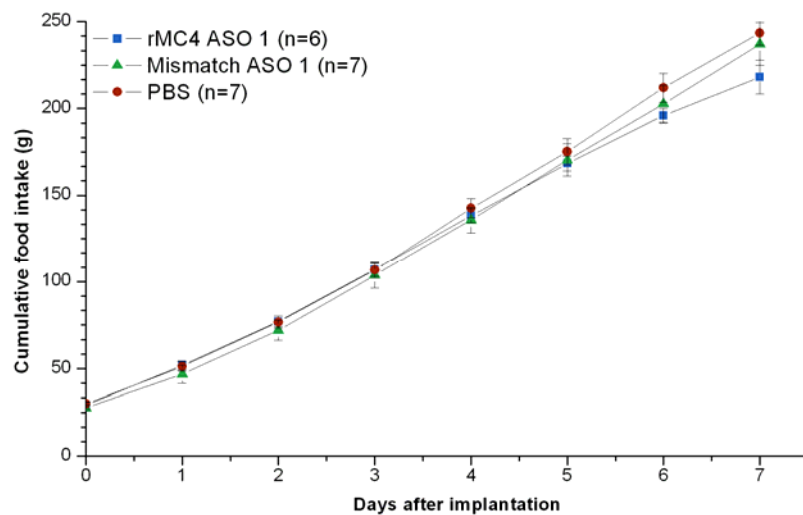


Fig. 22. Cumulative food intake after minipump application of ASO 1. On day 0 minipumps were implanted. Rats were infused into the lateral ventricle with rMC4 ASO 1, mismatch ASO 1 and PBS (control) via Alzet osmotic minipump (6.4 $\mu\text{g}/\text{h}$) for 7 days. Blue square: rMC4 ASO 1, (n = 6); Green triangle: Mismatch ASO 1, (n = 7); Red circle: PBS control, (n = 7). Mean values \pm S.D.

After 7 days of chronic ASO 1 infusion MC4-R mRNA levels were measured in the hypothalami of the rats by real time PCR. No inhibitory effect of the ASO 1 was measurable on MC4-R mRNA either in comparison to the mismatch control or its untreated control (Fig. 23).

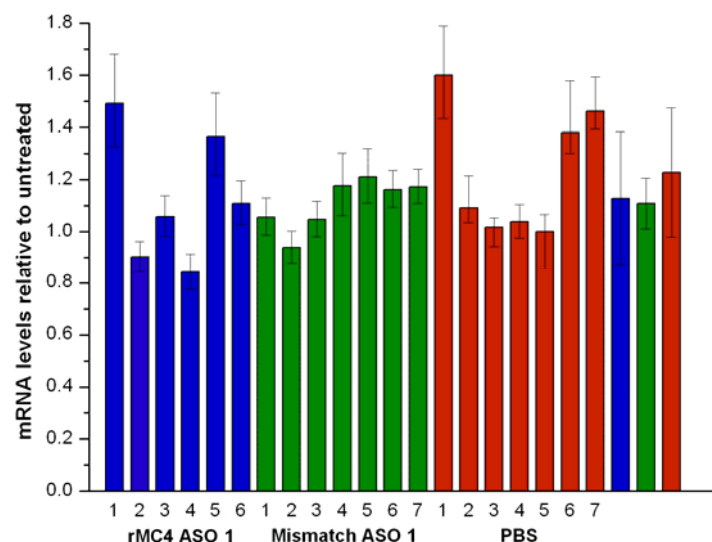


Fig. 23. rMC4-R mRNA levels after minipump application of ASO 1. SD rats were infused into the lateral ventricle with rMC4 ASO 1, mismatch ASO 1 and PBS (control) via Alzet osmotic minipump (6.4 $\mu\text{g}/\text{h}$) for 7 days. After day 7 the hypothalamus was dissected and total RNA was prepared. The relative quantification was done by using the comparative C_T -method. All values are compared to untreated hypothalamus standard and normalized to 18S rRNA expression. Each sample was measured in triplicate. Data obtained from 20 rats (Means and range) are shown. The last three bars are the mean values of each group \pm S.D.

Application of ASO 1 did neither promote down-regulation of MC4-R mRNA nor produce an increase in food intake or body weight in the treated animals.

3.4.3 Chronic Application of ASO 2

Similar experiments to those with ASO 1 were performed with ASO 2, which was shown to down-regulate rMC4-R *in vitro* (Figures 10-12). Treatment of ASO 2 *in vitro* resulted in down-regulation of >90% of the MC4-R mRNA levels and a reduction of >70% of rMC4-R dependent signaling. ASO 2 was chronically injected via Alzet minipumps at two different doses. Only the results of the higher dose (2 nmol/h/rat) are shown. The total amount of ASO 2 (MW 6225) delivered into the lateral ventricle after 24 h was 298 μ g. This was the highest dose of ASO we ever used. Infusion of ASO 2 had no significant effect on food intake, water intake and body weight (Fig. 24, 25). No toxic effects have been observed either suggesting the rats tolerated such high doses well.

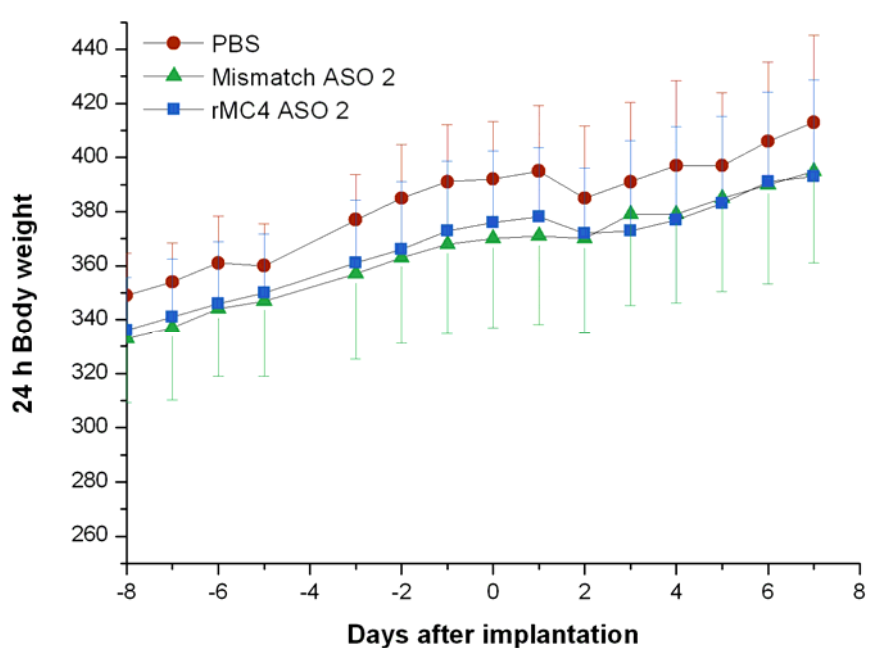


Fig. 24. Body weight after minipump application of ASO 2. On day 0 minipumps were implanted. Rats were infused into the lateral ventricle with rMC4 ASO 2, mismatch ASO 2 and PBS control via Alzet osmotic minipump (2 nmol/h) for 7 days. Blue square: rMC4 ASO 2, (n = 5); Green triangle: Mismatch ASO 2, (n = 4); Red circle: PBS control, (n = 4), Mean values + or - S.D.

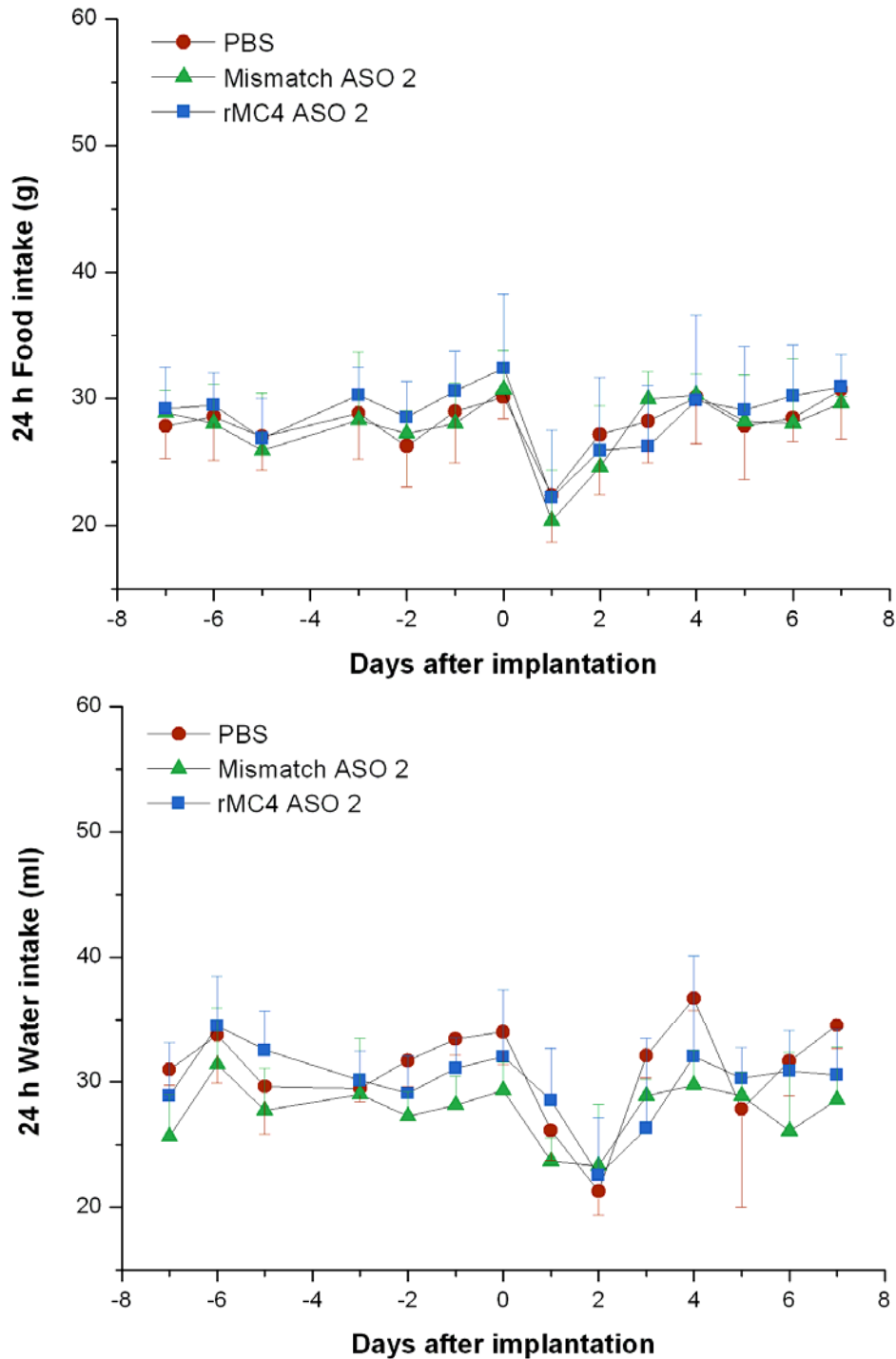


Fig. 25. 24 h Food and water intake after application of ASO 2. On day 0 minipumps were implanted. Rats were infused into the lateral ventricle with rMC4 ASO 2, mismatch ASO 2 and PBS control via Alzet osmotic minipump (2 nmol/h) for 7 days. Blue square: rMC4 ASO 2, (n = 5); Green triangle: Mismatch ASO 2, (n = 4); Red circle: PBS control, (n = 4), Mean values + or - S.D.

Upper Panel: 24 h Food intake

Lower Panel: 24 h Water intake

After 7 days of ASO 2 application hypothalami were collected and MC4-R mRNA measured. The ASO 2 treated animals showed no differences as compared to their controls (Fig. 26).

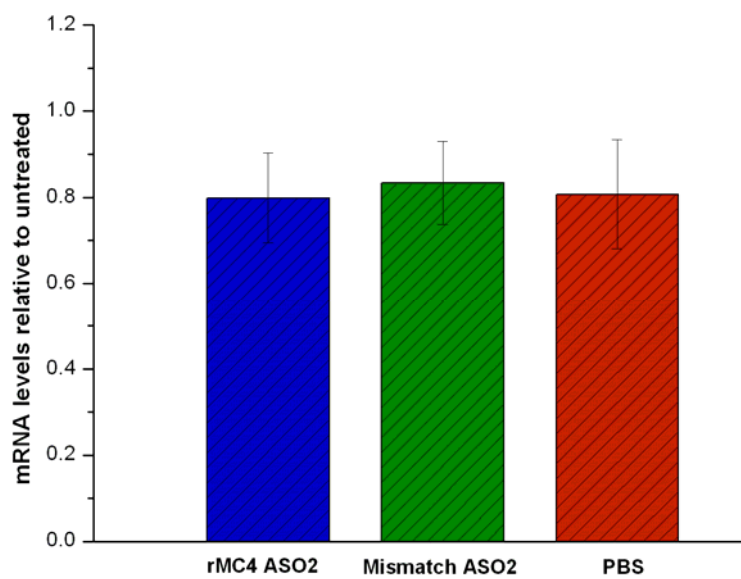


Fig. 26. Relative Quantification of rMC4-R mRNA levels after application of ASO 2 via minipump. After 7 days of chronic icv treatment with rMC4 ASO 2 into SD rats hypothalami were dissected and total RNA prepared. The relative quantification of rMC4-R mRNA by TaqMan was done by using the comparative C_T -method. All values are compared to untreated hypothalamus standard and normalized to 18S rRNA expression. Each sample was measured in triplicate. rMC4 ASO 2 (n=5), Mismatch ASO 2 (n=4), PBS (n=4). Mean values of each group \pm S.D.

3.5 Selection of Active siRNA for the Rat MC4-R

Three independent siRNAs prepared against rMC4-R were tested for their ability to reduce rMC4-R expression in the Hek293rMC4 cell line. The three siRNAs encompassed different regions of the rMC4 receptor coding sequence (Fig. 7). HEK293 cells expressing rMC4 receptor were transfected with siRNA1-3 with the help of Lipofectamine 2000. 24 hours after the transfection, total RNA was extracted and analyzed by real-time RT-PCR (Fig. 27). siRNA 2 caused the strongest reduction of rMC4-R mRNA levels (<15% of untreated cells). The siRNA 1 exhibited moderate silencing ability, whereas the siRNA 3 resulted in an increase of the rMC4-R mRNA. Transfection with 200 nM siRNA did not result in additional reduction in mRNA levels as compared to 100 nM.

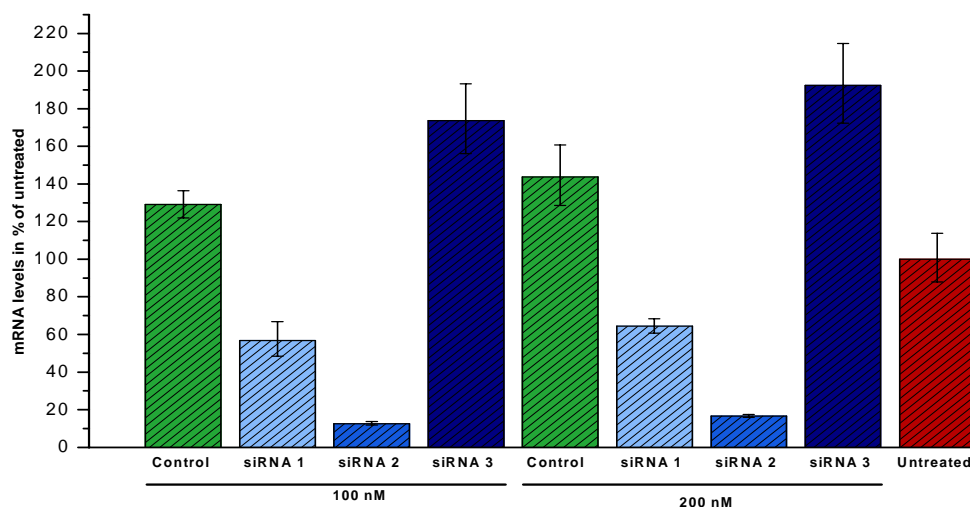


Fig. 27. Down-regulation of rat MC4-R mRNA in HEK293 after transfection with siRNA as revealed by TaqMan Analysis. HEK293-rMC4 cells were transfected (Lipofectamine 2000) with rMC4 siRNA 1, 2, 3 or the control siRNA. After 24 h RNA was extracted and analyzed with Real-time RT-PCR. mRNA levels in % of untreated cells \pm S.D. of two independent transfections (measured in triplicate) are shown.

The siRNA effects were also tested in a functional assay (Fig. 28, 29). As for the ASO, the NDP- α -MSH mediated intracellular Ca^{2+} release in the HEK293 cells co-expressing rMC4-R and $\text{G}_{\alpha 16}$ was analyzed with the FLIPR[®]. 100 (Fig. 28) and 200 nM (Fig. 29) of siRNA were used for the transfection, producing essentially the same results. The strongest effect was observed with siRNA 2, which completely abolished agonist mediated Ca^{2+} mobilization. This siRNA construct encompasses the same sequence as the rMC4 ASO 2. The siRNA 1 and 3 reduced the signal in FLIPR[®] to 30 %, respective to 40% of untreated cells. The siRNA control did not show any reduction at 100 nM, while at 200 nM a slightly decrease in the signal intensity was visible. The viability of the cells and their general capacity to release Ca^{2+} was not affected by the siRNA treatment since after the addition of 10 μM ATP treated and untreated cells showed the same signal, suggesting that the siRNA had only an effect on the rMC4-R mediated signaling (data not shown).

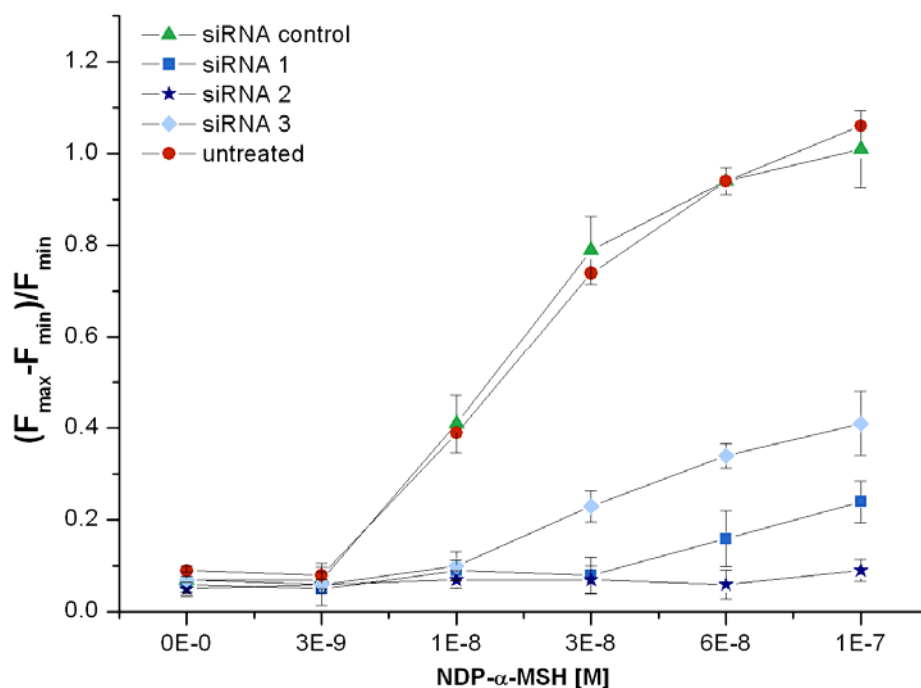


Fig. 28. The effect of siRNA on the activity of rMC4 receptor expressing HEK293 cells. HEK293 cells expressing rMC4 receptor were transfected with 100 nM siRNA or controls. 48 hours later the agonist induced transient Ca^{2+} release was measured with FLIPR. Scale not linear. Mean values \pm S.D. of two independent experiments (measured in triplicate).

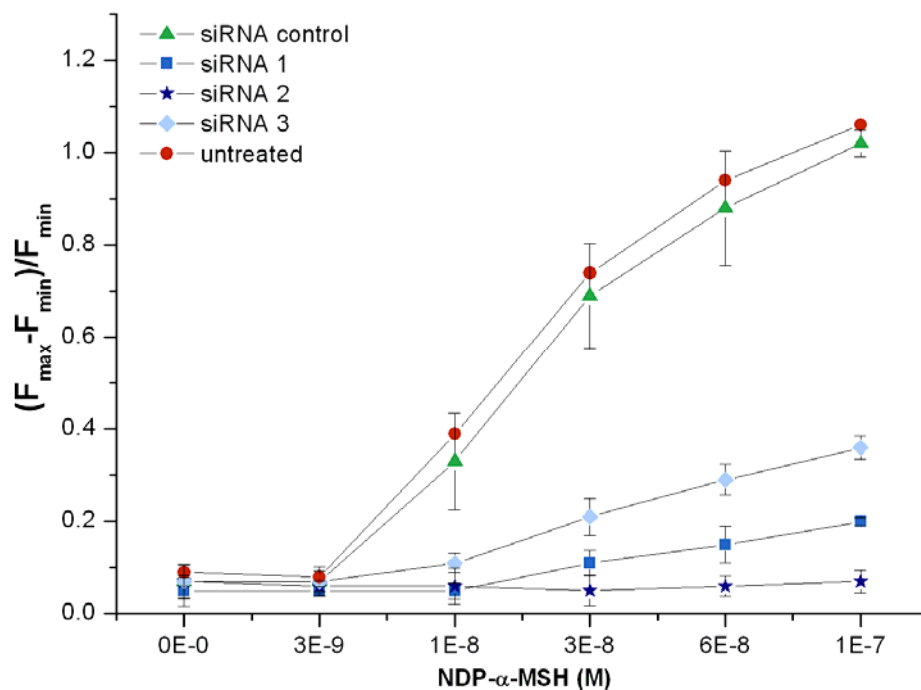


Fig. 29. The effect of siRNA on the activity of rMC4 receptor expressing HEK293 cells. HEK293 cells expressing rMC4 receptor were transfected with 200 nM siRNA or mock treated. 48 hours later the agonist induced transient Ca^{2+} release was measured with FLIPR. Scale not linear. Mean values \pm S.D. of two independent experiments (measured in triplicate).

3.5.1 Acute Application of siRNA 2

Since siRNA showed strong effects in the functional assays and appeared to be superior to ASO 2, it was tested *in vivo*. MC4-R siRNA 2 and siRNA control were administered icv in rats at two consecutive days in the afternoon (1400 - 1500 h): 50 or 100 µg/rat/injection. 24 hour food intake was measured over three days. Neither low (50 µg) nor high (200 µg) concentrations of siRNA 2 had an effect on food intake (Fig. 30), water intake (not shown) and body weight (Fig. 31). At the end of experiment all hypothalami were collected and MC4-R mRNA measured. No difference between the siRNA treated animals and their controls was observed (Fig. 32). Attempts using fluorescent labeled siRNA to analyze delivery and uptake provided negative results (data not shown). After icv application (chronic and acute) no staining of neurons was visible.

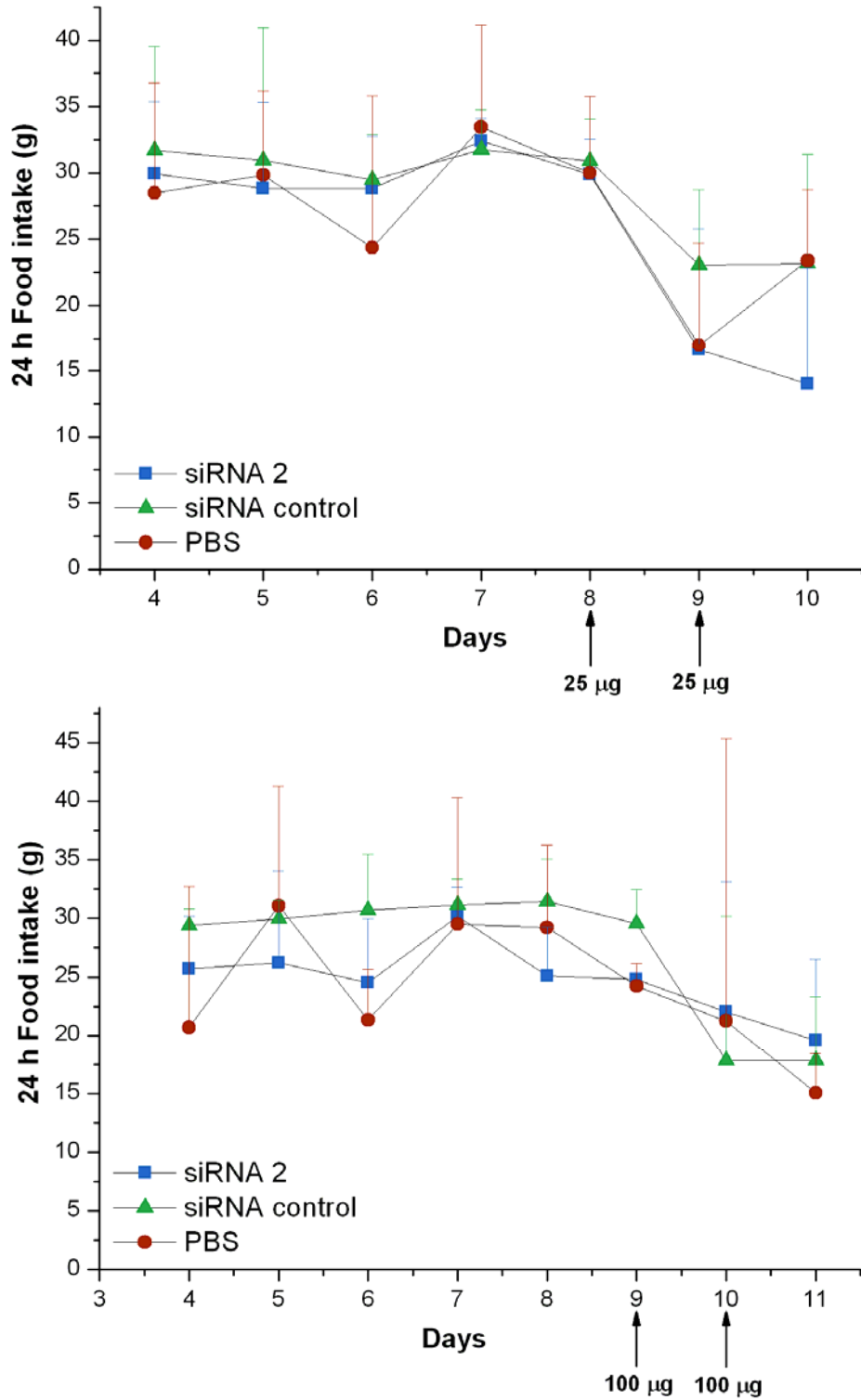


Fig. 30. 24 h food intake after treatment with siRNA. Arrows: Application time points: siRNA (25 µg, upper panel; 100 µg, lower panel) or control siRNA were applied icv 1 h before onset of dark phase. Blue square: rMC4 siRNA 2, (n = 5); Green triangle: siRNA control, (n = 5); Red circle: PBS control, (n = 5), Mean values + or - S.D.

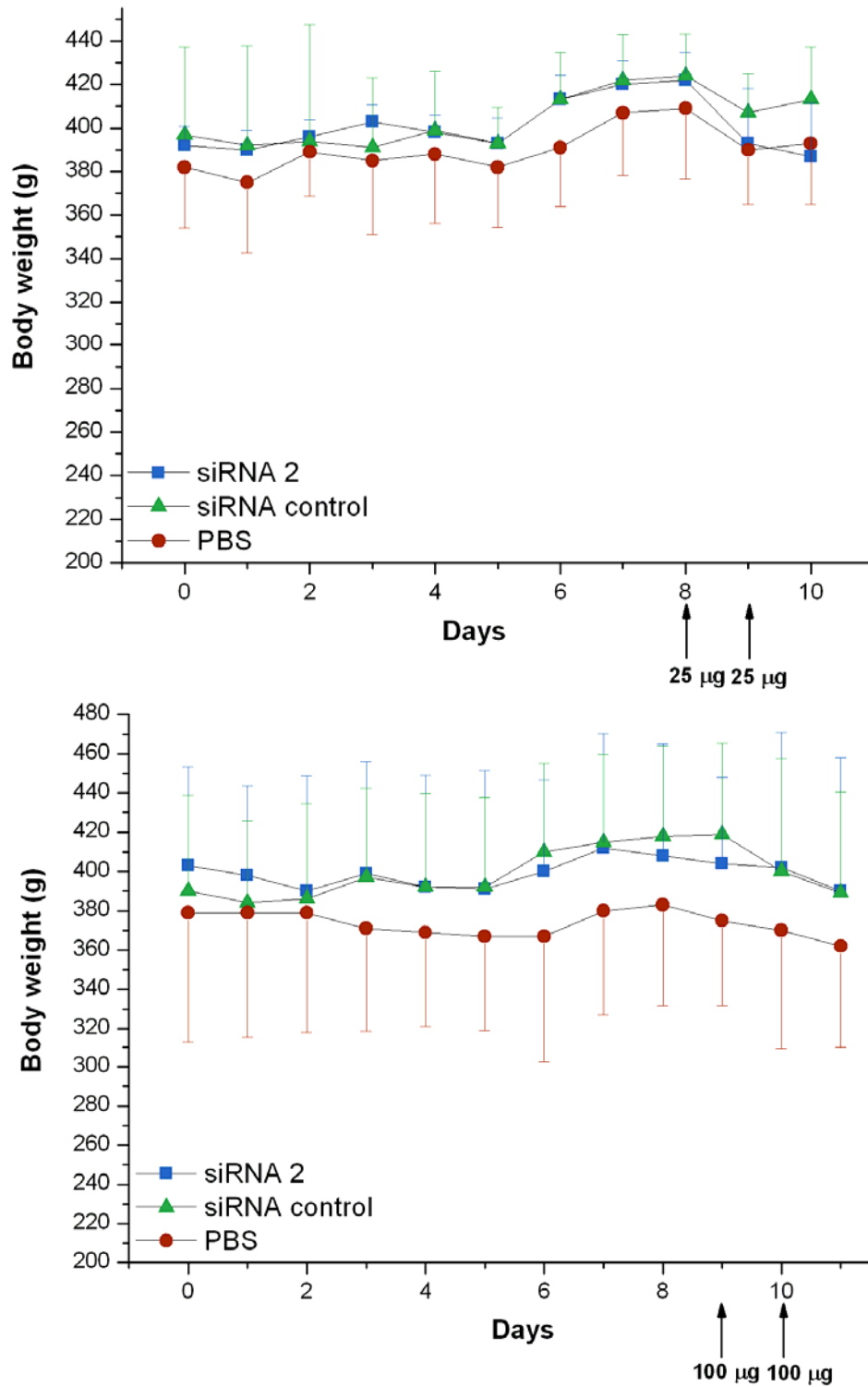


Fig. 31. 24 h body weight after treatment with siRNA 2. Arrows: Application time points: siRNA (25 µg, upper panel; 100 µg, lower panel) or control siRNA were applied icv 1 h before onset of dark phase. Blue square: rMC4 siRNA 2, (n = 5); Green triangle: siRNA control, (n = 5); Red circle: PBS control, (n = 5), Mean values + or - S.D.

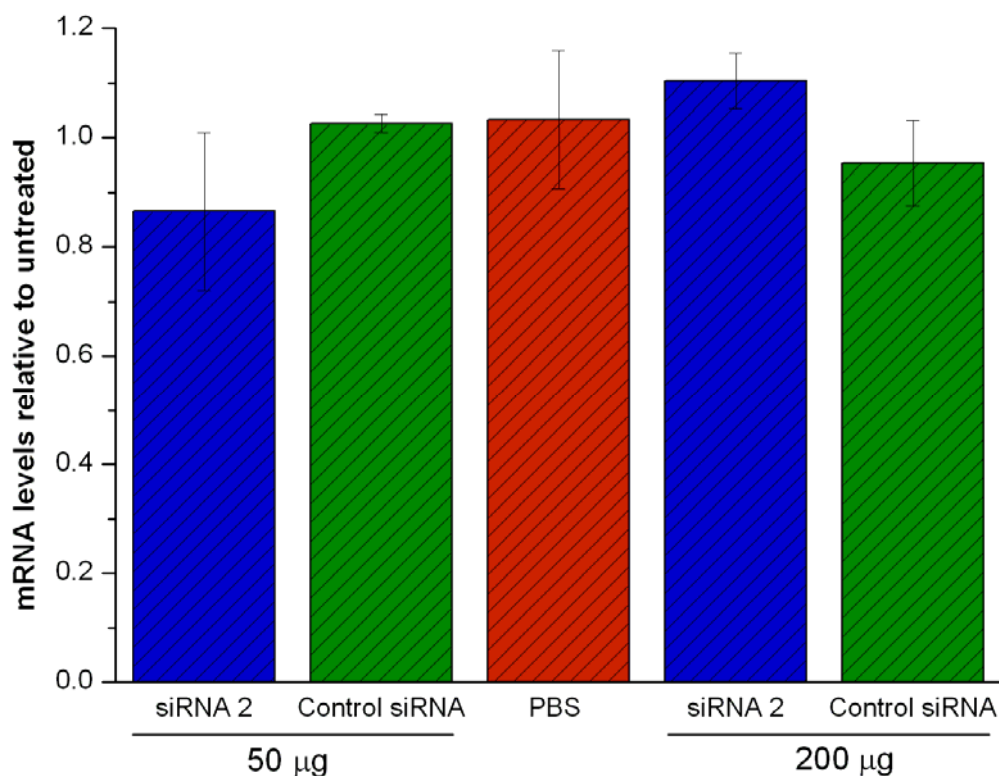


Fig. 32. Quantification of rMC4-R mRNA levels after acute icv treatment with rMC4 siRNA. rMC4 siRNA and control siRNA treatments of SD rats for two consecutive days (two times 25 µg or 100 µg) as indicated. Hypothalami were dissected and total RNA was prepared as described in the material and methods section. Relative quantification of mRNA amounts was done by using the comparative C_T -method. All values are compared to untreated hypothalamus standard and normalized to 18S rRNA expression. Each sample was measured in triplicate. Mean values of each group \pm S.D.

3.6 Preparation of an Antibody against the C-terminal Portion of Rat MC4-R

In addition to measurements of mRNA it is desirable to determine the effects on the rMC4-protein. Commercially available antibodies against rat MC4-R from different companies have been evaluated. These antibodies were tested on stable cell lines expressing the full-length rMC4-R, transiently transfected cells and rat hypothalamic brain homogenates. Unfortunately, neither C-terminal nor N-terminal commercially available antibodies gave a satisfactory result (data not shown). One antibody which we tested was withdrawn from the market shortly after we had purchased it. Therefore it was decided to generate a new antibody against the rMC4-R. Initially it was planned to generate antibodies against the N- and the C-terminal portion of the receptor protein. But due to the difficulties in expressing the N-terminal peptide in *E. coli* we

pursued only the C-terminal construct. The cloning strategy to generate the C-terminal construct is described in the Material & Methods section (Fig. 8). The 36 amino acids at the C-terminal end of the rMC4 receptor were fused to a His tag and were expressed in *E. coli* (BL21 (DE3)). After induction with IPTG the C-terminal peptide was produced in high amounts (Fig. 33, lane 4 and 5) whereas the construct encompassing the N-terminal peptide was not visible (Fig. 33, lane 2 and 3).

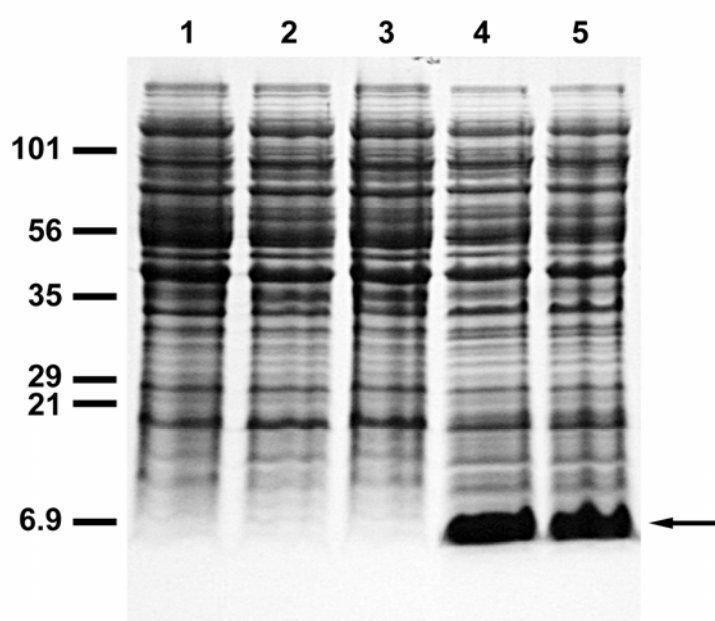


Fig. 33. N- and C-terminal rMC4 peptide expression in *E. coli*. Coomassie Brilliant Blue stained SDS-gel. Marker in kDa. Total bacterial protein loaded per lane ~ 30 μ g. Lane 1: Non-transformed bacteria, Lane 2/3: Bacteria transformed with the N-terminal rMC4-R construct after induction with IPTG, Lane 4/5: Bacteria transformed with C-terminal rMC4-R construct after induction with IPTG. Arrow: Size of positive clones expressing C-terminal rMC4 peptide (~ 7.1 kDa). This Western blot is representative of three independent experiments.

The culture of bacteria expressing the C-terminal peptide was scaled up and the C-terminal peptide was purified with the help of a Ni-NTA Superflow column. The resulting peptide was analyzed on a SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 34). The eluted peptide showed good purity and had the expected molecular mass of about 7 kDa.

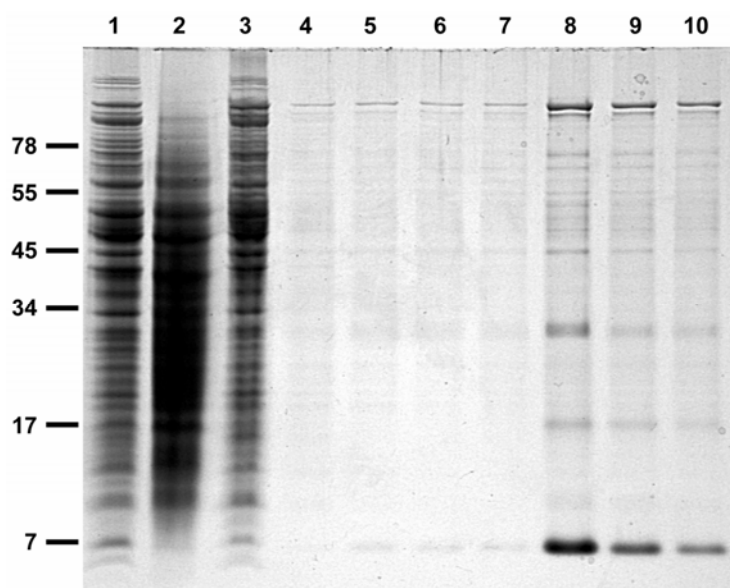


Fig. 34. rMC4-R C-terminal peptide purification on a Ni-NTA Superflow column. His-tagged C-terminal rMC4-R peptide was purified from cleared lysate (5 ml) derived from 100 ml induced *E. coli* culture on 1 ml of Ni-NTA Superflow. Total yield was 5.5 mg. Marker in kDa. Lane 1: starting material, induced culture, Lane 2: flow-through, Lane 3/4: wash, Lane 5-7: elution pH 5.9, Lane 8-10: elution pH 4.5. The Commissie Brilliant Blue gel is representative of two independent experiments.

The purification of the correct peptide was verified with an anti-His antibody (Fig. 35). The Western blot indicated additional bands with a higher molecular mass than that expected for the C-terminal peptide. Molecular mass calculations of this bands leads to the suggestion, that these were dimers, trimers or multimers of the 7 kDa peptide.

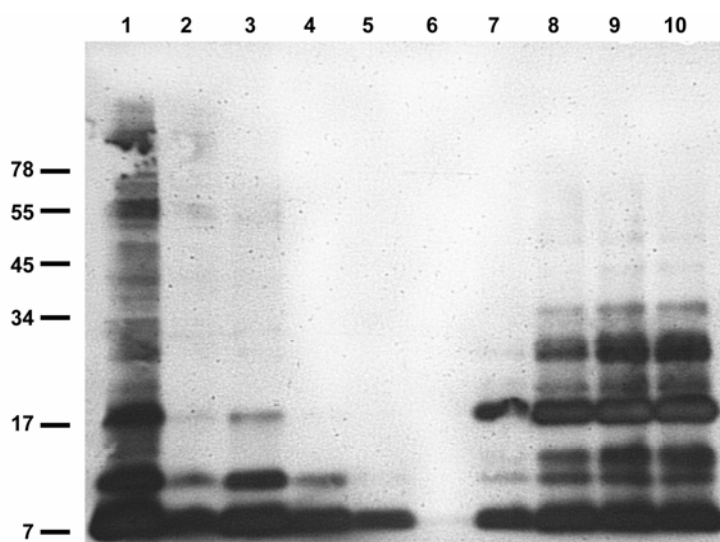


Fig. 35. Western blot of the rMC4-R C-terminal peptide purification on a Ni-NTA Superflow column. His-tagged C-terminal rMC4-R peptide was purified from cleared lysate (5 ml) derived from 100 ml induced *E. coli* culture on 1 ml of Ni-NTA Superflow. Total yield was 5.5 mg. Marker in kDa. Lane 1: starting material, induced culture, Lane 2: flow-through, Lane 3/4: wash, Lane 5-7: elution pH 5.9, Lane 8-10: elution pH 4.5. (Identical samples as used for gel in Fig. 34). Used antibody: pentaHis-MAb 1:500.

To increase the immunogenicity of the purified C-terminal rMC4-R peptide, it was linked to the carrier protein, KLH (keyhole limpet hemocyanin). Two rabbits were immunized and the two resulting sera were tested on Western blots of the purified rMC4-R C-terminal peptide.

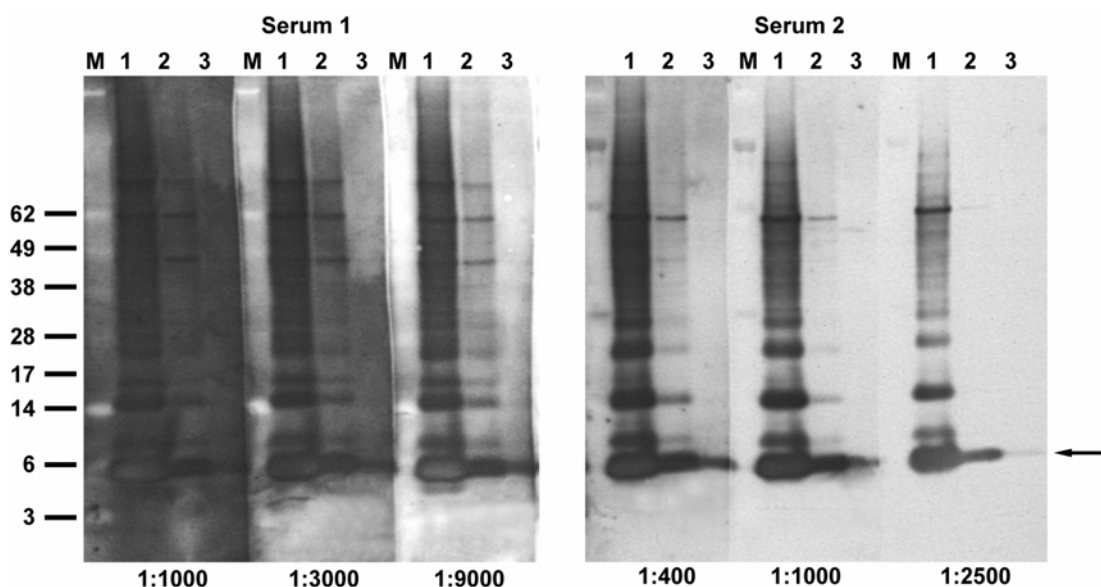


Fig. 36. Western blot with the anti-rMC4-R serum 1 and serum 2. Purified C-terminal rMC4-R peptide was loaded on a SDS-gel (1/0.1/0.01 μ g per lane). The indicated serum dilutions were used. Left panel: serum 1, right panel: serum 2. Arrow: size of the C-terminal peptide, M: Marker.

10 ng of purified rMC4-R C-terminal peptide was detectable with both sera (Fig. 36), although in the case of serum 1 a much higher dilution was necessary. Due to high background serum 1 was affinity purified with C-terminal peptide.

Western blots with the full length receptor, expressed in Hek293 cells were performed. A total protein and a membrane preparation were used. The preparations were tested with both sera (Fig. 37, 38).

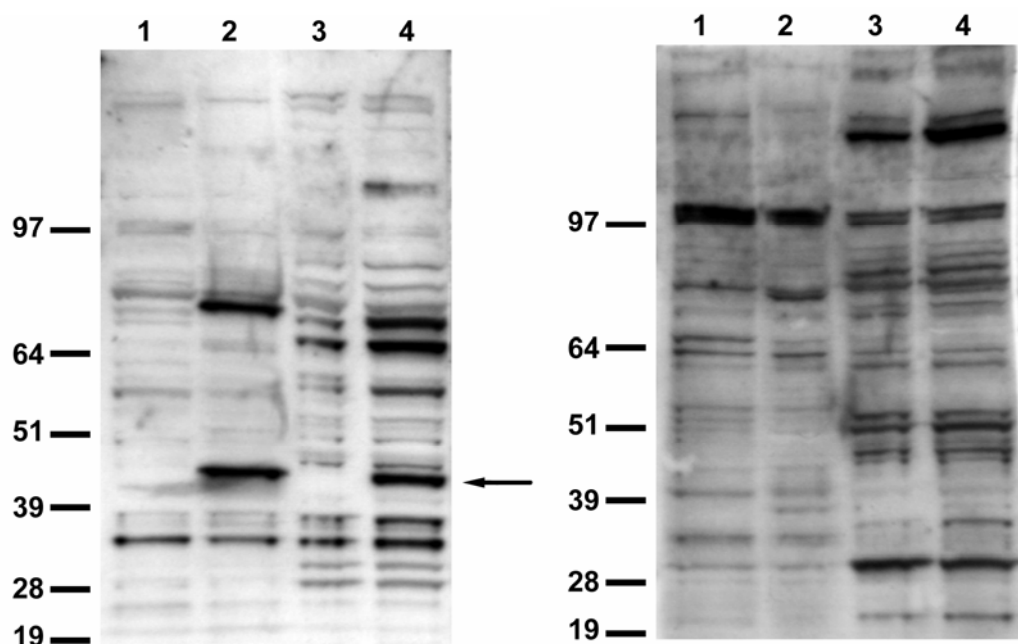


Fig. 37. Western blot with the anti-rMC4-R serum 1 and serum 2. Total protein preparations and membrane protein preparations of HEK293 and HEK293rMC4 were separated on a SDS-PAGE (25 μ g per lane) and transferred to membranes before being processed with antibodies. Marker in kDa. Lane 1: Membrane proteins of HEK293, Lane 2: Membrane proteins of HEK293rMC4, Lane 3: total protein of HEK293, Lane 4: total protein of HEK293rMC4. Left panel: Serum 1 was diluted 1:10'000. Right panel: Serum 2 was diluted 1:1'000. Arrow: full length rMC4-R (~42 kDa). This Western blot is representative of two independent experiments.

The full length rMC4-R was detectable with serum 1 (Fig. 37, left panel). The membrane protein fraction of HEK293rMC4 showed two predominant bands at ~42 and ~80 kDa (Fig. 37, left panel, lane 2), which were not present on membrane proteins prepared from HEK293 cells (Fig. 37, left panel, lane 1). Total protein extracts (Fig. 37, left panel, lane 4) contained the same two bands but also additional bands that had strong staining. The band at ~42 kDa corresponds to the expected molecular mass of the transfected rMC4-R construct. Using the same material, the anti rMC4 serum 2 failed to detect any specific band (Fig. 37, right panel).

Serum 1 was tested with two further independent cells lines expressing full length rMC4-R, confirming the results of Fig. 37 (not shown). Although both serum 1 and 2 recognized the purified rMC4 peptide (Fig. 36), only serum 1 was able to recognize full-length rMC4-R.

Affinity purification was performed to reduce unspecific signals. After affinity purification fractions from the purification steps were analyzed by Western blot (Fig. 38). The antibody binding was acid-labile and was eluted in the first four elution steps at pH 2.5 (data not shown). The 'purified' antibody recognized an additional band at 39 kDa in HEK293 as in HEK293rMC4 while a specific band at ~10 kDa was only visible in HEK293rMC4 preparation. The affinity purification apparently had not increased the antibody specificity but the background was reduced. The affinity purified antibody and the unpurified antibody was in addition tested with FACS and immunocytochemistry (data not shown) with the HEK293rMC4 cell line. With both methods the full length rMC4-R could not be detected in these assays. Further the serum 1 was tested in a Western blot with rat brain extracts from cortex and hypothalamus (Fig. 39).

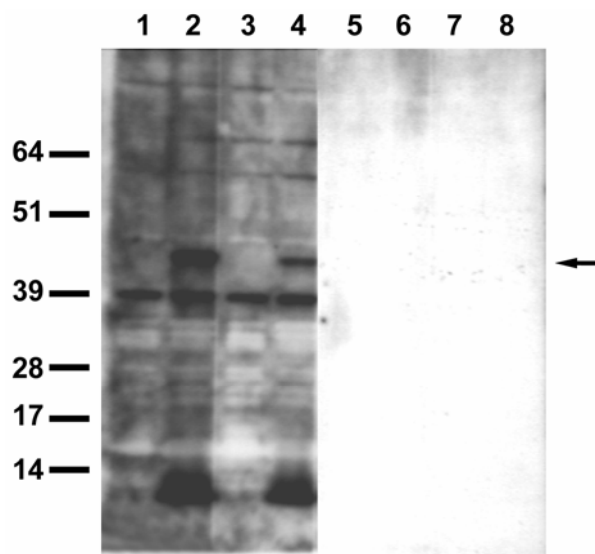


Fig. 38. Western blot of the antibody purification of serum 1. Membrane preparations of HEK293 (lane 1, 3, 5, 7) and HEK293 rMC4 (lane 2, 4, 6, 8) were separated on a SDS-PAGE (20 μ g per lane). Lanes 1-4 were tested with 1:1000 diluted fraction eluted at pH 2.5, Lanes 5-8 were tested with 1:1000 diluted fraction eluted at pH 11. Arrow: full length rMC4-R (~42 kDa).

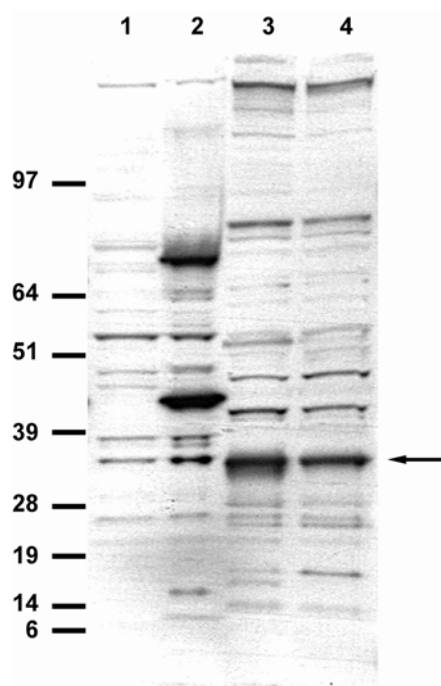


Fig. 39. Western blot with the anti-rMC4-R serum 1. Membrane protein preparations of HEK293, HEK293rMC4, rat cortex and rat hypothalamus were separated on a SDS-PAGE (25 μ g per lane) and transferred to membranes before being processed with antibodies. Marker in kDa. Lane 1: membrane proteins of HEK293, Lane 2: membrane proteins of HEK293rMC4, Lane 3: membrane proteins of rat cortex, Lane 4: membrane proteins of rat hypothalamus. Purified serum 1 was diluted 1:5'000. Arrow: full length rMC4-R (~36 kDa). This Western blot is representative of two independent experiments.

In both membrane protein preparations (cortex and hypothalamus) the most prominent band was visible at ~36 kDa which corresponds to the expected molecular mass of the unglycosylated wild type rMC4-R.

4 DISCUSSION

Since synthetic agonists and antagonists are not widely available and often lack specificity for receptor subtypes, antisense oligonucleotides (ASO) might be an alternative for drug target validation. ASO allow the determination of protein function by selectively knocking-down their expression. It has been shown that ASO have pharmacological activity in variant animal models and clinical trials in humans are currently underway. Up to now only one treatment with ASO has been approved for the use in men by the Food and Drug Administration (FDA) (Pirollo et al., 2003). ASO against central neuropeptide receptors have already been used by other investigators to study the effects on feeding behavior and insulin secretion (Akabayashi et al., 1994; Schaffhauser et al., 1997).

4.1 ASO: *In Vitro* Results

In order to evaluate the role of the melanocortin-4 receptor (MC4-R) in the regulation of food intake, ASO against this receptor subtype and mismatch controls were designed. To identify ASO with high efficacy, an *in vitro* testing system had to be developed. Consequently, stable cell lines expressing the rat MC4-R were generated. These cell lines were used to screen different ASO for their effect in reducing MC4-R mRNA levels.

Rat MC4-R was co-expressed with the $G_{\alpha 16}$ protein. $G_{\alpha 16}$, a promiscuous GTP-binding protein, enables a pure $G_{\alpha S}$ receptor like the MC4-R to couple to the PLC pathway and to signal via Ca^{2+} release. This makes it possible to use FLIPR[®] based methods as a fast and efficient method to identify new agonists or antagonists in a high-throughput format.

The two most promising ASO, both showing in preliminary studies (not shown) a good hybridization to the MC4-R mRNA, were tested in the HEK293 $G_{\alpha 16}$ -rMC4 cells. The strongest down-regulation (> 95%) was obtained with rMC4 ASO 2 at a concentration of 600 nM. The maximal down-regulation at the functional level was around 80% compared to sham treated cells and around 60% compared to the mismatch ASO. So far it has not been possible to find

conditions, where the NDP- α -MSH dependent Ca^{2+} signal could be decreased to the same degree, as it was possible for mRNA.

The gene region encompassing the ASO 2 showed a high accessibility for oligonucleotides, making it an ideal target for this type of approach. The transfection with mismatch controls resulted sometimes in higher mRNA levels compared with the untreated controls. Mismatch ASO bind weakly to rMC4-R mRNA, this would not be sufficient to promote RNase-H mediated degradation, but sufficient to stabilize RNA and interfere with its translation. A stabilization effect of the mRNA after binding to the ASO might also be the cause of the small (20%) effect observed in *in vitro* functional essays.

Three observations suggest that the ASO was highly specific: the effect was concentration dependent; the effect was saturable; no cellular toxicity was observed.

While RNA down-regulation was observed after 24 h, functional effects were only evident after about 48 h. This is what is normally observed for ASO, since down-regulation of mRNA precedes the effect on the corresponding proteins by 12-24 h, because it is dependent on the turnover of the corresponding protein.

These studies showed that ASO against rMC4-R are useful tools to down-regulate the MC4-R and thereby block the MC4-R signaling pathway.

4.2 ASO: *In Vivo* Distribution Studies

In recent years ASO have been widely used in *in vivo* studies (Opalinska and Gewirtz, 2002). Studies of ASO administered into the cerebral ventricles of rats have shown that phosphodiester ASO are rapidly degraded, whereas phosphorothioate ASO are resistant to degradation. They are cleared in a manner consistent with the bulk flow of cerebrospinal fluid (Whitesell et al., 1993). Phosphorothioate ASO given icv for a week did not show any evidence of toxicity, yet penetrated the brain extensively and were taken up by neurons. Other investigators have confirmed the superiority of phosphorothioate compared to other chemical modifications of ASO for CNS administration, due to

the improved cellular uptake and biodistribution, and an apparent lack of adverse effects (Chauhan, 2002; Szklarczyk and Kaczmarek, 1995; Yaida and Nowak, 1995).

ASO may be more stable within the CNS than in other body regions (Akhtar and Agrawal, 1997). To analyze distribution and cellular uptake after icv administration a fluorescence labeled ASO was used and monitored up to 24 h. The brain structures expressing high amounts of MC4-R (PVN, ARC) were reached within 3 h after application. ASO injected into the lateral ventricle probably moved with the bulk flow of cerebrospinal fluid (CSF) from the injection site to the third ventricle. Up to 10-15% of the cells in these areas showed uptake of the ASO. The sulfur based backbone modification apparently conferred stability up to 24 h of the ASO. The method used to label the ASO was chosen, since the 5' or the 3' end are susceptible to nuclease degradation in the cell, making it difficult to distinguish fluorescence associated with ASO from the free fluorescence label. ASO was therefore labeled with FITC at the 5' end via a phosphorothioate linkage, which is known to be nuclease resistant.

The pattern of the intracellular distribution of the ASO that we observed in our experiments was variable, though in the most cells both the cytoplasm and the nucleus were labeled. There were only few cells in which the ASO were exclusively visible in the cytoplasm. Sommer *et. al* (Sommer et al., 1993) have also demonstrated that FITC-labeled phosphorothioate ASO are localized in the cytoplasm and in the nuclei of neurons. Other groups have found ASO primarily in the nucleus (Leonetti et al., 1991) whereas Caceres et al. (Caceres and Kosik, 1990) found ASO to be localized mainly in the cytoplasm. These differences could probably be explained by the different manner of administration of the ASO (microinjection into cultured cells versus icv injection), different ways to label them (FITC and ^{32}P versus biotin and digoxigenin label) and different backbone modifications (phosphorothioate versus phosphodiester).

Whether the amount of labeled cells is enough to mediate an effect *in vivo* was tested with ASO in functional studies *in vivo*.

4.3 ASO: *In Vivo* Functional Studies

Obici et al. (Obici et al., 2001) published that icv infusion of ASO to MC4-R generated a marked decrease (~50%) in the hypothalamic MC4-R protein as assessed by Western blot analysis. This promising result prompted us to try to reproduce their observations. Since the sequence used to generate the ASO lies upstream of the starting codon, which was not present in the cDNA construct used to generate the rMC4 HEK293 cell line, the efficacy of this ASO could not be tested in these cells.

Neither acute nor chronic icv applications of ASO 1 lead to the expected increase in food intake and body weight. In contrast, in acute experiments a slight reduction in cumulative food intake was observed. Whether this is an indication that the animals did not tolerate the ASO is uncertain, because this reduction was not maintained during chronic treatment. During the first 24 h after the first application all three groups (ASO, mismatch and PBS) showed a loss of body weight, probably as a consequence of the manipulations associated with the icv treatment. The doses applied during chronic treatment are similar to those used by Whitesell et al. (Whitesell et al., 1993) (1.5 nmol/h for 7 days) which resulted in homogenous distribution throughout the brain and were well tolerated, and to those of Obici et al. (Obici et al., 2001) which were reported to cause a 50% reduction of rMC4 protein. We tried to measure these effects of ASO 1 using the same antibody as described by Obici et al. (Obici et al., 2001) but without success. In fact, this antibody even failed to detect the rMC4-R over-expressed in rMC4-R HEK293 cells. Shortly after we concluded the experiment, the antibody was withdrawn from the market due to inadequate performance. Our results with the ASO 1 construct lead to the conclusion that ASO 1 failed to cause a substantial reduction of rMC4-R mRNA and therefore track no effect on food intake.

Our *in vitro* results show that rMC4 ASO 2 efficiently down-regulate MC4-R. However, *in vivo* no significant effects were detectable. The only reproducible change in food intake that could be measured occurred during the recovery period after the implantation of the minipumps, but this effect is not MC4-R dependent. The high variability of food intake and body weight parameters after ASO application complicated the interpretation of the results. After the

negative results with ASO 2 in acute and chronic models we came to the conclusion that ASO have serious limitations for studying MC4-R effects *in vivo*.

Whether the number of cells which have taken up the ASO is high enough to mediate an effect is unknown since it seems that distribution and cellular uptake is the limiting step for the *in vivo* usage of ASO. The poor cellular uptake and nuclear accumulation remains as the most important challenge for delivery systems. The fact that antisense effects have been obtained *in vivo* with free ASO does not exclude that in some organs more robust effects might be achieved with the help of delivery systems. It is worth to note that there is a major discrepancy between the *in vitro* and *in vivo* studies of antisense effects. In the *in vitro* setting ASO are always administered with delivery vehicles whereas *in vivo* they were used without. Whether this discrepancy is based on a possible artefact is unknown.

4.4 Small interfering RNA: *In Vitro* / *In Vivo* Results

RNA interference is currently considered as the method of choice to knock-down a gene. There is a long list of *in vitro* data with mammalian cells which confirms that the siRNA mechanism is not limited to plants, *C. elegans* and *D. melanogaster*. Three different siRNA sequences against rMC4-R were generated and tested *in vitro*. siRNA 2, which encompass the same sequence as ASO 2 effectively knocked-down rMC4-R *in vitro* (>90% in the functional assay). This suggests that this region of the rMC4-R mRNA is highly accessible for DNA-RNA or RNA-RNA interaction. The specificity of the siRNA effect was confirmed by the concentration dependence and saturability of the effect. It was possible to knock-down MC4-R within 36 h, but the duration of the effect was not determined. Our results confirmed that siRNA is a powerful tool for knocking-down genes *in vitro*. Since the siRNA and the ASO 2 encompassed practically the identical sequence, our results suggest that siRNA was much more effective than ASO in down-regulation the rMC4-R (>90% compared to 60-70% in the functional assay).

The siRNA 2 was used to evaluate its effects *in vivo*. There are only few data available about the application of siRNA *in vivo*. In addition very little is known

about the stability of siRNA in body fluids. As for the ASO the limiting step for siRNA efficacy *in vivo* is probably the organ distribution and cellular uptake. They primarily act in the cytoplasm of cells, which is easier to access from the 'outside' by using nonviral methods than the nucleus. Nevertheless, achieving efficient uptake and long-term stability *in vivo* in relevant tissues is probably difficult (Dorsett and Tuschl, 2004). Synthetic RNAi have been applied *in vivo* in adult mice, but the most promising method is to express siRNAs from plasmid (shRNA, short hairpin RNA) and viral vectors (Lewis et al., 2002; McCaffrey et al., 2002; Xia et al., 2002). In the most successful *in vivo* study reported so far, high amounts of either shRNA or siRNA were administered via injections in the tail-vein of mice. Silencing was particularly high in the liver where about 80% inhibition of the specific gene was observed.

Our results show that siRNAs administered by direct icv injection into rat brain ventricles was not capable of down-regulating rMC4-R at RNA or protein level, although they were highly efficacious *in vitro*. Similar observations were published by Isacson et al. (Isacson et al., 2003), who found that siRNA targeted to dopamine D1 receptors applied via icv injection into rat brains did not reduce dopamine D1 receptor messenger RNA levels or receptor protein. As mentioned previously, siRNA probably failed to reach the cytoplasm of neurons. Using a fluorescent labeled siRNA we were not able to demonstrate staining after icv injection. It would therefore seem that 'naked' ASO have an advantage over 'naked' siRNA as some success with ASO have been reported (Wahlestedt et al., 1993). In particular, as shown in our experiments with labeled ASO, they seem to have a higher chance to reach the intracellular compartments. Probably they are also more resistant to nuclease degradation than siRNA. Chemical modifications of siRNAs to enhance *in vivo* stability might still be necessary to fully exploit the potential of these reagents. In parallel, the development of optimized vector systems specifically designed for the expression of shRNAs (like viral vectors) could enhance the feasibility of RNAi as an experimental tool. In addition there exists the same discrepancy with delivery methods. For siRNA studies *in vitro* new delivery systems have been developed to increase their effects. It has been demonstrated that plasmid DNA and siRNA formulate very differently with cationic liposomes and lip-

ids, and that, mechanistically, the two species of nucleic acids vary substantially in both the intracellular uptake and accumulation into defined intracellular vesicles (Spagnou et al., 2004). Whether these delivery systems can be used with high efficiency and without any toxicity *in vivo* is still a matter of investigation. Therefore, prior to using a specific delivery system for any application of siRNA, more research into optimized formulations and delivery conditions is needed.

4.5 Generation of an Antibody Against Rat MC4-R

Several commercially available antibodies against rMC4-R were tested in our studies, but none of them performed satisfactorily. In particular, these antibodies were not able to detect rMC4-R that was over-expressed in HEK293 cell line. Therefore we attempted to generate new antibodies. A C-terminal peptide of rMC4-R was successfully expressed with high yield in *E. coli*, whereas the N-terminal peptide failed. The reason for this result is not known. No mutation could be found in the sequenced construct. Consequently, the C-terminal peptide was chosen for the generation of antibodies. After purification of the peptide with the help of a His tag column and conjugation to KLH two rabbits were immunized. Both resulting sera were able to detect the recombinant peptide, but only serum 1 was capable to detect full-length MC4-R in Western blot. After affinity purification against the C-terminal peptide of the rMC4-R, the rMC4 antibody 1 can be used to detect and measure in a Western blot full length rMC4-R in cells over-expressing the recombinant protein and in rat hypothalami. This antibody will not be effective as 'functional' inhibitor, because the antigenic epitope is located on the C-terminal portion of the GPCR, so that they are not able to interfere with the binding of an agonist.

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CHAPTER B:

The Phosphorylated Immunomodulator FTY720 have Potent Effects on Internalization and Desensitization of Sphingosine 1-Phosphate Receptors that are not Observed with the Endogenous Agonist Sphingosine-1-Phosphate.

SUMMARY

FTY720 is a novel lipid immunomodulator that has been shown to be efficacious in allograft protection in Phase II trials of kidney transplantation in humans. FTY720 is phosphorylated by sphingosine kinase *in vitro*, which is believed to be necessary for the *in vivo* action of the parent compound. The cell surface receptors of the physiological ligand sphingosine-1-phosphate (S1P) were shown to bind the phosphorylated form of FTY720. We have evaluated the effects of the natural ligand sphingosine-1-phosphate, FTY720, and its phosphorylated derivatives on the activation, internalization and desensitization of a subset of sphingosine-1-phosphate receptors. The phosphorylated form of FTY720 and its derivative AFD(R) promoted extensive and long lasting internalization of the sphingosine-1-phosphate receptor 1, while the endogenous agonist S1P had only weak and transient effects. The internalization by FTY720 was accompanied by the persistent loss of receptor dependent signaling, an effect that was not observed with sphingosine-1-phosphate. FTY720 phosphate only caused partial internalization of the sphingosine-1-phosphate receptor 3, which did not result in effects of the receptor dependent activity. Sphingosine-1-phosphate receptor 4 showed a weak response. The differential effects, e.g. the strong and long lasting internalization of sphingosine-1-phosphate receptor 1 and the much weaker effect at the other receptors, may explain the unique cellular (*in vivo*) effects of phosphorylated FTY720 compared to sphingosine-1-phosphate. Our results further show that, in contrast to earlier reports the internalization requires FTY720 to act as an agonist. As the derivatives of FTY720 confer varying levels of receptor internalization and desensitization it is possible to screen for compounds with altered downstream signaling responses, leading to unique therapeutic benefits.

ABBREVIATIONS

AC	Adenylate cyclase
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cells
AP2	Adapter protein 2
C	Complement system
cAMP	Cyclic adenosine monophosphate
CB1, 2	Cannabinoid receptor 1, 2
CHO	Chinese hamster ovary cells
CTL	Cytolytic T lymphocytes
CsA	Cyclosporin A
CTL	Cytolytic T lymphocytes
DAG	Diacylglycerol
EDG-1	Endothelial differentiation gene-1
ERK	Extracellular signal-related kinase
FACS	Flow activated cells sorting
FCS	Fetal calf serum
FLIPR	Fluorescence image plate reader
GEF	Guanine nucleotide exchange factor
GTP	Guanine nucleotide triphosphate
GRK	G-protein coupled receptor kinases
GPCR	G-protein coupled receptor
GTP γ S,	Guanosine 5 γ -3-O-(thio)triphosphate
HEK	Human embryonic kidney cells
HEV	High endothelial venules
HLA	Human leukocyte antigens
HUVEC	Human umbilical vein endothelial cells
IFN γ	Interferon γ
IL-2,4,5	Interleukin 2, 4, 5
JNK	C-Jun N-terminal kinase
LN	Lymph node
LPA	Lysophosphatidic acid

NFAT	Nuclear factor of activated T-cells
NK	Natural killer cells
MHC	Major histocompatibility complex
MΦ	Macrophage
mS1P4	Mouse sphingosine-1-phosphate receptor 4
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLN	Peripheral lymph nodes
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
PTX	Pertussis toxin
Rac, Ras, Rho	Small GTPases
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
SLE	Sinus-lining endothelium
SRF	Serum response factor
S1P _{1,2,3,4,5}	Sphingosine-1-phosphate receptor 1,2,3,4,5
S1P	Sphingosine-1-phosphate
T _{act}	Activated T-cells
T _C	Cytotoxic T-cells
TCR	T-cell receptor
T _H	Helper T-cells
T _n	Naïve T-cells
TOR	Target of rapamycin
TNFβ	Tumor necrosis factor β

6 INTRODUCTION

6.1 Immunosuppression and Transplantation

Clinical organ transplantation only became a viable treatment option after the advent of immunosuppressants. The function of our immune system is to defend the organism against viruses, bacteria and parasites. Therefore our immune system has evolved a system to distinguish between 'self' and 'non-self'. Each individual has a unique and evolving immune system that results from inherited genes and previous exposure to infectious and other potentially harmful agents. In addition, everybody expresses a unique combination of proteins on every cell (only shared by genetically identical twins). These proteins are named human leukocyte antigens (HLA) and are members of the major histocompatibility complex (MHC). With the aid of HLA's the organism can recognize 'non-self' cells and will attack them. Such cells are present in transplanted organs which are not genetically identical with the recipient (allograft). To circumvent the destruction of the transplanted organ, the so called graft rejection, the host immune system has to be permanently immunosuppressed. Organs routinely used for transplantation include bone marrow, kidney, liver, heart, lung, pancreas, intestine and skin.

6.2 Mechanism of Graft Rejection

A variety of mechanisms participate in allograft rejection (Kubis, 1994). The most common is cell-mediated involving cytolytic T lymphocytes (CTL) mediated cytotoxicity (Fig. 1). Less common mechanisms involve antibody directed to the graft and activation of the complement system, which leads to destruction of the transplanted organ by antibody-dependent cell-mediated cytotoxicity (ADCC). The hallmark of graft rejection is an influx of T-lymphocytes and macrophages into the graft. Recognition of foreign MHC class I alloantigens on the grafts by host CD8⁺ cells through the T cell receptor (TCR) can lead to CTL-mediated killing. Nevertheless, the strongest response is mediated by alloantigens coming from the graft, which are degraded and presented as peptides by the major histocompatibility complex II (MHC-II). This complex is present on the surface of antigen presenting cells (APC) in the secondary lymphoid organs of the host and activates CD4 T cells preferentially. A subset of graft activated T cells, called helper (T_H) cells, start producing in the

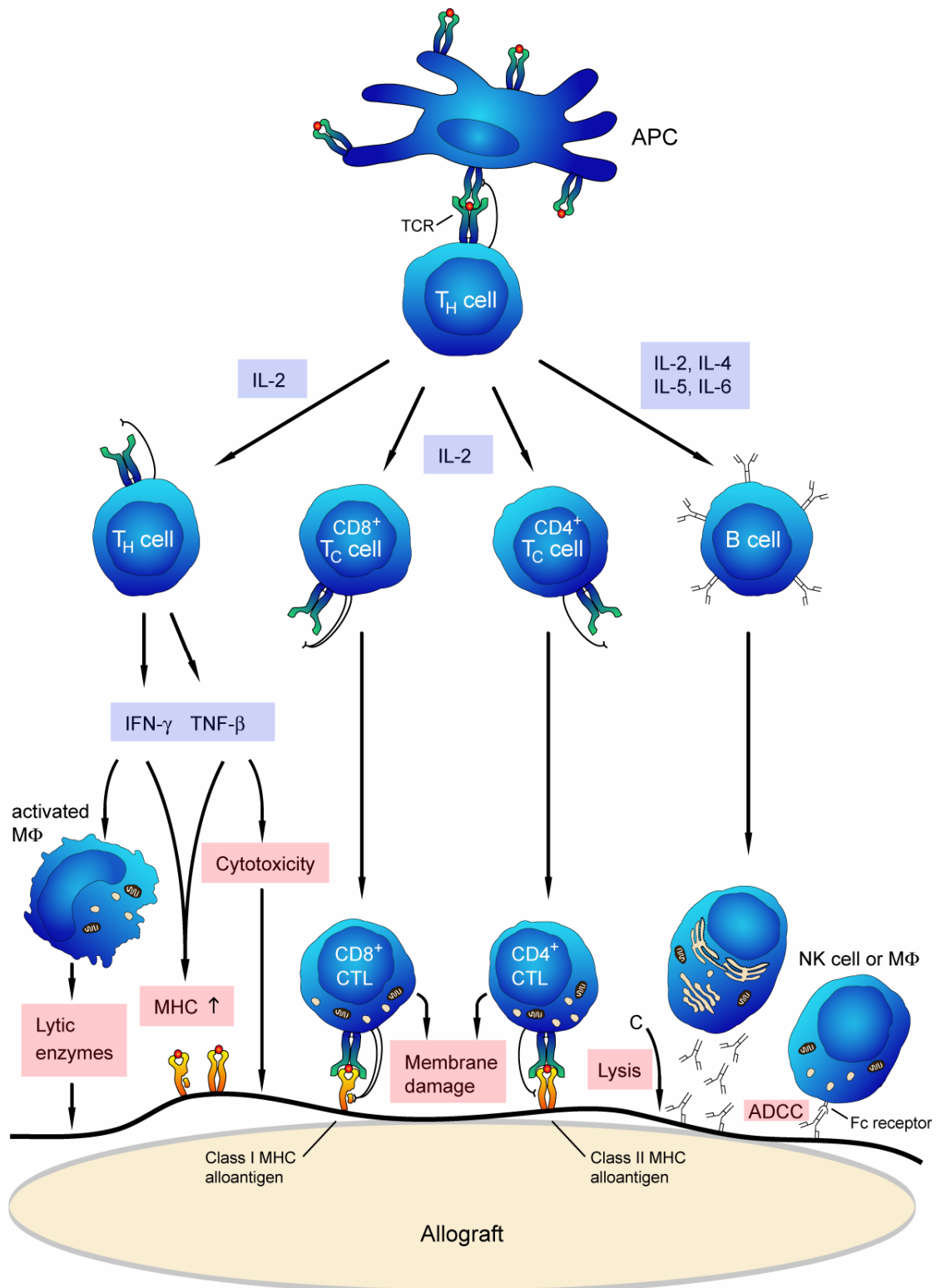


Fig. 1. Effector mechanisms (described in the red boxes) involved in allograft rejection. The generation or activity of various effector cells depend directly or indirectly on cytokines (blue boxes) secreted by activated T_H cells. ADCC: antibody dependent cell mediated cytotoxicity, APC: Antigen presenting cell, C: Complement system, M Φ : macrophage, NK: natural killer cell, TCR: T cell receptor, T_C : Cytotoxic T-cells. Modified after Kubis (Kubis, 1994).

peripheral lymph nodes (PLN) a broad set of cytokines (IL-2, -4, -5, IFN- γ , TNF- β). These lymphokines stimulate a broad spectrum of cells. IL-2 will support the proliferation of cytotoxic T cells (T_C) which will activate cell-mediated cytotoxicity. Other cytokines (IL-2,-4,-5,-6) will stimulate B-cells leading to the production of anti-graft antibodies. With the aid of these antibodies the complement system, macrophages or NK cells recognize the foreign cells and destroy them by attacking the membrane and cause its lysis. Cytokines like IFN- γ and TNF- β activate macrophages to secrete lytic enzymes to destroy foreign cells.

6.3 Drugs Used for Immunosuppression

From the early 1960s until the introduction of cyclosporin A (CsA) in 1983, the combination of azathioprine and corticosteroids was the standard regimen for maintenance of immunosuppression. Azathioprine is an imidazole derivative of 6-mercaptopurine. After application, absorption and metabolization it interferes with purine synthesis and inhibits *de novo* purine synthesis. This results in suppression of the proliferation of B- and T- lymphocytes (Barshes et al., 2004). Mycophenolate mofetil a prodrug of mycophenolic acid has a similar mode of action and inhibits also *de novo* purine synthesis. Azathioprine and mycophenolate mofetil (CellCept[®]) are classified as inhibitors of nucleotide synthesis.

In 1983 the launch of cyclosporin A under the brand name of Sandimmun[®] and later as microemulsion (Neoral[®]) revolutionized the field of transplantation because of its high efficacy in reducing acute graft rejection. CsA is primarily responsible for the success of the survival of transplanted organs and it has been the standard treatment for over 20 years. CsA inhibits calcineurin, which cannot dephosphorylate the nuclear factor of activated T-cells (NFAT) leading to inhibition of interleukin-2 (IL-2) transcription. Consequently, signaling by IL-2 controlling the production of cytotoxic T lymphocytes and of specific antibody is strongly attenuated. A similar mode of action has been demonstrated by tacrolimus or FK506 (Prograf[®]), another calcineurin inhibitor. CsA and FK506 are inhibitors of cytokine synthesis.

Sirolimus or rapamycin (Rapamune[®]) is similar in structure to tacrolimus but

blocks T-cell activation at a later point in the pathway than tacrolimus and CsA. Rapamycin binds to the mammalian target of rapamycin (mTOR) protein, a kinase, which is important for the proliferation of T cells. The major effect of this interaction is an inhibition of the IL-2 mediated signal transduction pathway and leads to suppression of T-cell activation and proliferation. Sirolimus is therefore a member of inhibitors of signal transduction.

The beneficial effects of these drugs in transplantation and autoimmunity are the consequences of their broad immunosuppressive potential. Most of the immunosuppressive treatments that have been developed have the disadvantage of being nonspecific, which results in generalized immunosuppression, which places the recipient at increased risk for infection. Moreover all available drugs show side-effects and some long-term toxicity. Over the years, even though acute rejection can be efficiently controlled, grafts can be lost through a process known as chronic graft rejection, which results in arteriosclerosis. This rejection is organ dependant, for instance transplanted livers and kidneys survive longer than hearts and lungs. Calcineurin inhibitors (FK505, CsA) in addition have nephrotoxic effects. The half-lives of grafts vary from twenty years to less than two years.

For those reasons there is an ongoing effort to find new and effective immunosuppressants that have fewer side effects, do not evoke a general immunosuppression and allow a prolonged organ survival. Several pharmaceutical companies, including Novartis, are active in identifying such compounds. At the moment FTY720 is one of the most promising candidates as a novel immunosuppressant.

6.4 FTY720, a New Immunosuppressant

FTY720 is a synthetic analogue (2-amino-2-1,3-propanediol hydrochloride) of myriocin (Forrest et al., 2004a) (Fig. 4). This novel immunosuppressant was derived by chemical synthesis from myriocin, a metabolite isolated from culture filtrates of the ascomycete *Isaria Sinclairii* (Fujita et al., 1994) (Fig 4). In traditional Chinese medicine *Isaria Sinclairii* extracts were widely used as drug for eternal youth with severe gastrointestinal side-effects (Fujita et al., 1994).

These side-effects were in part due to myriocin that at the same time turned out to be a potent immunosuppressant. In order to generate compounds with less toxicity, modified compounds were synthesized. One of the improved compounds (Adachi et al., 1995), which was developed by Prof. T. Fujita (Taito Co. Ltd.) in collaboration with Yoshitomi Pharmaceuticals Ltd., was called FTY720. In 1997 the compound FTY720 was licensed-in by Novartis from the Japanese company Yoshitomi. FTY720 represents the prototype of a new class of immunosuppressants that, instead of inhibiting cytokine production, signal transduction or lymphocyte proliferation, alter the migration and homing of lymphocytes.

6.5 Graft Rejection and FTY720

FTY720 prolongs allograft survival with great potency and efficacy. This effect has been documented in rats (skin, heart, liver, small bowel) (Chiba et al., 1996; Suzuki et al., 1996b), dogs (kidney) (Kawaguchi et al., 1996) and monkeys (kidney) (Kahan, 1998). It has no antiproliferative activity at therapeutically relevant concentrations (Pinschewer et al., 2000) and therefore synergizes effectively with the calcineurin inhibitor CsA. (Chiba et al., 1996; Hoshino et al., 1996; Kahan, 1998; Kawaguchi et al., 1996). FTY720 prevents also perivascular inflammation and graft arteriosclerosis (Nikolova et al., 2000). In addition it does not inhibit T-cell cytokine production and/or B-cell antibody production in striking contrast with other immunosuppressants like cyclosporine A and FK506. All this data suggests that FTY720 has not a direct effect on lymphocyte function, but it has a completely novel mechanism of action.

6.6 Mechanism of Action of FTY720

The hallmark of the mode of action of this drug is a markedly reduced number of circulating T- and B-cells in the blood (lymphopenia) within hours after its oral administration (Chiba et al., 1998; Enosawa et al., 1996; Pinschewer et al., 2000). The sequestration of lymphocytes from blood and spleen into lymph nodes (LN) and Peyer's patches (PP) is completely reversible. This prompted the speculation that the drug regulates the homing of lymphocytes by influencing homing receptors (cytokine receptors). Subsequent studies

showed that the sequestration was independent of homing receptors and adhesion molecules. In the mean time independent publications demonstrated that the immunosuppressive action of FTY720 was indeed the consequence of its lymphopenic effect, leading to a suppression of T-cell infiltration into the grafted organs.

Recent publications have shown that the phosphorylated form (Fig. 4) of FTY720 (Mandala et al., 2002b) behaves similar to the structural homologue sphingosine-1-phosphate (S1P), a natural platelet derived lysophospholipid that is present at high nanomolar concentrations in serum (Kimura et al., 2001).

Naïve T-cells (T_n) regularly circulate between blood and lymphatic tissue during their process of antigen finding. The blood T-cells enter the lymph nodes via high endothelial venules (HEV) and egress through the sinus-lining endothelium (SLE) via thoracic duct lymph into blood (Sallusto and Lanzavecchia, 2000). It has been found that T- and B-cells require the $S1P_1$ receptor to egress from peripheral lymphoid organs and thymocytes to egress from the thymus (Matloubian et al., 2004b). At the moment it is believed that the internalization of $S1P_1$ receptors is the key step in the mode of action of FTY720 (Fig 2). These finding provided indications that sphingosine-1-phosphate is a critical physiological and pharmacological regulator of lymphocyte egress.

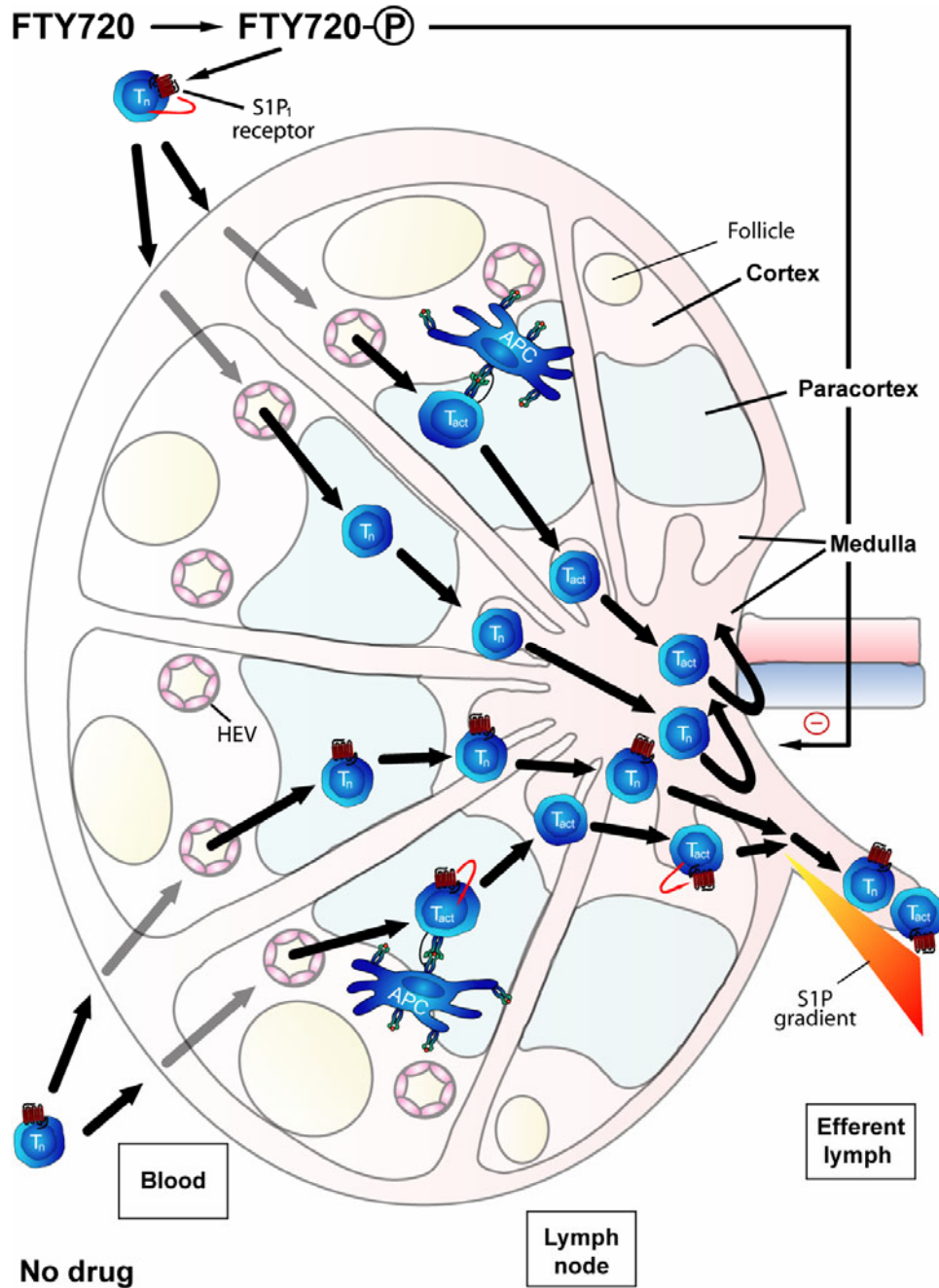


Fig. 2. S1P/S1P₁-dependent egress of T-cells from lymph nodes: effect of FTY720.

Naïve T-cells (T_n) regularly circulate between blood and lymphatic tissue. T_n enter lymph nodes (LN) via high endothelial venules (HEV) and egress in an S1P/S1P₁-dependent step via the efferent lymph into the blood. In case of productive antigen encounter in the LN, T_n become activated (T_{act}) by antigen presenting cells (APC) and transiently down-modulate S1P₁; this renders cells unresponsive to the obligatory egress signal provided by S1P, and, as a consequence, the proliferating cells remain in the LN. At the end of the proliferation phase, T_{act} up-regulate S1P₁ and egress from LN in an S1P/S1P₁-dependent step.

FTY720, after phosphorylation, acts as agonist at S1P₁ on T_n and T_{act}. In contrast to S1P it induces long lasting internalization of the receptor. This renders all T-cells unresponsive to sphingosine-1-phosphate (S1P), depriving them from the obligatory egress signal provided by S1P. As a result, all T_n and T_{act} are 'trapped' in LN, being unable to recirculate to peripheral tissues. Similarly, FTY720 down-modulates S1P₁ on thymocytes and B-cells, retaining them in thymus and LN, respectively (not shown). Modified after Brinkmann et al. (Brinkmann et al., 2004a) and Janeway (Janeway and Travers, 1995).

6.7 Sphingolipids

Sphingolipids are the major structural components of all eukaryotic membranes. They are composed of a ceramide backbone, containing sphingoid base and an amide-linked fatty acid tail, and have a polar head group. The most common sphingolipid is sphingomyelin, which contains a phosphocholine head group. (Fig. 3) Ceramide, the backbone of all sphingolipids, has a hydroxyl head group and an acyl chain that can vary in length. Sphingosine also has a hydroxyl head group but lacks the amide linked fatty acid side chain. Other sphingolipids, such as cerebrosides and gangliosides, can have head groups that consist of a single sugar or oligosaccharide chains of varying length and complexity (Spiegel and Milstien, 1995). The diversity of sphingolipid structure plays a role in the diverse functions they play in cells, serving as both structural entities and bioactive molecules.

Sphingolipid metabolites such as sphingosine, ceramide, and sphingosine-1-phosphate (S1P) are also important bioactive molecules that are involved in many cellular functions. Changes in metabolism and biosynthesis of sphingolipids has been observed upon oncogenic transformation, suggesting that they may play a role in cell growth, tumor formation, cellular differentiation, and cell cycle control (Pyne and Pyne, 2000). Sphingolipid metabolites have also been shown to affect cellular morphology by inducing changes to the cytoskeletal architecture (Van Brocklyn et al., 1999).

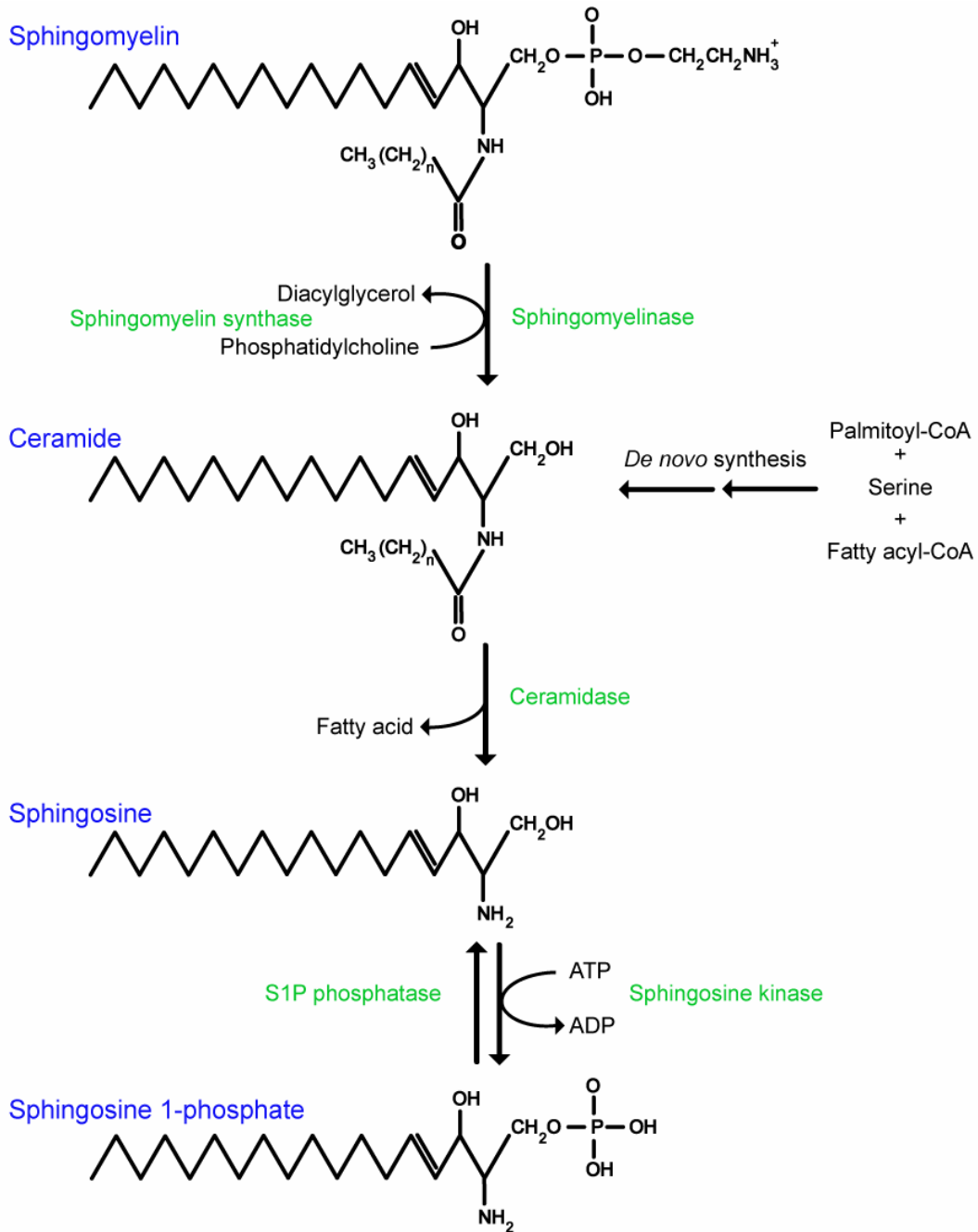


Fig. 3. A simplified scheme of sphingosine-1-phosphate biosynthesis. Modified after Pyne (Pyne and Pyne, 2000).

6.8 Sphingosine-1-Phosphate (S1P)

Sphingosine-1-phosphate (S1P) (Fig. 3) is a sphingolipid metabolite which was suggested to behave either as an intracellular second messenger or as an extracellular signaling initiator (Spiegel and Milstien, 2000). S1P is present at high nanomolar concentrations in serum (Kimura et al., 2001) and is re-

leased by platelets during platelet activation. The degradation of sphingomyelin to ceramide is the first step in the formation of S1P by the degradation pathway (Fig. 3). This step is catalyzed by the actions of a family of enzymes called sphingomyelinases that cleave off the phosphocholine head group resulting in the formation of ceramide. Ceramide can also be *de novo* synthesized by an independent pathway. The amide linked fatty acid group of ceramide is then cleaved by ceramidase to form sphingosine. Once formed, sphingosine is not only readily taken up by cells but is also phosphorylated on its primary hydroxyl group to form S1P through the action of sphingosine kinase. S1P can then be further degraded to ethanolaminephosphate and palmitaldehyde (Zhou and Saba, 1998) or it can be de-phosphorylated back to sphingosine by S1P phosphatase (Le Stunff et al., 2002).

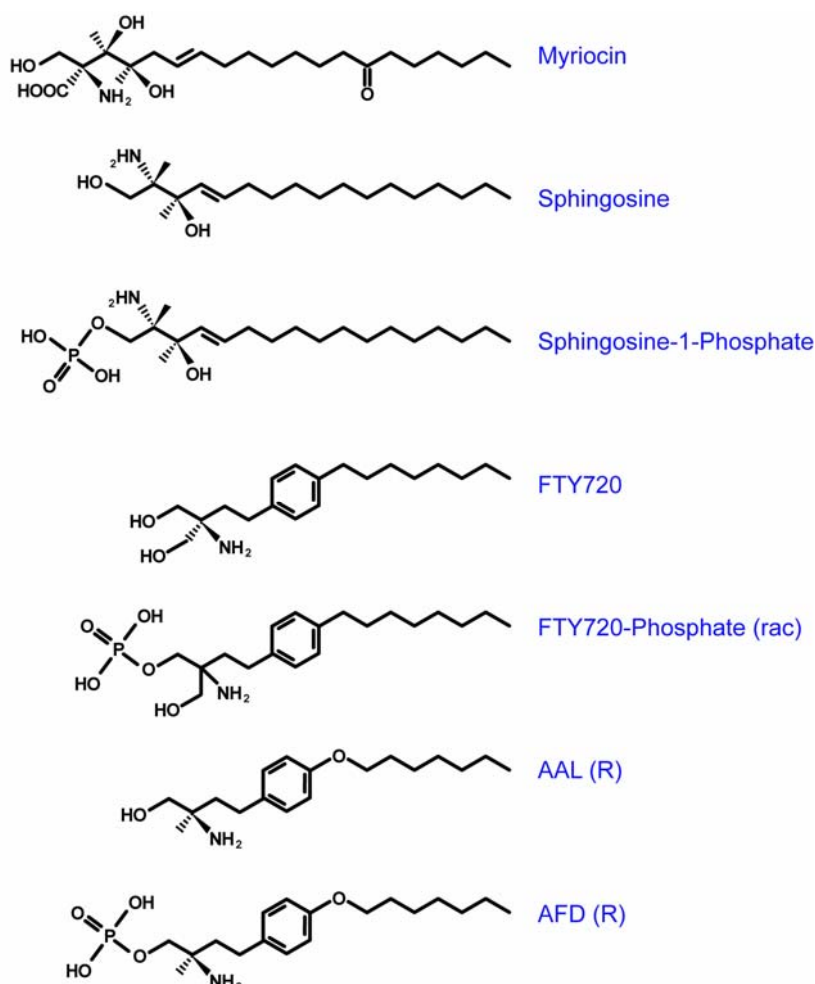


Fig. 4. Chemical structure of myriocin, sphingosine, sphingosine-1-phosphate, FTY720, FTY720-phosphate, AAL(R) (an active prodrug), AFD(R)(a FTY720 derivative)(Brinkmann et al., 2002a).

S1P acts as a survival factor for many cell types including melanocytes, hepatoma cells, neutrophils, macrophages, acute leukemia cells, HUVECs, keratinocytes, and hepatic myofibroblasts. There is some evidence for both receptor-mediated and intracellular effects of S1P on cell survival and protection from apoptosis (Radeff-Huang et al., 2004). Because of the interconvertibility of ceramide, sphingosine and S1P and the opposing effects on cell fate of ceramide and sphingosine (anti-growth, pro-apoptotic) compared to S1P (pro-growth, anti-apoptotic), the dynamic balance between S1P and ceramide/sphingosine has been proposed to determine cell survival or death (Pyne and Pyne, 2000). These observations have prompted by the authors to propose the hypothesis of the ceramide/S1P rheostat.

6.9 S1P is a Ligand for the endothelial differentiation gene (EDG)

Since many of the observed effects of S1P were pertussis toxin (PTX) sensitive, it was for a long time hypothesized that a S1P surface receptor exist (Yamamura et al., 1997). One candidate was an orphan gene, cloned from phorbol ester differentiated human umbilical vein endothelial cells (HUVEC) and called endothelial differentiation gene (EDG) (Hla and Maciag, 1990a). This gene shared a high degree (~20%) of similarity with the cannabinoid (CB) receptors, CB1 and CB2. Binding studies using human embryonic kidney cells (HEK 293) cells overexpressing EDG-1, now named S1P₁ (Chun et al., 2002), showed that S1P and dihydro-S1P could displace bound [³²P]S1P. A large battery of other sphingolipids and lysophospholipids were also tested but none of them showed high affinity for the binding to S1P₁/EDG-1 receptor (Lee et al., 1998b).

To date, five S1P receptors have been found: S1P₁ (EDG1), S1P₂ (EDG5), S1P₃ (EDG3), S1P₄ (EDG6), S1P₅ (EDG8) (Siehl and Manning, 2002). Comparison of the amino acid sequences reveals 46–54% identity among the S1P receptors (Takuwa et al., 2002). They have overlapping as well distinct patterns of expression in different tissue. S1P₁, S1P₂, and S1P₃ receptors are widely expressed, whereas S1P₄ is confined to lymphoid and hematopoietic cells and S1P₅ to glial cells in the CNS. In addition, the coupling of these receptors to different G proteins (Fig. 5) explains their differential signal trans-

duction properties, and also the broad cellular effects of S1P (Sanchez and Hla, 2004).

6.10 G-Protein Coupled Receptor (GPCR)

The S1P/EDG receptor family as well as the melanocortin receptor family are members of the superfamily of G protein coupled receptors (GPCRs) (Review: Gether, 2000). All GPCRs have the same basic structure. They contain seven alpha helical transmembrane domains. GPCRs have been named based on their ability to recruit and regulate the activity of intracellular heterotrimeric G proteins. Upon binding of the ligand there is a conformational change in the receptor that allows for the interactions with the heterotrimeric G proteins, which are composed of three subunits, one alpha subunit (α) and one beta/gamma ($\beta\gamma$) subunit. G proteins are classified on the basis on their α -subunits, and there are 15 known α -subunits that have been categorized into four subfamilies ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$) based on sequence and functional similarities. Up to date five β and fourteen γ proteins have been described. The α -subunit contains the guanine nucleotide binding site, whereas β and γ form a tightly associated complex. When inactive, the α -subunit is bound to GDP and to the $\beta\gamma$ -complex forming a trimeric protein complex. Activation occurs by catalyzing the exchange of guanine nucleotide diphosphate (GDP) for guanine nucleotide triphosphate (GTP). One function of the receptor is to act as a guanine nucleotide exchange factor, or GEF. Once the heterotrimeric G protein is in its GTP bound active state, it dissociates from the receptor and then into α and $\beta\gamma$ subunits. Both the GTP bound α -subunit and the released $\beta\gamma$ -dimer can modulate several cellular signaling pathways. These include, among others, stimulation or inhibition of adenylate cyclases, activation of phospholipases, as well as regulation of potassium and calcium channel activity. The α -subunit quickly gets inactive after the intrinsic nucleotide triphosphate phosphatase (GTPase) activity catalyzes the cleavage of GTP to GDP.

Further complexity of GPCR signaling has recently become apparent after the publication of data indicating that GPCRs may not solely act via heterotrimeric G proteins. In particular, it has been suggested that agonist-promoted phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs) and subsequent sequestration (see Internalization) of the receptors

from the cell surface are not only important for turning off signaling, but also play a key role in switching the receptor from G protein-dependent pathways to signaling cascades normally used by growth factor receptors (Lefkowitz, 1998).

Several bacterial toxins can modify the activity of GPCRs. Two of the best known are pertussis toxin and cholera toxin. Pertussis toxin is a 76-kD protein that ADP-ribosylates the alpha subunit of the $G_{\alpha i}$ protein. The ribosylation prevents the exchange of GDP to GTP and therefore inhibits signaling from the GPCR. Cholera toxin catalyzes a similar reaction, leading to the transfer of an ADP-ribose unit from NAD^+ to an arginine side chain in the alpha subunit of G_S proteins. Ribosylation of the $G_{\alpha s}$ protein inactivates the intrinsic GTPase activity of the alpha subunit causing it to become constitutively active. Both toxins are widely used to study GPCRs (Gilman, 1987).

6.11 S1P₁ Receptor

Sphingosine-1-phosphate receptor 1 (S1P₁/EDG-1) was originally identified as an early/immediate gene that was upregulated upon treatment of human umbilical vein endothelial cells (HUVECs) with phorbol 12-myristate 13-acetate (PMA), a phorbol ester (Hla and Maciag, 1990a). Since PMA promotes differentiation of endothelial cells into tubular structures, it was hypothesized that S1P₁ played a role in this process therefore the gene was called endothelial differentiation gene (EDG). S1P₁/EDG-1 has been found to couple specifically to the $G_{\alpha i}$ pathway (Lee et al., 1996b), while no detectable coupling was found with $G_{\alpha s}$, $G_{\alpha q}$ or $G_{\alpha 12/13}$ using an Sf9 insect cell system (Windh et al., 1999a). Subsequent overexpressing S1P₁/EDG-1 experiments in the human embryonic carcinoma cell line HEK293 (cell line is devoid of intrinsic S1P₁) suggested that sphingosine-1-phosphate (S1P), a specific component of fetal calf serum growth medium, is responsible for S1P₁ receptor dependent rearrangement of the cell shape (Lee et al., 1998b). Binding studies confirmed that S1P was indeed a high affinity ligand for S1P₁/EDG-1 with a K_d of 8 nM. The cloning of S1P₁/EDG-1 and the subsequent identification of its ligand facilitated the characterization of many signaling pathways and cellular re-

sponses that are mediated by S1P.

In cells overexpressing S1P₁, S1P was capable to stimulate extracellular signal-regulated kinase (ERK), important proteins mediating cellular proliferation (Kon et al., 1999b; Lee et al., 1998b; Lee et al., 1999), as well as phosphoinositide 3-kinase (PI3K β), another proteins with important effects on cellular growth (Kluk and Hla, 2002). Forskolin induced cyclic adenosine monophosphate (cAMP) accumulation was inhibited (Zondag et al., 1998) by S1P₁ activation. All these responses were pertussis toxin sensitive, indicating that they were mediated via G_{oi}. It was further observed that S1P₁ mediates S1P stimulation of small cellular GTPase Rac via G_{oi} and that this activation was dependent on PI3K (Takuwa et al., 2002).

S1P₁ plays a critical role in the maturation of vascular smooth muscle cells. Homozygous S1P₁ knock-out mice die as a consequence of leaky vessels and extensive bleeding during the embryonal development (Allende et al., 2003a; Liu et al., 2000b). S1P₁/EDG-1 is widely expressed. Several mouse, rat and human tissues such as heart, brain, lung, thymus, kidney, spleen, adipose tissue, skin, uterus, testis and liver have been found to express mRNA for S1P₁/EDG-1 (Kluk and Hla, 2002). S1P₁ was suggested to play a critical role in the motility and directional migration of a variety of cells (Le Stunff et al., 2002).

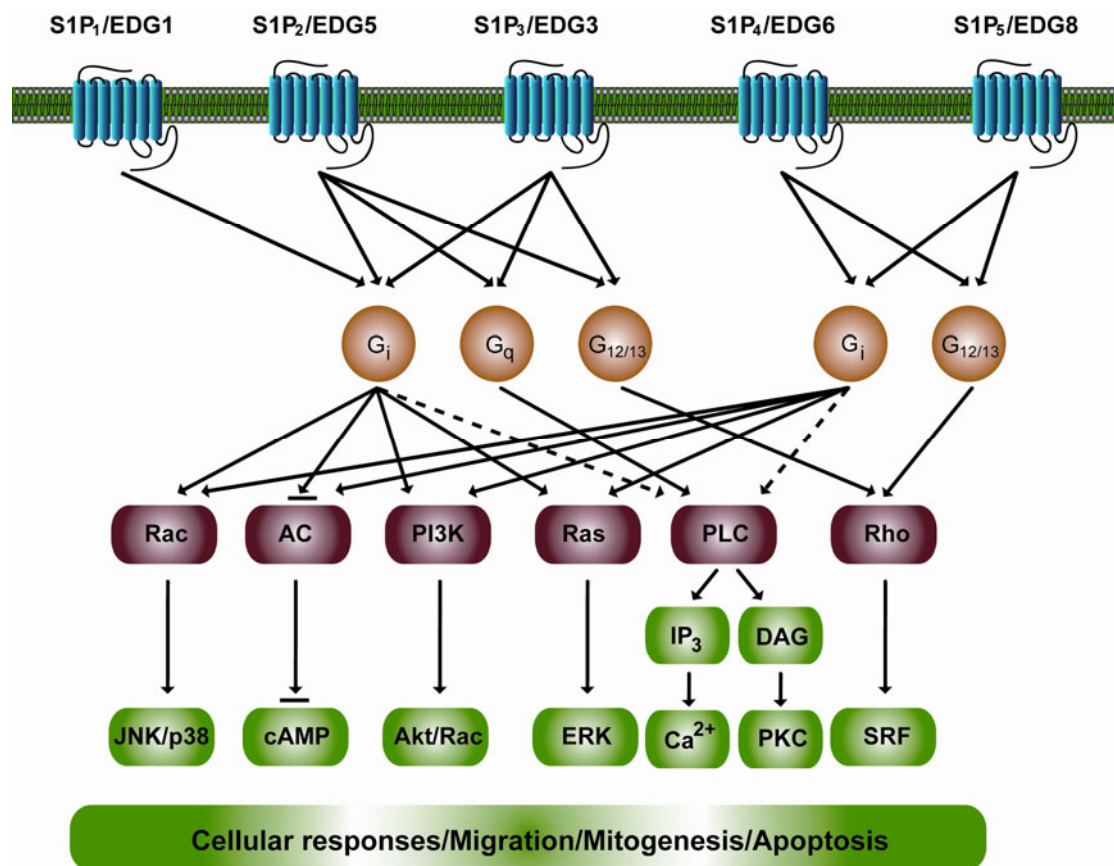


Fig. 5. S1P is a ligand for five G-protein-coupled receptors. Sphingosine-1-phosphate (S1P) which is present in serum, often being secreted by mast cells, platelets and monocytes, binds to specific members of the S1P receptor family, which are coupled to different G proteins (for example EDG1/S1P₁ and EDG6/S1P₄ couple mainly to G_{α_i}; both EDG5/S1P₂ and EDG3/S1P₃ activate G_{α_i}, G_{α_q} and G_{α_{12/13}}; and EDG8/S1P₅ is linked to G_{α_i} and G_{α_{12/13}}) leading to activation or inhibition of the indicated downstream signaling pathways. Only a few examples of these pathways are illustrated - in particular, adenylyl cyclase–cyclic AMP (AC); extracellular signal-regulated kinase (ERK); Jun amino terminal kinase (JNK); the small GTPases of the Rho family (Rho and Rac); p38 Mitogen-activated protein kinase (p38); phospholipase C (PLC); phosphatidylinositol 3-kinase (PI3K); protein kinase B (Akt); protein kinase c (PKC); serum response factor (SRF); inositol 1,4,5-triphosphate (IP₃); diacylglycerol (DAG). Modified after Spiegel (Spiegel and Milstien, 2003), Anliker (Anliker and Chun, 2004) and Sanchez (Sanchez and Hla, 2004).

6.12 S1P₂/EDG-5

The S1P₂/EDG-5 gene was originally isolated from rat cardiovascular and rat nervous tissues by two independent groups and was given the names ARG 16 and H218, respectively (MacLennan et al., 1994; Okazaki et al., 1993). The genomic structure of S1P₂/EDG-5 is the same as the rest of the members of the S1P receptor family. It consists of two exons with the entire coding sequence located in the second exon (Liu and Hla, 1997). This receptor, like S1P₁/EDG-1, is a high affinity receptor for S1P. Binding studies with this receptor demonstrated that it has a K_d of 20-25 nM for S1P (Gonda et al.,

1999).

S1P₂/EDG-5 couples to a G_{αi}, G_{αq} and G_{α12/13} proteins (Windh et al., 1999a) to elicit a wide range of biological effects like activation of PLC and Ca²⁺ release (Ancellin and Hla, 1999; Kon et al., 1999b). S1P₂/EDG-5 promotes the phosphorylation of p42/44 ERK proteins, implying a role for S1P₂/EDG-5 in cell growth (Gonda et al., 1999). It was also found that S1P₂/EDG-5 suppressed growth factor-stimulated Rac activity *via* a PTX-insensitive G- protein (Takuwa et al., 2002). Although S1P₂ was proposed to have a function in heart development (Kupperman et al., 2000), knockout mice lacking S1P₂ showed no obvious phenotypic abnormalities and are viable (Ishii et al., 2001b).

6.13 S1P₃/EDG-3

The S1P₃ receptor is functionally very similar to S1P₂/EDG-5. Like the other S1P receptors the binding affinity of S1P₃/EDG-3 to S1P was 20-25 nM (Van Brocklyn et al., 1999). It has a broad G_α coupling profile (G_{αi}, G_{αq} and G_{α12/13}) and activates as many pathways as S1P₂/EDG-5 does (Windh et al., 1999a). Cellular responses elicited from this receptor are also similar to those mediated by S1P₂/EDG5. These include activation of phospholipase C (PLC), activation of ERK, and Ca²⁺ mobilization (Kon et al., 1999b; Okamoto et al., 1999; Windh et al., 1999a). S1P₃/EDG-3 was found to activate Rac via G_i (Takuwa et al., 2002) and Rho via G_{α12/13}, leading to the clustering of integrins and stress fiber formation (Paik et al., 2001). Recent studies suggest a role for S1P₃ receptor in blood pressure regulation (Forrest et al., 2004a; Sanna et al., 2004b). An unexpected observation was that transgenic mice lacking S1P₃ were viable and showed no apparent anatomical or physiological defects (MacLennan et al., 2001b).

6.14 S1P₄/EDG-6

S1P₄/EDG-6 is the fourth member of the S1P receptor family. This gene was originally isolated from lymphoid and hematopoietic tissues, where it is highly expressed (Gräler et al., 1998). The binding affinity to S1P was determined to be lower than that of the previously known members, with a K_d of 63 nM (Gräler et al., 1998). S1P₄/EDG-6 couples primarily to the G_{αi} pathway since all of the observed effects have been pertussis toxin sensitive. Upon stimulation of S1P₄/EDG-6, there is a strong activation of ERK. Stimulation with S1P

was reported to activate PLC and promote intracellular Ca^{2+} release (Yamazaki et al., 2000).

Upon S1P stimulation, Chinese hamster ovary cells (CHO-K1) ectopically expressing S1P₄ potently activate the small GTPase Rho. As a consequence these cells undergo cytoskeletal rearrangements resulting in peripheral stress fiber formation and cell rounding (Graler et al., 2003).

6.15 S1P₅/EDG-8

The fifth and most recently discovered member of the S1P receptor family is S1P₅/EDG-8 (Glickman et al., 1999). The signaling associated with this receptor has to be elucidated. It seems that this receptor couples to the $G_{\alpha i}$ pathway, like S1P₁/EDG-1 and S1P₄/EDG-6 (Im et al., 2001). Binding experiments have shown that S1P₅/EDG-8 is another high affinity receptor for S1P with a K_d determined to be 2 nM (Malek et al., 2001).

Northern blot analysis has revealed that in humans S1P₅/EDG-8 is mainly expressed in the skeletal muscle, heart and kidney whereas in rats S1P₅/EDG-8 is exclusively expressed in brain, spleen and skin (Niedernberg et al., 2002). These differences in expression profiles suggest that these receptors may play different roles in human and rat physiology. In S1P₅ expressing CHO cells S1P₅/EDG-8 is coupled to $G_{\alpha i}$ and $G_{\alpha 12}$ and inhibits ERK activation (Malek et al., 2001a).

6.16 Internalization of G- Protein Coupled Receptors

The exposure of GPCRs to agonists often results in the rapid decrease of receptor responsiveness. This process is named desensitization and includes uncoupling of the receptor from heterotrimeric G proteins (Hausdorff et al., 1990), internalization (Fig. 6) of cell surface receptors into intracellular compartments (also termed receptor sequestration or endocytosis) and downregulation of the mRNA and protein synthesis (Ferguson, 2001). Desensitization is thought to be necessary to prevent signaling from a permanently activated receptor subsequent to the agonist binding (Pierce and Lefkowitz, 2001b). One of the first steps in this process involves the functional “uncoupling” of the G proteins from the receptors. This process is fairly rapid (seconds to minutes) and occurs after receptor phosphorylation at the carboxy-terminal tail or at the third intracellular loop by intracellular second-messenger regulated kinases (PKA and PKC) and by G protein-coupled receptor kinases (GRKs). GRKs are recruited to and specifically phosphorylate only agonist occupied receptors leading to “homologous” desensitization (Hausdorff et al., 1990). Seven mammalian GRK genes have been identified. Once phosphorylated by a GRK, the receptor is bound by a member of the arrestin protein family. Arrestins recognize both GRK phosphorylation sites on the receptor and the active conformation of the receptor, driving the formation of a tight complex (Luttrell and Lefkowitz, 2002; Perry and Lefkowitz, 2002a). GRK-phosphorylation is not sufficient for desensitization, but rather serves to create high affinity sites to promote the binding of arrestin proteins which guarantees desensitization by preventing further coupling to G proteins (Gurevich et al., 1995). Four distinct mammalian arrestins are known, two of them (visual and cone arrestins) are restricted to the phototransduction pathway. Two somatic forms, β -arrestin 1 (arrestin 2) and β -arrestin 2 (arrestin 3) are ubiquitously expressed and are thought to regulate signaling as well as internalization of many different GPCRs. Following agonist-dependent GRK-phosphorylation of receptors, the β -arrestin proteins, present in the cytosol, are recruited to the plasma membrane of the site of activated receptors. The arrestins themselves contain a binding site for the clathrin adaptor protein AP2. This leads to the

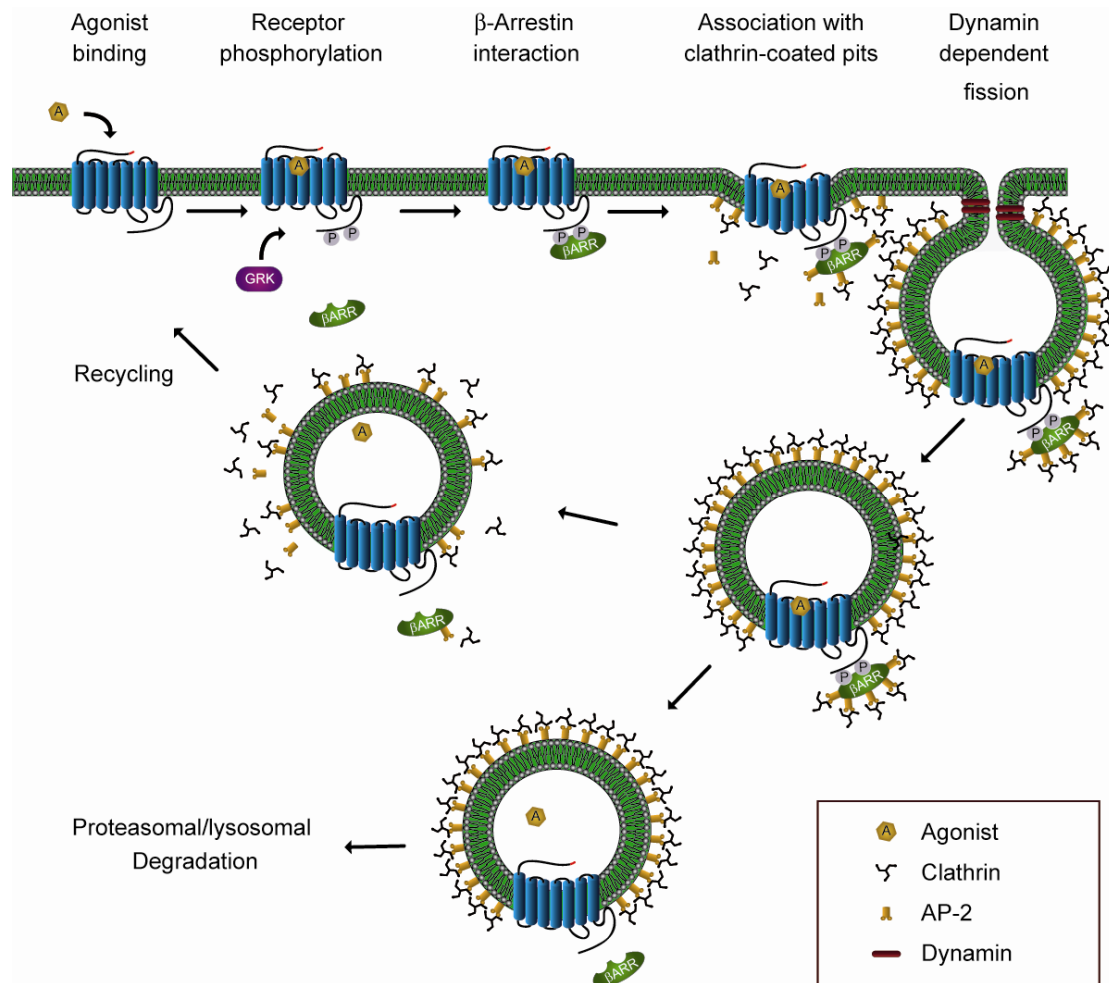


Fig. 6. Internalization of GPCRs. β-arrestin dependent internalization of G-protein-coupled receptors (GPCR) occurs through clathrin-coated pits. After agonist binding to GPCR, GPCR kinases (GRK) phosphorylate residues in the third intracellular loop and carboxyl tail of GPCRs, leading to the recruitment of β-arrestin. The β-arrestin recruits AP-2 complex and clathrin, which target GPCR to clathrin-coated pits. The receptors are internalized when these pits are 'pinched off' from the plasma membrane by the action of the GTPase dynamin to form coated vesicles. The endocytosed GPCR can then either recycle back to the cell surface or undergo proteasomal/lysosomal degradation. Modified after Pierce and Lefkowitz (Pierce and Lefkowitz, 2001b).

recruitment of clathrin to the complex arrestin-receptor. This facilitates the entry of the receptors into clathrin-coated pits for subsequent internalization (Goodman et al., 1996; Laporte et al., 1999). Furthermore, GRKs and arrestins appear to play a direct role in signaling by acting as scaffold proteins and bringing specific signaling molecules into proximity of the activated receptor (Luttrell and Lefkowitz, 2002). This leads to modifications of the activity in the complex components.

Once internalized, receptors can either be targeted to specialized intracellular

compartments where they are dephosphorylated and recycled back to the surface or they can undergo degradation via the proteasomal/lysosomal pathway (Claing et al., 2002).

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8 S1P RECEPTOR INTERNALIZATION (SUBMITTED PAPER)

The Phosphorylated Immunomodulator FTY720 have Potent Effects on Internalization and Desensitization of Sphingosine 1-Phosphate Receptors that are not observed with the Endogenous Agonist Sphingosine-1-Phosphate.

Christoph Hangartner, N. Rao Movva, Thi-Thanh-Thao Tran and Danilo Guerini*, Transplantation and Immunology, Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland

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*To whom correspondence should be addressed

Abbreviations

BSA	Bovine serum albumin
EC ₅₀	Effective concentration resulting in 50% of maximal activity
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorter
FCM	Flow cytometry
FCS	Fetal calf serum
FLIPR	Fluorescence image plate reader
GRK	G-protein coupled receptor kinase
GPCR	G-protein coupled receptor
GTPγS,	Guanosine 5γ-3-O-(thio)triphosphate
HBSS	HEPES based salt solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(ethanesulfonic acid)
IC ₅₀	Concentration needed for 50% inhibition of maximal activity
MAPK	Mitogen activated protein kinase
MOPS	3-(N-morpholino) propanesulfonic acid
mS1P ₄	Mouse sphingosine 1-phosphate receptor 4
PTX	Pertussis toxin
PVT	Polyvinyl toluene
NET	Nonhidet EDTA Tris
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
S1P _{1,2,3,4,5}	sphingosine 1-phosphate receptor 1,2,3,4,5
S1P	sphingosine 1-phosphate
Tris	Tris (hydroxymethyl)aminomethane
WGA	Wheat germ agglutinin

Abstract

FTY720 is a novel lipid immunomodulator that has been shown to be efficacious in allograft protection in Phase II trials of kidney transplantation in humans. FTY720 is phosphorylated by sphingosine kinase *in vitro*, which is believed to be necessary for the *in vivo* action of the parent compound. The cell surface receptors of the physiological ligand sphingosine-1-phosphate were shown to bind the phosphorylated form of FTY720. We have evaluated the effects of the natural ligand sphingosine-1-phosphate, FTY720, and its phosphorylated derivatives on the activation, internalization and desensitization of a subset of sphingosine-1-phosphate receptors. The phosphorylated form of FTY720 and its derivative AFD(R) promoted extensive and long lasting internalization of the sphingosine-1-phosphate receptor 1, while the endogenous agonist had only weak and transient effects. The internalization by FTY720 was accompanied by the persistent loss of receptor dependent signaling, an effect that was not observed with sphingosine-1-phosphate. FTY720 phosphate only caused partial internalization of the sphingosine-1-phosphate receptor 3, which did not result in effects of the receptor dependent activity. Sphingosine-1-phosphate receptor 4 showed a weak response. The differential behaviors, e.g. the strong and long lasting internalization of sphingosine-1-phosphate receptor 1 and the much weaker effect at the other receptors, may explain the exquisite cellular (*in vivo*) effects of phosphorylated FTY720 compared to sphingosine-1-phosphate. Our results further show that, in contrast to earlier reports the internalization requires FTY720 to act as an agonist. As the derivatives of FTY720 confer varying levels of receptor internalization and desensitization it is possible to screen for compounds with altered downstream

signaling responses, leading to unique physiological and therapeutic benefits.

Introduction

Sphingosine-1-phosphate receptors, formerly called EDG receptors (Lynch, 2002) are novel G-protein coupled receptors (GPCR) belonging to the lysophospholipids subfamily. The first member of this family (S1P₁ or EDG-1) was discovered in human endothelial cells (Hla and Maciag, 1990). Subsequent experiments suggested that sphingosine-1-phosphate (S1P), a specific component of serum, was responsible for S1P₁ receptor dependent activity (Lee et al., 1998). Currently the S1P receptor family consists of five well characterized members S1P₁, S1P₂, S1P₃, S1P₄ and S1P₅ in mammals with high homology to each other (Hla et al., 2001; Spiegel et al., 2002).

S1P₁ plays a critical role in the maturation of vascular smooth muscle cells and homozygous S1P₁ knock-out mice die as a consequence of the incomplete sealing of blood vessels (Allende et al., 2003; Liu et al., 2000). Recent studies suggest a role for the S1P₃ receptor in blood pressure regulation (Forrest et al., 2004; Sanna et al., 2004), while S1P₂ may have a function in the maintenance of cardiovascular system (Hla et al., 2001). Transgenic mice lacking S1P₃ and S1P₂ (Ishii et al., 2001; MacLennan et al., 2001) or both (Ishii et al., 2002; Kono et al., 2004), however, show no dramatic phenotype and are viable. Despite their rather restricted tissue expression, the function of S1P₄ and S1P₅ receptors is not yet established (Graeler et al., 1999; Im et al., 2001; Im et al., 2000; Malek et al., 2001; Van Brocklyn et al., 2000).

The novel immunomodulator FTY720 has recently been shown to be very efficacious in human clinical trials and opening the door to novel therapies pre-

venting organ rejection in transplant patients (Budde et al., 2002). The observation that the phosphorylated form of the FTY720 is a high affinity agonist of four S1P-receptors (Brinkmann et al., 2002; Mandala et al., 2002), suggested a role for some of these receptors in the control of the migration of lymphopoietic cells between various cellular compartments. In order to understand the specific functions of these S1P receptors, stable CHO cell lines were generated expressing each of these proteins. As the parent CHO cells are largely devoid of intrinsic S1P dependent activity, they are suitable to study the unique biological responses generated by the introduced receptors. CHO cells have been shown to allow GPCR coupling with different G protein subsets (Kon et al., 1999; Newman-Tancredi et al., 2002), making them amenable to different GPCR specific assays such as the measurement of intracellular calcium, the production of cAMP, the binding of agonists to isolated membrane proteins and functional assays based on the GTPase activity present in their membranes (Brinkmann et al., 2002; Mandala et al., 2002).

S1P₁ is coupled primarily via a G_{αi} protein, which controls among other the cascade of mitogen kinases (MAPK) (Lee et al., 1996; Windh et al., 1999). Previous studies (Kon et al., 1999) indicated a minimal PTX dependent mobilization of Ca²⁺ in CHO cells overexpressing EDG-1, but the receptor was not considered capable of eliciting efficient signals through the phospholipase C and Ca²⁺ pathway. Recent observations have, nevertheless, suggested it is possible to find conditions where efficient Ca²⁺ transients can be generated by G_{αi} coupled receptor (Werry et al., 2003), (Bouhlal, R, pers. comm.).

GPCRs undergo agonist dependent desensitization and this process is

thought to be necessary to prevent the permanently activated state of the receptor subsequent to the agonist binding (Pierce and Lefkowitz, 2001). Desensitization is initiated by the phosphorylation of the receptor by GPCR specific kinases (GRKs) and is further followed by the association of specific scaffolding proteins called arrestins (Shenoy and Lefkowitz, 2003). The receptor is internalized after binding with arrestin (Perry and Lefkowitz, 2002) and is no longer available for agonist binding. Thus, the desensitization process plays an important role in the cessation of the signaling events initiated at the surface of the receptor by the specific ligand. The internalized GPCR becomes after a certain time dephosphorylated and it reappears on the cell surface by vesicle mediated transport, where it is ready for the next round of agonist binding. Thus, the rate of the recycling of the receptor to the cell surface plays a critical role in modulating signaling responses. GPCR's can be classified based on fast and slow recycling kinetics (Perry and Lefkowitz, 2002; Shenoy and Lefkowitz, 2003). The internalized receptors in some cases undergo degradation and this has been shown to strongly affect the density of the receptor on the cells surface after its activation (Marchese and Benovic, 2001). These observations underline the importance of studying the post-activation fates of GPCR. It has been reported that the S1P₁ receptor undergoes phosphorylation following agonist binding (Watterson et al., 2002) as well as internalization (Kohno et al., 2002) and that the glycosylated N-terminal domain plays an important role in this process (Kohno and Igarashi, 2003).

In the present study, we have developed assays and measured the internalization of SIP receptors (S1P₁, S1P₃ and S1P₄) in response to natural agonist S1P and the immunomodulatory drug FTY720 and its phosphorylated deriva-

tives. We have noted significant differences in the behavior of these receptors to these various ligands, in particular to the endogenous agonist S1P. Our results indicate that compounds can be found that affect, to different degrees, the internalization and reappearance of the S1P receptors. These characteristics might be an important component of the pharmacological properties of FTY720 that are crucial to its efficacy in the clinic.

Material and Methods

Materials

Sphingosine-1-Phosphate (S1P) was purchased from BioMol, (Juro Supply, Luzern, Switzerland). LipofectAMINE and Opti-MEM were from Corporation (Gibco, Invitrogen Corporation, Paisley PA4 9RF, UK). Oligonucleotide primers were purchased from Microsynth (Balgach, Switzerland). The protease inhibitors cocktail tablets were purchased from Roche Applied Science (Roche Diagnostics AG, Rotkreuz, Switzerland). Media and antibiotics were from Gibco, Invitrogen Corporation (Gibco, Invitrogen Corporation). SuperFect™ Transfection Reagent was purchased from Qiagen (Qiagen AG, Basel, Switzerland). For details on FTY720, FTY720-P, AFD(S), AFD(R), AAL(S) and AAL(R) see Brinkmann et al. (Brinkmann et al., 2002).

Culturing of the Cells

HeLa cells were cultured in DMEM (Gibco, Invitrogen) 10% FBS (heat inactivated, Gibco, Invitrogen), 50 µg/ml gentamicin (Gibco, Invitrogen) at 37°C, 5% CO₂, 95% air. CHO cells were cultured in RPMI 1640 or αMEM (Gibco, Invitrogen), 10% FBS (heat inactivated, Gibco, Invitrogen Corporation), 50 µg/ml

gentamicin (50 mg/ml, Gibco, Invitrogen) or 10000 units/ml penicillin and 10 mg/ml streptomycin (Gibco, Invitrogen), at 37°C, 5% CO₂, 95% air. Selection and maintenance of the HeLa and CHO cell clones were done in the same medium in the presence of 500 µg/ml G418 (Gibco, Invitrogen).

Generation of myc Tagged Human S1P Receptor Constructs

The pcDNA3.1 (Invitrogen Corporation) expression vectors encoding N-terminally myc-tag human S1P₁ and human S1P₃ were a gift from J. P. Hobson (Van Brocklyn et al., 2000). The expression vector (cloned in the mammalian expression vector pRc/CMV (Invitrogen Corporation) harboring the human S1P₄ gene and fused to the c-myc peptide tag at the carboxyl terminal was kindly provided by J. P. Hobson (Van Brocklyn et al., 2000). Mouse S1P₄ (GenBank Accession Number: MMU6074) was cloned by a PCR based method using following oligonucleotides: 5'GAGAGATCTAACATCAGTACCTGGTCC and 5'CTCTAGGTGCTGCGGACGCTGG and mouse genomic DNA. The amplified mouse S1P₄ cDNA was cloned in the pcDNA3.1 vector with a myc-tag inserted at the N-terminus of the protein. Human S1P₂ (GI: 4090955) was cloned in mammalian expression vector pcDNA 3.1 Topo V (Invitrogen Corporation). The lung cDNA used in the PCR reaction was obtained from BD Biosciences Clontech and amplified using following oligo nucleotides: 5'CACCATGGGCAGCTTGTACTCGGAGTACCTGAACCCCAACAAGGTCCAG and 5'GATTCAGACCACCGTGTTGCCCTCCAG.

Transfection and Selection of G418 Resistant Clones

The day before transfection, 0.5×10^6 cells were seeded in 100 mm diameter cell culture dishes. On the day of transfection, 10 μg of the cDNA vector were diluted in a total volume of 300 μl of fresh medium without serum and antibiotics. 60 μl of SuperFect Transfection Reagent was mixed with the DNA solution and incubated for 10 min at room temperature. The cells were washed once with phosphate-buffered saline (PBS) and 3 ml of fresh culture medium (containing 10% FBS and antibiotics) were added to the reaction tube containing the transfection complexes. The mixture was immediately transferred to the cultures and incubated for 3 hours at 37°C and 5% CO₂. After the incubation, the medium was removed and replaced with 10 ml of fresh culture medium. 72 hr after the transfection, the culture supernatants were removed and replaced with 10 ml of fresh culture medium containing 0.5 mg/ml of G418. Before G418 resistant cells reached confluence, they were sub-cloned in 96-well culture plates at a final density of one cell/well.

Preparation of Membrane Proteins

To obtain 100-150 mg of membrane proteins, cells were grown in 10 to 14 large culture dishes (500 cm²). The cells were harvested in 20 ml cold 10 mM HEPES (pH 7.5), 0.1% fatty acid-free bovine serum albumin (BSA), protease inhibitors cocktail (1/50 complete[®], Roche Applied Science), centrifuged at 750xg for 10 min at 4°C and re-suspended in 10 ml cold membrane buffer (20 mM HEPES, pH 7.4; 100 mM NaCl; 10 mM MgCl₂; 1 mM EDTA; 0.1% BSA and protease inhibitors cocktail). The cell suspension was homogenized on ice, using a Polytron homogenizer at 25000 rpm at three intervals of 20 sec-

onds each. The homogenate was centrifuged at 26'900xg for 30 min at 4°C and the pellet resuspended by vortexing in 2 ml of cold membrane buffer. The volume of the membrane protein suspension was adjusted to a final concentration of 2 to 3 mg protein/ml.

Activity Measurements of the Cells Expressing S1P Receptors: GTP γ ³⁵S and FLIPR

The FLIPR assay was performed as follow. CHO were plated in black Costar plate (96 or 384 well, 50'000 cells or 12.500 cells) in the culture medium described before and cultured at 37°C 20-24 h in a CO₂ incubator. Cells were then incubated in HBSS medium (Gibco, Invitrogen corporation) containing 2 μ M Fluo4AM (Molecular Probes, Cat. No, F-1241; 1 mg/ml stock in DMSO), 5 mM probenecid for 1 at 37°C, rinsed with HBSS buffer, 2.5 mM probenecid and overlaid with 75 μ l of the same medium (96 well plate). The plate was transferred to the FLIPR. In some cases the cells were pre-stimulated with 10 μ M ATP. Prior to the next addition, the cells were left at room temperature for 15-25 min. After measuring the baseline for 40 sec, the agonist was added in 25 μ l aliquot of HBSS (4x times concentrated) and the fluorescence was measured at interval of 2 sec for 3 to 5 min.

To characterize the GTP γ ³⁵S-binding to membrane proteins from CHO cells expressing S1P receptors, WGA coated scintillant PVT beads (SPA-bead, Amersham Biosciences, Little Chalfont, Buckinghamshire, HP7 9NA, UK) were used. The assay was performed in 96-well Optiplates (Packard instruments, PerkinElmer, 45 William Street, Wellesley, MA 02481-4078, USA). Af-

ter a short homogenization, the membrane proteins were resuspended at 75 µg/ml in 50 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 20 µg/ml saponin, 0.1% fat free BSA (pH 7.4), mixed with 1 mg/well SPA-bead, 10 µM GDP, different concentrations of agonists and incubated for 10-15 min at RT. The GTPγ³⁵S binding reaction was started by the addition of 200 pM GTPγ³⁵S (Amersham, >1000Ci/mmol). The Optiplates were sealed and incubated at RT for 110-120 minutes with constant shaking. The plates were then centrifuged for 10 minutes at 2000 rpm and counted with a TopCount instrument (Packard Instruments). Calculations of the EC₅₀ were performed with a non-linear regression fit program as available in the Origin 7 RS2 software package (Origin Lab Corporation, One Roundhouse Plaza, Northampton, MA 01060, USA).

Agonist Treatment/Internalization

Stable or transiently transfected cells were grown in six-well plates to 60-80% confluence (about 10⁶ for CHO cells and 3.10⁶ for HeLa cells) in DMEM, RPMI1640 or αMEM, 10 % charcoal-stripped lipid-depleted FBS, 50 µg/ml Gentamicin. For stable clones 0.5 mg/ml G418 were included. The cells were incubated for the indicated time with a 1 µM agonist solution at 37°C in a CO₂ incubator in complete medium followed by one PBS wash step. In case of a wash-out, after 1 h the supernatant was removed, the cells were washed by PBS and incubated for the required time in fresh complete medium.

FCM (flow cytometry) Analysis of Expression of S1P-Receptors.

After the incubation and treatment with the compounds the cells were washed with phosphate-buffered saline (PBS) and detached by treatment with PBS/1 mM EDTA. 10^6 cells were incubated either with 4 $\mu\text{g/ml}$ of monoclonal mouse anti c-myc IgG1 (Roche Applied Science) antibody, 1/100 diluted S1P₁ antibody, (Exalpha Biologicals Inc., 84 Rosedale Road, Watertown, MA 02472, USA) or with isotype control mouse IgG1 (Pharmingen, BD Biosciences, Basel, Switzerland) in balanced salt solution (BSS)–5% FCS (BSS is 0.14 M NaCl, 1 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄, 0.3 mM NaH₂PO₄, and 0.4 mM KH₂PO₄ [pH 6.9]) for 60 min at 4°C. When using the S1P₁ antibody, recognizing a C-terminal (cytosolic) epitope, the cells were permeabilized using the Cytoperm solution (Becton and Dickinson, 1 Becton Drive, Franklin Lakes, NJ USA 07417) prior to the addition of the antibody solution. The cells were washed once in BSS/5% FCS, and incubated 60 min at 4°C in the dark with 1 $\mu\text{g/ml}$ of Alexa488-labeled goat anti mouse secondary conjugates (Molecular Probes, Juro Supply, Luzern, Switzerland) The cells were washed again before being subjected to cytofluorometric analysis (FACS Calibur, BD Biosciences, Basel, Switzerland). Flowcytometry (FCM) measurements were performed with 10'000 viable cells per sample. The viability was tested by the addition of 3 μl of a 1 μM DNA binding TO-PRO-3 iodide solution (Molecular Probes, Juro Supply, Luzern, Switzerland) and analyzed by FCM.

Immunoblotting

Cells in six-well plates were harvested by scraping into 0.2 ml of PBS, centrifuged at 350xg and the pellet resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.8-8.0). After a 15 min on ice, protease inhibitor mix (Roche Applied Science) was added and the cells were homogenized by three times freezing (dry ice) and thawing. The homogenate was centrifuged for 15 min at 14000xg at 4°C. The pellet was resuspended in 50 µl of 5 mM Tris-HCl pH 8.0, mixed with an equal volume of electrophoresis sample buffer and fractionated by SDS-PAGE using 12% (w/v) Tricine polyacrylamide resolving gels (Schagger and von Jagow, 1987).

Following transfer to a nitrocellulose membrane, nonspecific protein binding sites were blocked by incubating 60-min in 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS). Membranes were then incubated overnight at 4 °C with monoclonal mouse anti c-myc (Roche Applied Science) (1 in 500 dilution) in 5% (w/v) non-fat dry milk/0.05% Tween 20. After rinsing, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Bio-Rad, Reinach, Switzerland) for 60 min at 4 °C in the dark. The membrane was washed two times 5 for min and one time for 15 min with 5% (w/v) non-fat dry milk/0.05% Tween 20. Immunoreactive proteins were identified by ECL-plus Western blotting detection kit and hyperfilm ECL (Amersham Biosciences, Otelfingen, Switzerland).

Immunoprecipitation

48h after transfection CHO cells in six-well plates were rinsed with Methion-

ine-free MEM (GibcoBRL, Basel, Switzerland) and left 20 min in Met-free MEM at 37°C. The cells were incubated with [³⁵S]-Methionine (150 µCi/ml) in Met-free MEM for 2 h at 37°C in a humidified atmosphere containing 5% CO₂. The [³⁵S]-Methionine containing medium was replaced by complete medium containing a 10000 fold excess of cold Methionine and the desired compounds. After a further incubation for 1 to 3 h at 37°C the cells were harvested into 0.2 ml of PBS centrifuged at 350 x g and resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.8-8.0). After a 15 min incubation time on ice, protease inhibitor mix (Roche Applied Science) was added and the cells were homogenized by three times freezing (in dry ice) and thawing (at 37°C). The homogenate was centrifuged for 15 min at 14000 xg at 4°C. The pellet was resuspended in 100 µl of 5 mM Tris-HCl pH 8.0. The labeled membrane proteins (5x10⁶ cpm) were dissolved in 400µl 10 mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5% SDS followed by a 2.5 fold dilution in NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25 % gelatine, 0.1% NP-40, 1 mM EDTA). Triton X-100 and sodium deoxycholate were added to a final concentration of 0.3% and 0.5% respectively, prior incubating for 30 min at 4°C. After centrifugation, the supernatant was incubated with 4 µg antibody monoclonal mouse anti c-myc IgG1 (Roche Applied Science) overnight at 4°C. Complexes were precipitated by protein A Sepharose (Amersham Biosciences, Otelfingen, Switzerland) for 4 h at 4°C. The precipitate was washed four times in 20 volumes of NET buffer and two times in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40. The bound material was released by resuspending and boiling the pellet in 50 µl SDS-PAGE sample buffer. The immunoprecipitates were analyzed by SDS-PAGE gel electrophoresis and autoradiography.

Indirect Immunofluorescence Microscopy

The CHO cells were plated on Biocoat (collagen I coated coverslips, Becton and Dickinson Labware, Bedford, MA 01730, USA). After 1 or 2 days in the incubator, the cells were washed twice with PBS (150 mM NaCl, 20 mM NaH₂PO₄, pH 7.4, 0.1 mM CaCl₂, 0.1 mM MgCl₂), fixed for 20 min in 3% para-formaldehyde, washed four times with PBS and then incubated for 30 min in 0.1 M glycine in PBS. After four washes with PBS the cells were in some cases permeabilized in 0.1% Triton X-100 for 3 min, followed by four PBS washes, and incubated in blocking buffer (5% FCS, 0.1% bovine serum albumin, 5% glycerol, 0.04% NaN₃ in PBS) for 1 h. The coverslips were overlaid with the primary antibody (antiMyc 1-9E10, Roche Applied Science) diluted in blocking buffer (1:100). After rinsing with the blocking buffer, the cells were incubated with secondary antibodies for 1 h (goat anti-mouse Alexa fluor 488-conjugated antibodies diluted 1:100 in blocking buffer, Molecular Probes, Eugene OR 97402-0469, USA). The coverslips were washed five times in blocking buffer prior to mounting in a medium containing 80% glycerol, 2.5% DABCO (2,4-diazabicyclo-(2,2,2)-octane) in PBS, pH 8.0. The cells were observed in an AXIOVERT 10 microscope (Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence illumination and a 25x, 63x or a 100x oil immersion plan-neofluar objective, and images were collected by an ultra cooled digital camera.

Results

Stable CHO cell lines expressing functional human, receptors, hS1P₁, hS1P₂, hS1P₃, hS1P₄ and the mouse receptor mS1P₄ were generated as described in

the Experimental Procedures section and shown in Fig. 1. The hS1P₁, hS1P₃, hS1P₄ and mS1P₄ were tagged with myc-tag to facilitate their monitoring with myc specific monoclonal antibodies (Fig. 1A). This was necessary as several of the commercial antibodies to S1P receptors performed sub-optimally in our monitoring experiments.

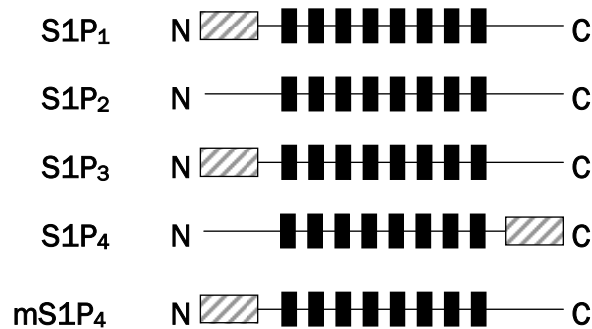


Figure 1A. Characterization of S1P -receptors expressing CHO cell lines. The 5 constructs that were used during this study. The black boxes indicate the location of the transmembrane domains. The striped box indicates the location of myc-epitope. The N-Terminus (N) is located in extracellular space, while the C-terminus (C) intracellular space.

The expression of the tagged receptors was verified by immunocytochemistry (Fig. 1B). Although the transient expression of these receptors in CHO cells yielded high protein levels as judged by Western blot (shown for S1P₁, Fig. 1C), stable CHO cell lines proved to be more suitable for FCM and functional analysis as they resulted in a homogeneous cell population expressing similar amounts of recombinant protein (Figs. 1B and D). Similar results as with the myc antibody (recognizing the N-terminus of the protein, Fig. 1Da) were obtained with a S1P₁ specific antibody recognising a C-terminal epitope of the protein following cell permeabilization (Fig.

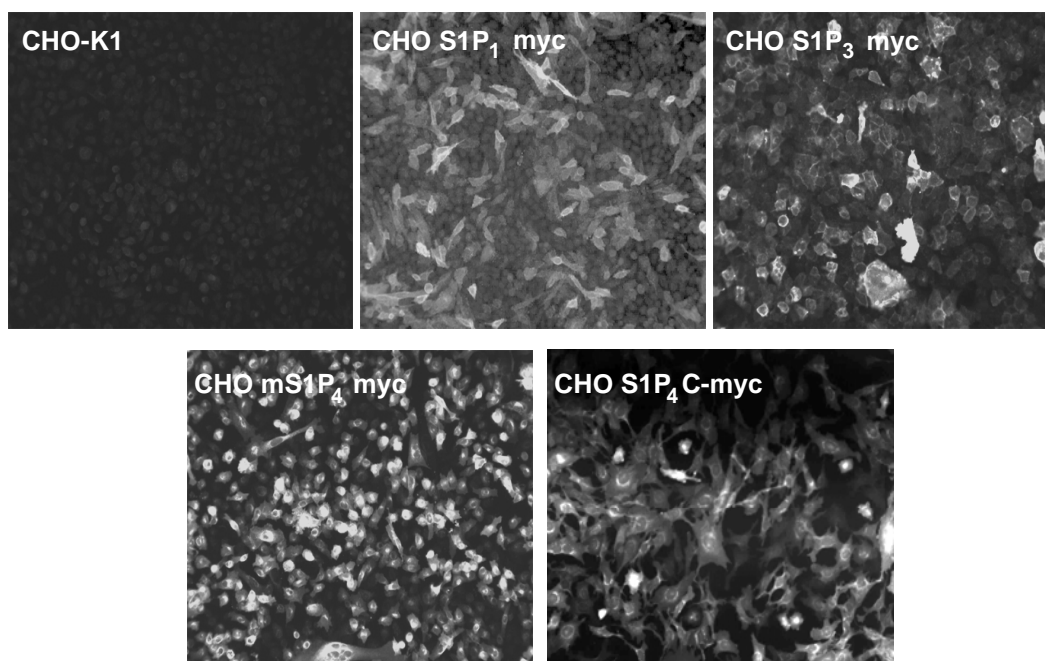


Figure 1B. Characterization of S1P -receptors expressing CHO cell lines. Immunocytochemistry of cells expressing hS1P₁, hS1P₃, mS1P₄ and hS1P₄ receptors. CHO K1 wt cells or cells expressing hS1P receptors were plated on coverslips and after 1-2 days in cultures, staining was performed with the myc monoclonal antibody. The hS1P₄ cells were permeabilized prior to the staining, since the C-terminal myc-epitope is located at the cytosol. The images were taken with a fluorescent microscope using a 10x magnification objective.

1Db). This suggested that in the stable CHO cell lines the S1P₁ receptor was expressed at the surface of the cell with the intact myc tag. The mS1P₄, hS1P₁ and hS1P₃ receptors all engineered with an N-terminal myc-tag, proved suitable for FCM analysis. The receptor hS1P₄ engineered with C-terminal myc tag could also be visualized by FCM, though the cell permeabilization techniques prevented the discrimination between the internal and the external localization of the receptor molecules. The recombinant receptors retained their functional activity in stable CHO cell lines as measured by the FLIPR or GTPγS-binding assays (Figs. 1E and 1F). The hS1P₂ receptor was expressed in active form without any tag (Figs. 1A, E and F) as it did not bind phosphorylated FTY720 derivatives (Brinkmann et al., 2002; Mandala et al., 2002), and only limited experiments could be performed with this receptor.

The *in vitro* properties of the compounds evaluated in this study and as meas-

ured in the Ca^{2+} mobilization assay (Fig. 1D) are summarized in Table 1. FTY720-P and AFD(R) were full agonists at hS1P₁, hS1P₄ and mS1P₄, partial

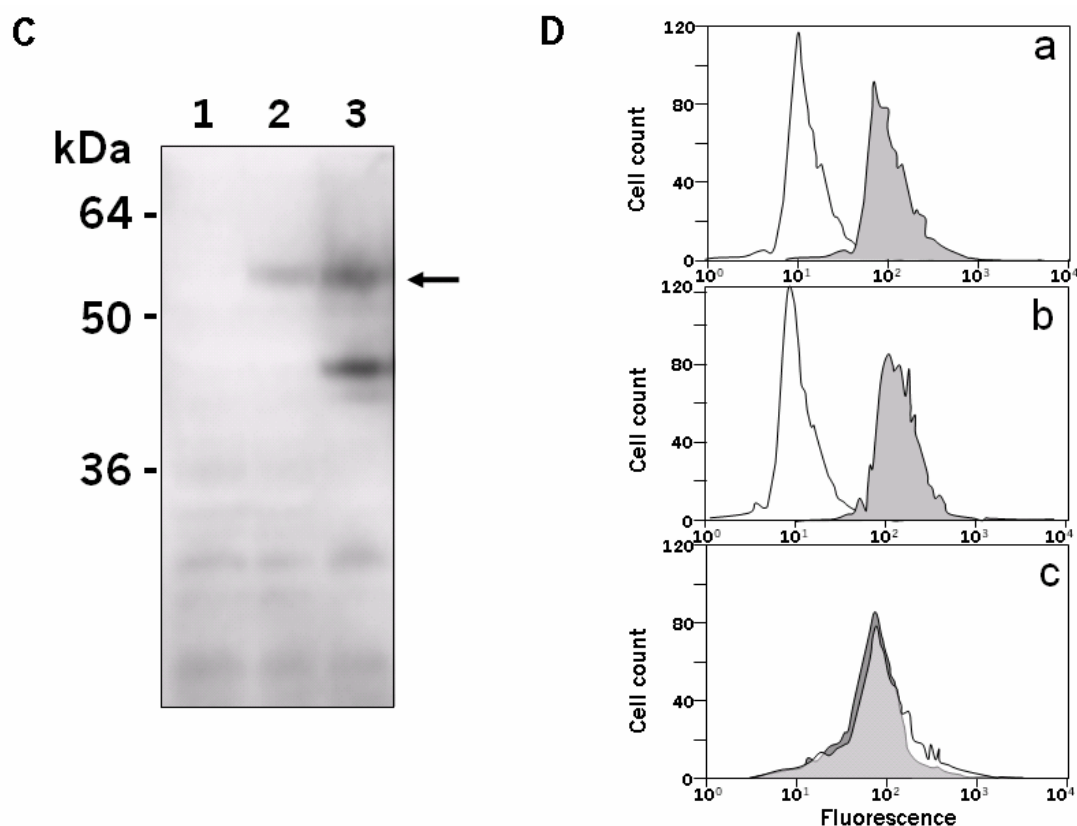


Figure 1C/1D. Characterization of S1P-receptors expressing CHO cell lines.

(C) Expression of myc-tagged S1P₁ receptor analyzed by Western blot with c-myc antibody. Cell extracts prepared from untransfected (lane 1), stably (lane 2) or transiently (lane 3) transfected CHO cells. Around 20 μg of total protein were loaded on the gel. Arrow indicates the predicted position for the mature glycosylated myc-S1P₁ protein (Kohno and Igarashi, 2003; Kohno et al., 2002). The band in transiently transfected CHO cells migrating with an apparent MW of 42-45 kDa likely represents a partially processed receptor.

(D) Comparison of anti c-myc antibody with anti S1P₁ antibody in FCM analysis. Panel a: Stable CHO cells expressing hS1P₁ were stained with (grey) or without (white) anti c-myc monoclonal antibody. Panel b: CHO cells expressing hS1P₁ were stained with (grey) or without (white) anti S1P₁-specific antibody, after permeabilization of the cells. Panel c: Stability of the expression levels of myc-S1P₁ during a standard experiment. CHO cells expressing S1P₁ were stained with anti c-myc antibody prior (grey) or after 1 hour (white) during which the cells were incubated and manipulated as normally done when the cells are exposed to compounds.

agonists at hS1P₃ and did not activate hS1P₂ (Table 1). Similar results were obtained in the GTP γ S assays and were consistent with the previously pub-

lished observations (see (Brinkmann et al., 2002; Mandala et al., 2002)).

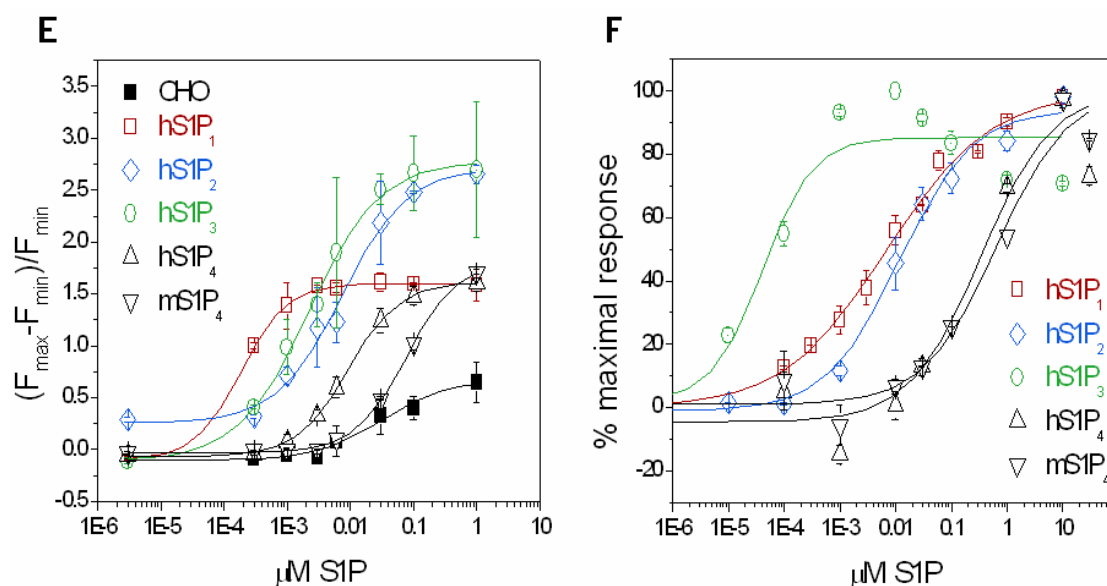


Figure 1E/1F. Characterization of S1P -receptors expressing CHO cell lines.

(E) Functional assay with cells expressing S1P receptors. Ca^{2+} mobilization was measured after the control cells and cells expressing S1P receptors were loaded with Fluo4AM for 1 hour. The activity was calculated by the following equation: $(F_{\text{max}} - F_{\text{min}}) / F_{\text{min}}$, where the F_{min} is the base line fluorescence before the addition of the agonist and F_{max} is the maximal height of the fluorescence signal after the addition of the agonist.

(F) Functional assay with membrane proteins expressing S1P receptors. The GTP γ S binding was measured at different concentrations of S1P.

	hS1P ₁		hS1P ₂		hS1P ₃		hS1P ₄		mS1P ₄		CHO	
	EC ₅₀ nM	E _{max} %	EC ₅₀ nM	E _{max} %	EC ₅₀ nM	E _{max} %	EC ₅₀ nM	E _{max} %	EC ₅₀ nM	E _{max} %	EC ₅₀ nM	E _{max} %
Sphingosine	>10000	<3	>10000	<3	>10000	<3	>10000	<3	ND	ND	ND	ND
S1P	0.47±0.5	100	22±19	100	4.6±0.6	100	6.5±5	100	11±5	100	145±170	25±7
FTY	>10000	30	>10000	<3	>10000	<3	>10000	<3	>10000	<2	>10000	<2
FTY-P	0.6±0.9	93±10	>10000	<3	23±8	21±1.2	48±30	106±38	ND	ND	>10000	<2
AFD(R)	1.7±1.5	93±11	>10000	<3	17±4	20±2.6	99±50	96±4	ND	ND	>10000	<2
AFD(S)	111±21	82±12	>10000	<3	>10000	0	43±21	98±3	ND	ND	>10000	<2

Table 1. Binding properties as determined in the FLIPR assay.

Agonist Mediated Internalization of S1P₁ in CHO Cells as Analyzed by FCM

The internalization of hS1P₁ was measured by FCM analyses following the addition of specific compounds. The natural agonist, S1P caused partial internalization of hS1P₁ (Fig. 2A, trace 2) as measured by the disappearance of approximately 50% of the receptor from the cell surface after 1 hour incubation (Fig. 2D). FTY720, FTY720-P (the phosphorylated mixed racemic form FTY720) as well as the phosphorylated, enantiomeric pure FTY720-derivative AFD(R) (for the structure see Suppl. Fig.1) efficiently promoted the internalization of the S1P₁ receptor (Fig. 2A, traces 3 and 4, Fig.2B, trace 2), resulting in the complete disappearance of the receptor from the cell surface. The phosphorylated stereoisomer ADF(S) (Suppl. Fig.1) did not internalize the hS1P₁ receptor as measured by FCM (Fig. 2B trace 3).

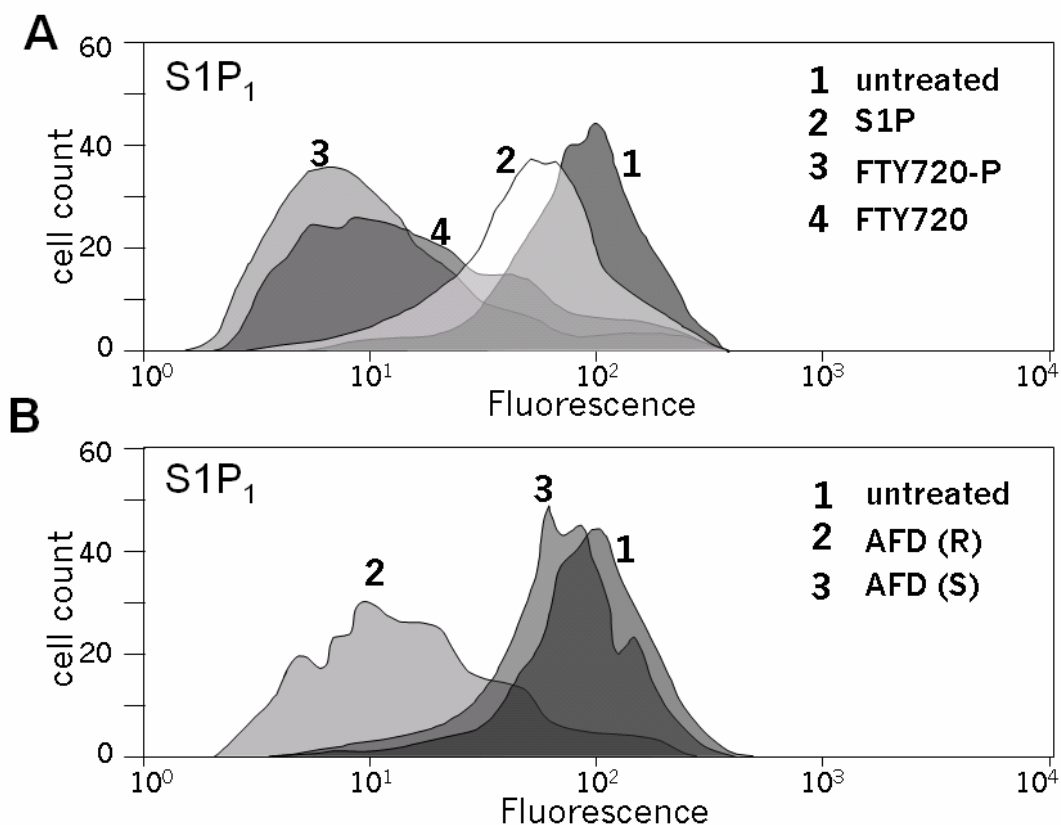


Figure 2. Agonists-induced internalization of S1P₁, measured by flow cytometry.

(A-B) Detection of hS1P₁ expressed on the surface of CHO cells. The panels A and B are representative of at least three independent experiments.

(A) CHO cells expressing S1P₁ were incubated for one hour with 1 μM S1P, FTY720, FTY720-phosphate or with medium alone.

(B) CHO cells expressing S1P₁ were incubated for one hour with 1 μM of AFD(R), AFD(S) (for the structure of the compounds see Suppl. Fig. 1) or with medium alone.

Non-phosphorylated molecules such as sphingosine, FTY720 and the compound AAL(R) (Suppl. Fig.1) were also capable of promoting internalization (Fig. 2D). Sphingosine, the compound AAL(R) (Suppl. Fig.1) and FTY720, were presumed to be promoting internalization as they had time to undergo phosphorylation in CHO cells during the one hour incubation time used in these FCM experiments. It has been previously reported that FTY720 and the compounds AAL(R) (Suppl. Fig.1) but not AAL(S) (Suppl. Fig.1) can be effi-

ciently phosphorylated *in vivo* (Brinkmann et al., 2002) and in cell culture systems (Sanchez et al., 2003). The Kinetic differences in internalization between FTY720 and FTY720-P are demonstrated by the experiments summarized in Fig. 2C. While at 1000 nM FTY720-P promoted fast internalization (>80% after 5 min), that mediated by FTY720 was delayed (60% after 30 min), suggesting that FTY720 needs to be phosphorylated to bind and internalize S1P₁. However, after 1 h incubation both compounds showed similar extents in internalization of S1P₁ (Fig. 2C, see also Fig. 2D). Sphingosine and AAL(R) behaved similarly (not shown). The EC₅₀ for the internalization (after 1 h) for phosphorylated FTY720 was estimated to be 30-50 nM, while the apparent EC₅₀ for FTY720 was at least 10 higher (>400 nM).

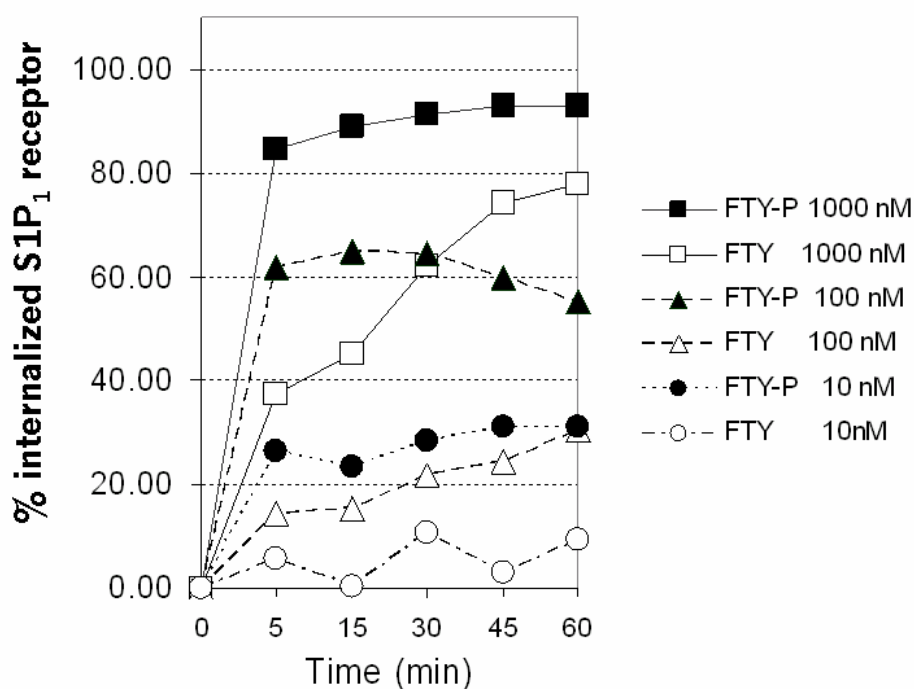


Figure 2C. Agonists-induced internalization of S1P₁, measured by flow cytometry.

Internalization of S1P₁ is concentration and time dependent and requires modification of FTY720: S1P₁ CHO cells were incubated with 10, 100 and 1000 nM of FTY720-P (FTY-P) or with FTY720 (FTY). At regular time samples were collected and processed for FCM analysis. The amount of internalized S1P₁ is plotted against the time of incubation. The percent of in-

ternalization was calculated by the following equation: $(1-(F_{cp}-F_{iso})/(F_o-F_{iso})).100$; F_{cp} =fluorescence peak after the addition of agonist, F_{iso} = fluorescence peak obtained with isotype control antibodies, F_o =fluorescence prior to the addition of compound. Complete (100%) internalization is reached when the fluorescence detected by FCM is equal to that obtained with isotype controls. Experiment is representative of three independent experiments.

A summary of the results with different compounds is shown in Fig. 2D. Thus, our data indicate that the binding at the S1P₁ receptor is a necessary requirement for S1P₁ receptor internalization. Our results also indicate that these compounds confer different degrees of internalization of the hS1P₁ (shown in Fig. 2D) in spite of their equivalent potencies in functional receptor binding (indicated in Table 1).

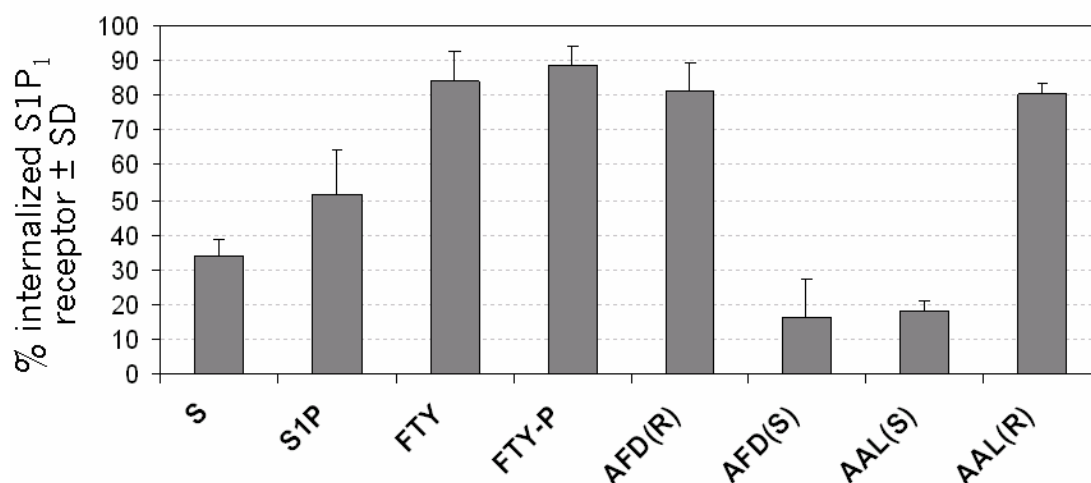


Figure 2D. Agonists-induced internalization of S1P₁, measured by flow cytometry.

Summary of the results of the flow cytometry analysis. The amount of internalized receptors was calculated after 1 hour treatment with agonist by determining the height of the peaks observed in FCM experiments. The percentile internalization is calculated as described in the legend for panel 2C. S=sphingosine. Means ± S.D. (n>3)

We have performed internalization experiments with S1P₁ in HeLa cells to assess if the strong internalization mediated by FTY720 derivatives is a peculiarity of CHO cells. As in the case of the CHO cells, the effect of S1P was much weaker than that observed with FTY720-P (Suppl. Fig. 2) and we conclude

that the strong internalization of FTY720-P is related to an intrinsic property of the receptor and not a peculiarity of the CHO cell lines used.

Agonist Mediated Internalization of CHO Cells Expressing S1P₃ Analyzed by FCM

We have measured the agonist's mediated internalization at hS1P₃ receptors to determine if this receptor behaved in a similar way to hS1P₁. These data are shown in Figs. 3A and B. S1P had a much stronger internalizing effect on hS1P₃ when compared to FTY720-P and the compound AFD(R) (Suppl. Fig.1). This is consistent with the observation that FTY720-P is a partial agonist at this receptor (see Table 1) (Brinkmann et al., 2002; Clark et al., 1999). S1P promoted complete internalization of S1P₃ receptor while FTY720, FTY720-P, AFD(R) and AAL(R) caused roughly 60% internalization (Fig. 3B). As is for hS1P₁, the inactive compounds AFD(S) and AAL(S) (Suppl. Fig.1) did not promote internalization of S1P₃ (Fig. 3B) supporting the conclusion that this effect was only mediated by biologically active compounds. In contrast to hS1P₁ and hS1P₃, mS1P₄ was not effectively internalized by S1P or by FTY720-P, the internalized fraction of the receptor never exceeding greater than 30% (see suppl. Fig. 3).

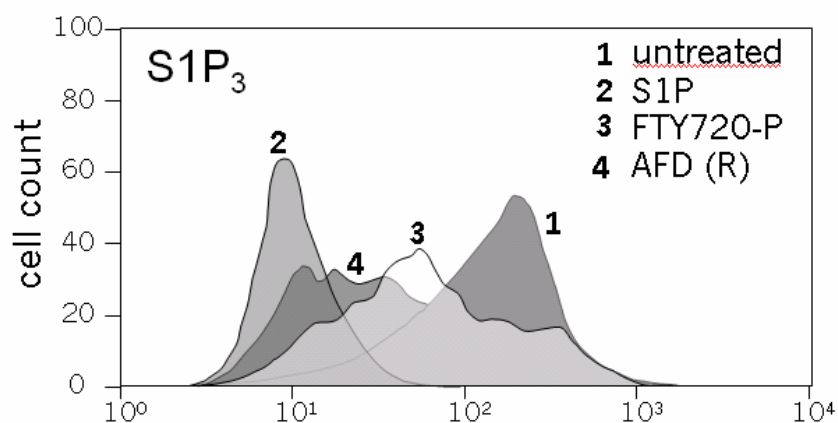


Figure 3A. Agonist-induced myc-S1P₃ internalization on CHO cells, analyzed by flow cytometry. Effect of agonists on the surface expression of myc-S1P₃: Cells were incubated for one hour with 1 μ M of S1P, FTY720-Phosphate, AFD(R) or medium alone.

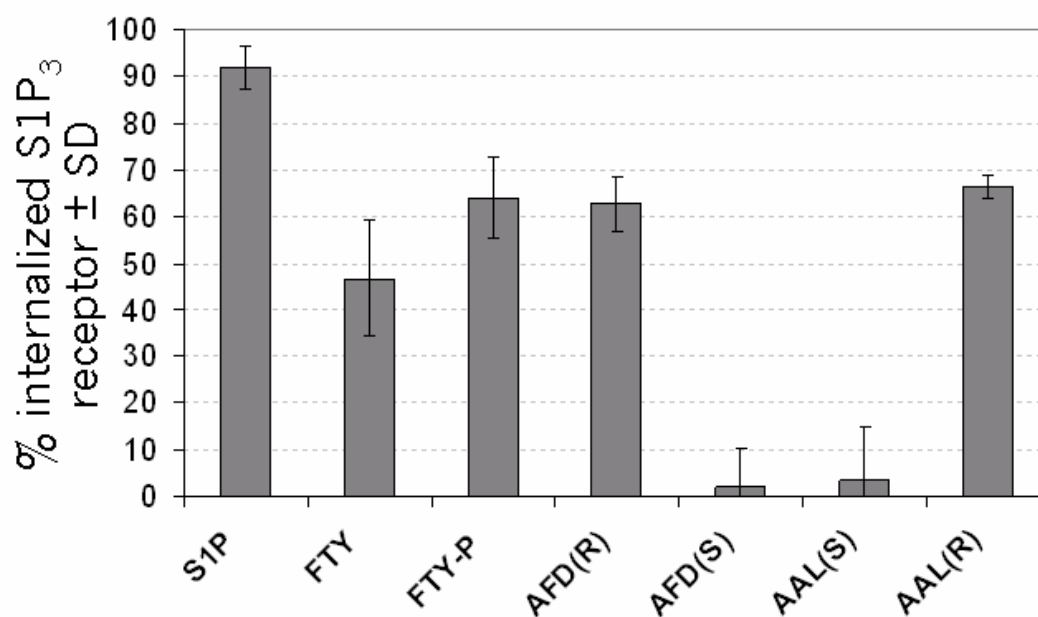


Figure 3B. Agonist-induced myc-S1P₃ internalization on CHO cells, analyzed by flow cytometry. Summary of the effects of agonists on S1P₃ as measured by FCM. The extent of internalization (as defined in the legend of Fig.2C) is shown for myc-S1P₃ after 1 hour treatment with. Means \pm S.D (n>2)

Kinetics of Receptor Internalization

To further evaluate the effect of the compounds on the recycling of the receptors, compound-treated cells were analyzed at different times after the removal of the compound through a wash out period. The results indicated an excellent recovery of the surface expression of hS1P₁ of cells pre-treated with S1P (Fig. 4A, lower panel) as measured after 3 hours following the removal of agonist. In contrast, only 15-25% percent of the initial receptor surface expression was detected (Fig. 4A, upper panel) following FTY720-P treatment. These experiments indicate that FTY720-P causes long lasting internalization of S1P₁ receptors compared to the natural ligand S1P. A complete recovery of the surface expression of human S1P₃ receptor was observed following S1P removal (Fig. 4B), while in the case of FTY720-P only a fraction of the initial receptor could be detected (Fig. 4B).

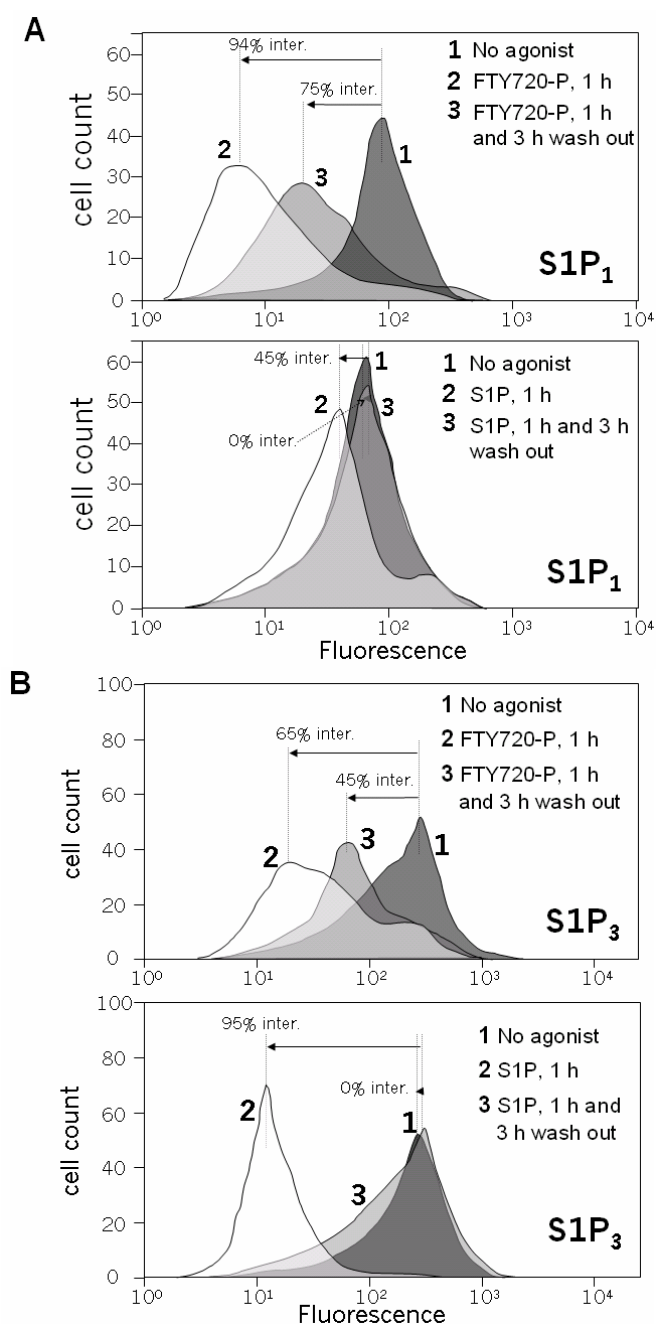


Figure 4A/4B. Internalization and recycling of myc-S1P receptors in CHO cells.

(A) Surface expression of myc-S1P₁ on CHO cells analyzed by FCM: Cells were incubated for one hour with 1 μ M of S1P, FTY720-Phosphate or medium alone (no agonist). After 1h incubation a portion of the cells was processed by FCM. The remaining of the cells were washed and incubated for 3 h without agonist prior to the FCM analysis. The FCM profile of S1P₁ CHO cells incubated with the medium is given by the dotted line.

(B) Surface expression of myc-S1P₃ on stably transfected CHO cells: Cells were incubated for one hour with 1 μ M of indicated agonists (S1P, lower panel or FTY720-Phosphate, upper panel). The cells were washed and incubated for 3 h in medium without agonist prior to the FCM analysis. Negative controls are untreated S1P₃ transfected CHO cells.

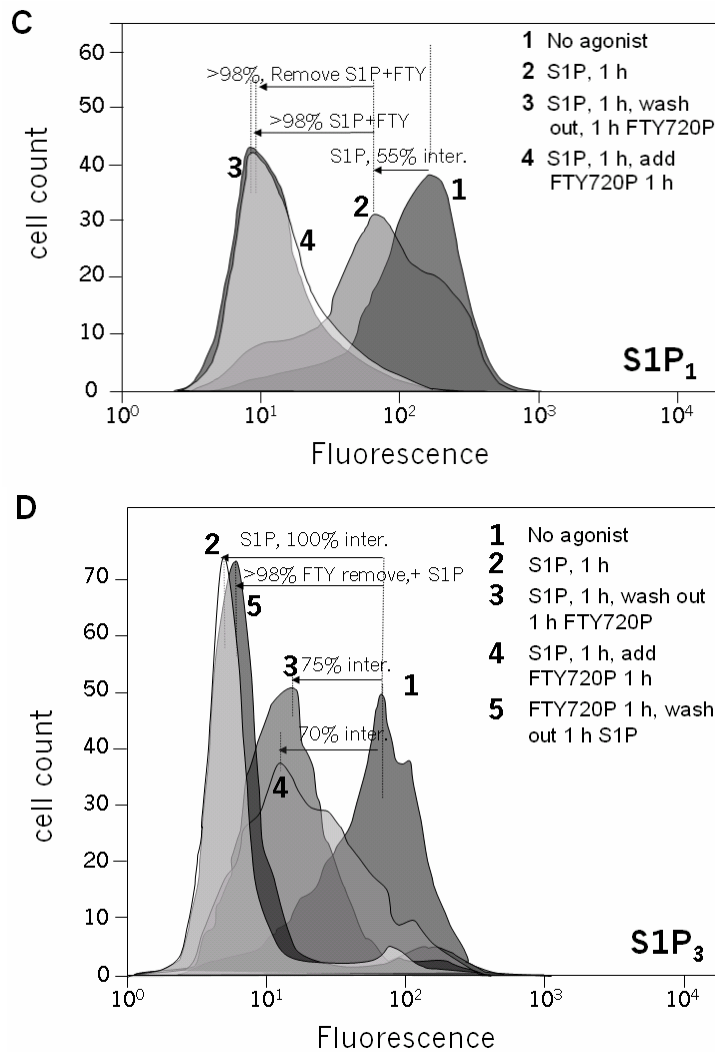


Figure 4C/4D. Internalization and recycling of myc-S1P receptors in CHO cells.

(C) FTY720-P supersedes the effect of S1P on internalization of S1P₁: Cells were incubated for one hour with 1 μ M of S1P and one third of cells were analyzed by FCM (trace 2). Another third of the cells was washed and then incubated for an additional hour with 1 μ M FTY720-P prior to FCM (trace 3). The last portion of the cells was incubated for one additional hour after the addition 1 μ M FTY720-P (the S1P was not removed, trace 4).

(D) FTY720-P effect of S1P on internalization of S1P₃ in the presence of S1P: Cells were incubated for one hour with 1 μ M of S1P and then analyzed by FCM (trace 2). A portion of the cells was washed and incubated for an additional hour with 1 μ M FTY720-P prior to FCM (trace 3). To the remaining cells 1 μ M FTY720-P was added (without washing) for 1 hour and analyzed by FCM (trace 4). In an independent experiment, the S1P₃ cells were pre-incubated with 1 μ M FTY720-P, washed and then further incubated with 1 μ M S1P (trace 5) prior to the FCM experiment. The signal of S1P₃ CHO cells incubated in the presence of medium alone is given (trace 1).

Finally, CHO cells expressing hS1P₁ were incubated for one hour with S1P and then for an additional hour with FTY720-P in the presence or in the absence of S1P. FTY720-P mediated effects were prevailing over those of S1P, promoting more than 98% internalization of S1P₁ in the presence of S1P (Fig. 4C).

Also in the case of S1P₃ prevalence of the effects of FTY720-P were observed (Fig. 4D) since partial internalization was observed when S1P and FTY720-P were added together. Full internalization of S1P₃ after the pre-treatment with S1P, was reversed by incubation with FTY720-P. Importantly, after the incubation with and the removal of FTY720-P, S1P was able to fully internalize S1P₃, suggesting that the exchange between S1P and FTY720-P at the receptor was occurring during the incubation.

FTY720-P Does not Promote Degradation of the S1P₁ Receptor

We have investigated if the addition of FTY720-P and its derivatives promoted the degradation of hS1P₁ receptor following their internalization. CHO cells expressing hS1P₁ receptor were labeled with ³⁵S-Met for 2h. During this period labeled proteins are synthesized and delivered to the plasma membrane (Guerini et al., 2002). After this labeling period, the radioactivity is chased away by dilution and cells were exposed to S1P, FTY720 and FTY720-P. There were no differences in the level of S1P₁ receptors under these various conditions as measured by immunoprecipitation (Fig. 5), suggesting that none of these compounds affected the stability of the S1P₁ receptor compared to the untreated cells and independent of their ability to internalize the receptors.

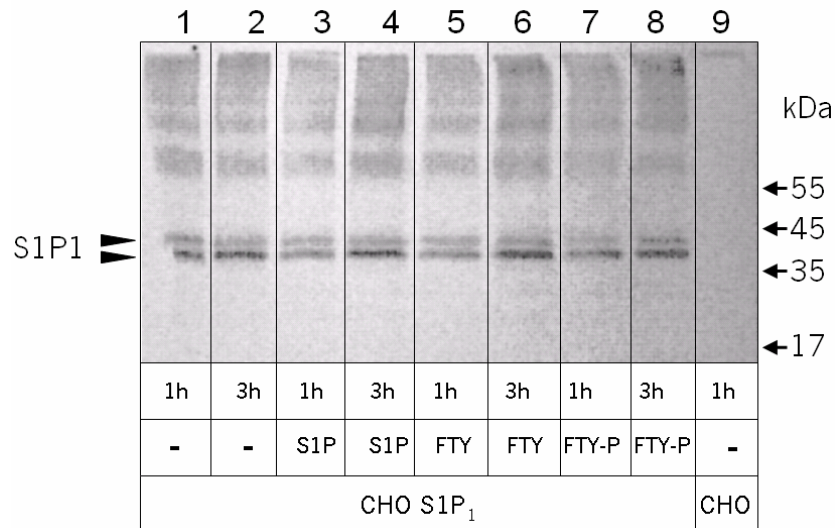


Figure 5. Internalization of S1P₁ receptor does not promote its degradation.

CHO cells expressing S1P₁ were pulsed and chased with ³⁵S-Met and the receptor was immunoprecipitated with the myc monoclonal antibody. The samples were separated by SDS-PAGE and the dried gel was exposed to X-ray Film. Samples are as following: 1: no treatment, 1h chase, 2: no treatment 3 h chase; 3 and 4 incubation with 1μM S1P for 1 or 3 h chase respectively; 5 and 6 incubation with 1μM FTY720 for 1 or 3 h chase respectively; 7 and 8 incubation with 1μM FTY720-P for 1 or 3 h chase respectively; 9: wild type CHO cells. The experiment was performed with transiently transfected CHO cells. Similar results were obtained with stable transfected cells.

Internalization Affects the Measurable Functional Activity of S1P₁

The effect on the internalization (i.e. the loss of cell surface fluorescence of the receptor as measured by FCM) of these receptors was correlated with desensitization (defined as the loss of response to the agonist by measuring agonist mediated intracellular Ca²⁺ release, see Fig. 1E). Cells were pre-incubated for 1h with different concentrations of agonists, after which the agonists were removed. The cells were then transferred to fresh medium for additional time (3h), during which the cells recovered from the first exposure of the agonist and were judged fully competent in their calcium release response (see suppl Fig. 4). In the FCM experiments 3h were sufficient to completely reverse internalization by S1P, but only weakly affected that of FTY720-P (Fig. 4A). At the end of the recovery phase, the S1P₁ dependent activity was

measured by exposing the cells to a concentration of S1P that is known to evoke the maximal signal. As shown in Fig. 6, pre-incubation of the

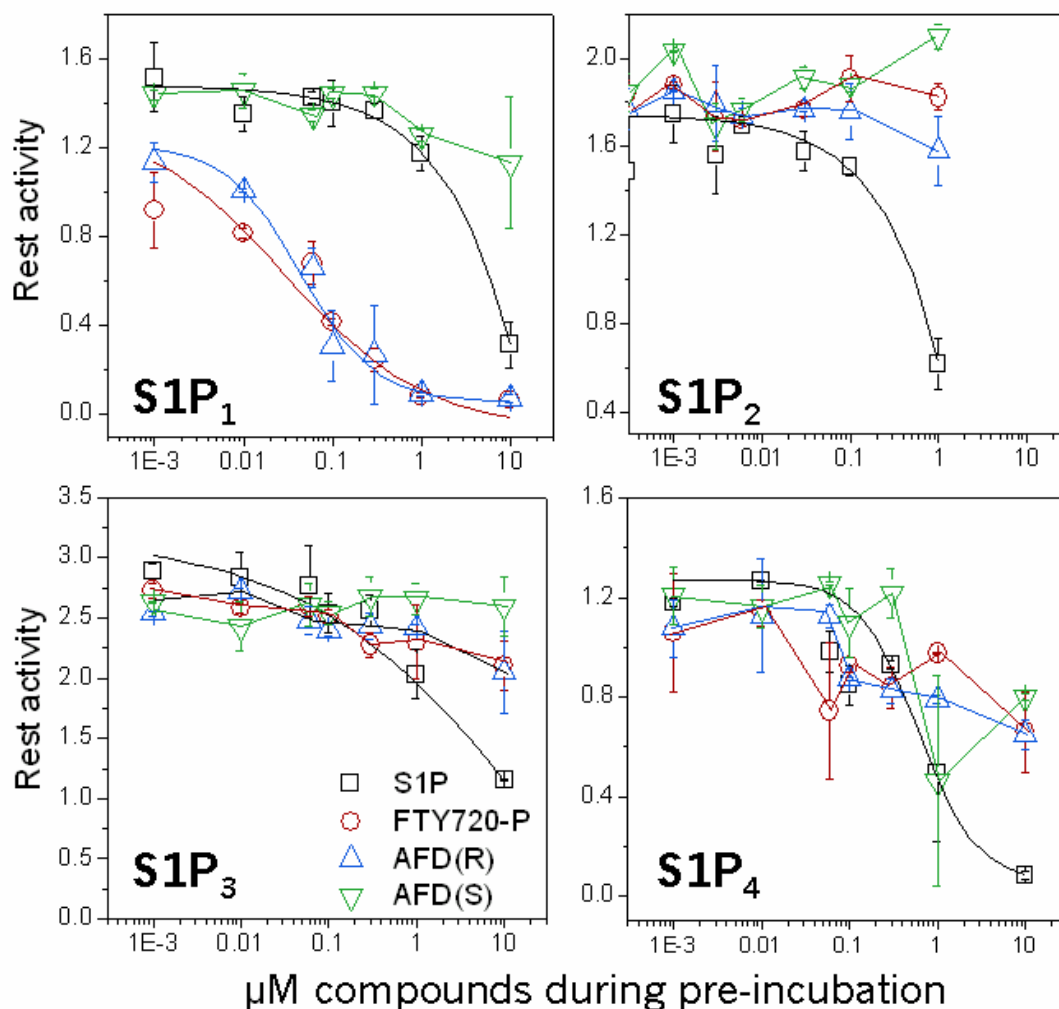


Figure 6. The effect of the pre-incubation of agonist on the activity of S1P receptors expressing CHO cells. CHO cells expressing S1P₁, S1P₂, S1P₃ and S1P₄ were preincubated with S1P, FTY720-P, AFD(R) and AFD(S) at different concentrations. The cells were washed after 3h incubation and further incubated for 3 h (without agonist) prior to measuring of agonists induced Ca²⁺ transient by FLIPR. 30 nM S1P for S1P₁ and to 100 nM S1P₂, S1P₃ for S1P₄ were used. To verify that the Ca²⁺ signaling of the cells had recovered, the ATP mediated intracellular Ca²⁺ release was recorded (see suppl. Fig.4). The concentrations of S1P were chosen to give the maximal signal without saturating it. The plots summarize the concentration dependent effect of agonists pre-incubation on the signal mediated by S1P. The rest activity was defined as $(F_{max}-F_{min})/F_{min}$, where F_{min} the height of fluorescence signal prior to the addition of S1P, while F_{max} is the maximal height of signal obtained after the addition of S1P.

CHO cells expressing S1P₁ with FTY720-P and AFD(R) caused a strong repression of the specific activity as measured by calcium release. The IC₅₀ values for the FTY720-P and AFD(R) are calculated to be 20-30 nM, i.e., after pre-incubation with 20-30 nM of agonist and a subsequent 3 h post-wash period only 50% of the maximal activity was recovered. Pre-incubation with higher concentrations of these two agonists, in good agreement with the loss of fluorescence from cell surface (shown in Fig. 4), completely suppressed the response to S1P (Fig. 6, panel S1P₁). The pre-incubation with S1P lead to a detectable reduction in calcium release only at the highest concentration used (10 μM). No changes on the calcium release activity were observed with AFD(S), even at 10 μM. Consistent with the weak internalization effects or the fast recycling, only limited effects on calcium release are observed with cells expressing the human S1P₃ and S1P₄, being the most effective compound S1P. S1P₂ receptor cell lines failed to show an effect with the compounds except for S1P.

Discussion

The receptor internalization effects of the S1P and different synthetic agonists were studied on specific S1P receptors. These experiments showed significant differences between physiological ligand, S1P, the immunomodulator FTY720 and its phosphorylated derivatives, in particular at the hS1P₁ receptor as measured by internalization and desensitization of the receptors. The effects at hS1P₃ correlated with the observation that FTY720 and its derivatives are partial agonists at this receptor. FTY720, FTY720-P and its biologically active derivatives ADF(R) as well as AAL(R), promoted the complete disappearance of hS1P₁ from the surface of the cells in contrast to the partial internalization of hS1P₁ mediated by S1P. Our results with S1P are also consistent with the previously published results of partial internalization of S1P₁ by S1P (Watterson et al., 2002).

The internalization mediated by FTY720 and its derivatives was a long lasting effect and the hS1P₁ needed a longer time, when compared to the treatment with equimolar S1P, to reappear back to the surface. In contrast, S1P promoted full internalization as well as faster recovery of hS1P₃ receptor, while only a fraction of the partially internalized hS1P₃ receptor by FTY720-P reappeared on the cell surface with delayed time interval.

The receptor activity measurements as determined by intracellular Ca²⁺ are consistent with the degree of internalization as well as the timing of reappearance of the receptor suggesting that the internalization of the receptor resulted in a proportional loss of a functional effect. The concentration of FTY720-P needed to promote 50% of the activity loss is around 20 nM, well in the range of the 30-120 nM of phosphorylated compounds found in blood plasma of rats

after administration (Mandala et al., 2002)

Several other important observations were made from our studies. First, FTY720-P mediated effects were dominant and occurred at equimolar concentrations of the natural agonist S1P (Figs. 4C and D). This suggests that in spite of high concentrations of S1P *in vivo* (up to 100-300 nM, (Yatomi et al., 1997)), FTY720 and its phosphorylated derivatives are able to promote extensive loss of the cell surface S1P₁ receptor leading to profound and longer lasting effects. In parallel, FTY720 would counteract the strong internalization of S1P₃ expected in the presence of S1P alone. Second, the internalized hS1P₁ receptor following the treatment with FTY720, is recycled with significant delay, which is not the case for natural ligand S1P, suggesting the existence of different (agonist specific) mechanisms of recycling. This might also be true for S1P₃ where despite the stronger internalization effect of S1P compared to FTY720-P, the recycling of the S1P influenced hS1P₃ receptor was fast and efficient. Third, the effects on internalization of the hS1P₁ seemed not to depend on the cellular background since two different cell lines gave essentially the same results. Since the phosphorylated form of FTY720 is formed at relevant levels in serum and it is stable *in vivo* (Brinkmann et al., 2004; Brinkmann et al., 2002; Mandala et al., 2002), daily administration of FTY720 may result in the permanent internalization of S1P₁ and loss of signaling by this receptor.

Different modes of receptor recycling have been described (Shenoy and Lefkowitz, 2003). GPCRs belonging to the class A recycle quickly while those belonging to class B are retained for longer time in the interior of the cell. Slow and fast recycling of the GPCR to the cell surface might be mediated by independent intracellular pathways (Perry and Lefkowitz, 2002). Specific opioid

receptor agonists have been described with different effects on the internalization kinetics the corresponding receptor (Whistler et al., 1999). However, the spectrum of differences observed for the S1P₁ agonists in this study were rather large and unexpected.

An important consequence of the persistence of the internalized form of the receptor could be a long lasting activation (or deactivation) of pathways that are initiated after the GPCR desensitization. A large body of experiments indicate that mitogen activated protein kinases (MAPK) form a multi-protein complex after GPCR and become associated with arrestin (Pierce and Lefkowitz, 2001). It would therefore be plausible that FTY720 signaling at S1P₁ would result in the modulation of pathways that are qualitatively and quantitatively different from those mediated by S1P. It is tempting to speculate that FTY720-P despite being an agonist would act as a functional antagonist at the S1P₁. In spite of its ability to generate initial agonistic responses, significant amounts of the receptor would remain intracellular and not accessible to further signaling by the agonist ligand. Finally, due to the specific effects on hS1P₁, treatment with FTY720-P is expected to affect the signaling by the physiological agonist S1P on this receptor. It might be that some of the clinical effects following FTY720 treatment (Budde et al., 2002) are mediated by the internalization and desensitization effects of this drug on the hS1P₁ receptor or other members of this receptor subfamily. Our studies suggest that screening for agonists or antagonists that influence internalization and reappearance in a specific way might lead to novel drugs with differential biological effects and clinical profiles.

Two recent publications demonstrated the importance of S1P₁ for egress of

lymphocytes in mice (Allende et al., 2004; Matloubian et al., 2004). In fact, T-cells lacking S1P₁ were not able to leave the lymph nodes, resulting in a general lymphopenia. Matloubian et al (Matloubian et al., 2004) suggested that internalization mediated by FTY720 might phenocopy the effect of deletion of S1P₁ in lymphocytes and provided indication that this drug strongly internalized S1P₁. These suggestions were in line with results in another recent publication (Graeler and Goetzl, 2004). Graeler and Goetzl suggested that FTY720 mediated the internalization of S1P₁ and other S1P receptors, by a novel non-competitive effect of the non-phosphorylated form of FTY720 (Graeler and Goetzl, 2004). It was suggested that FTY720 might act independently from the agonist effect of its phosphorylated form.

The results presented in our manuscript strongly suggest that the phosphorylated compounds (FTY720-P or AFD(R)) are mediating internalization of S1P receptors and that the effect of the non-phosphorylated forms occurred only after the compounds became phosphorylated by the cells. This is consistent with agonists mediated internalization of S1P-receptors, a classical effect observed at other GPCR (Pierce and Lefkowitz, 2001).

The phosphorylated forms of FTY720 influenced the internalization and the reappearance of S1P receptors in a qualitatively and quantitatively different manner than that mediated by S1P, likely contributing to its unique biological and therapeutic effects, in spite of significant presence of S1P levels *in vitro* and *in vivo*.

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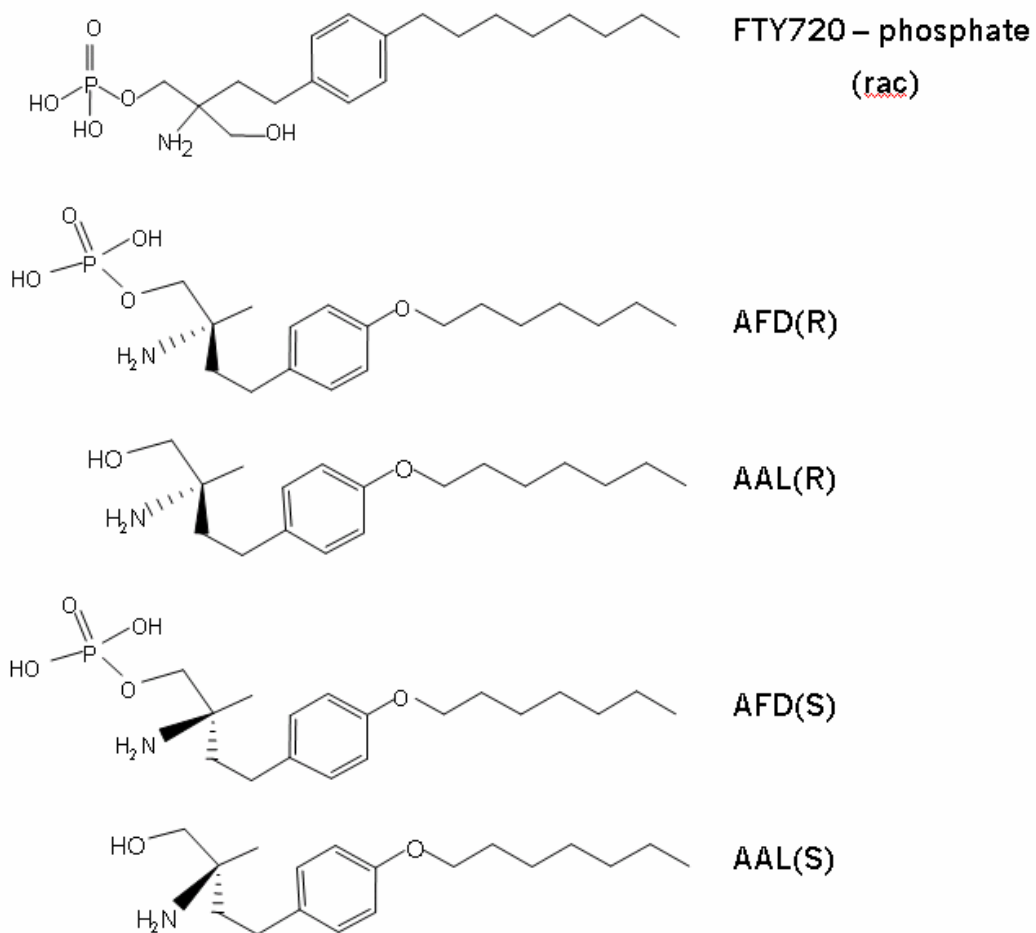
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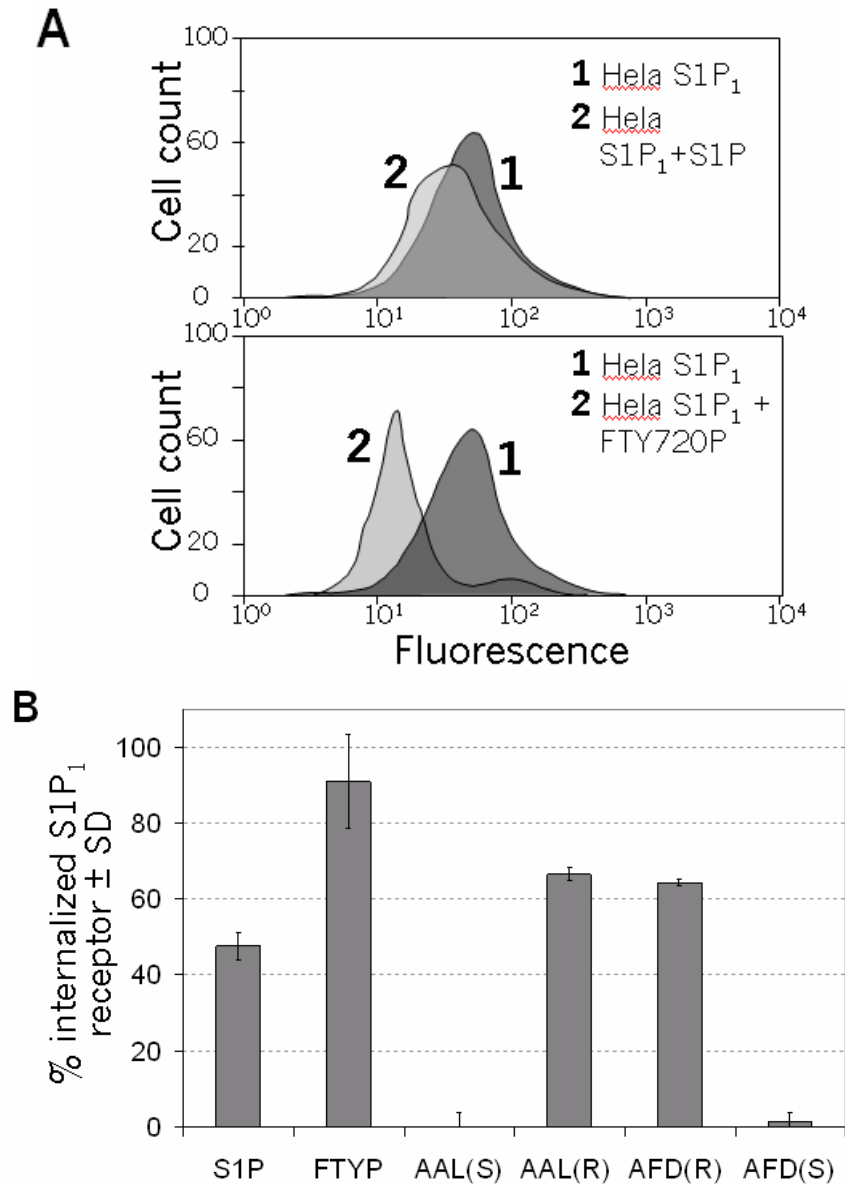
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Supplement



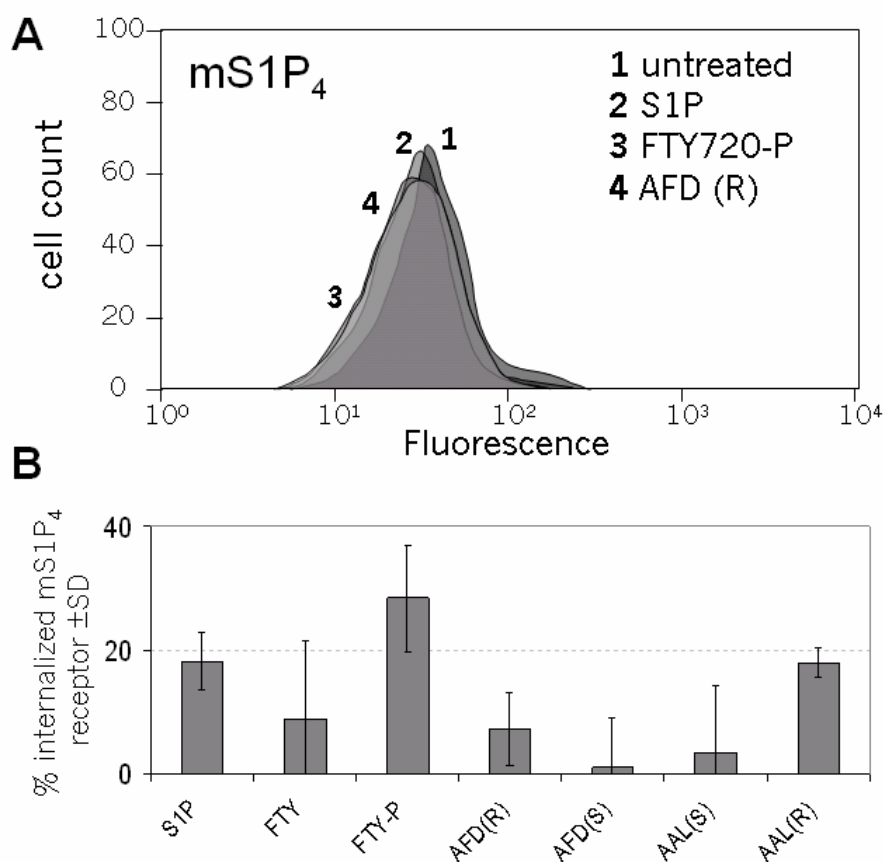
Supplementary Figure 1. The chemical structure of the compounds used in this study.



Supplementary Figure 2. Agonist-induced S1P₁ internalization on transfected HeLa cells determined by flow cytometry.

(A) Upper and lower panel: HeLa cells expressing myc-S1P₁ were incubated for one hour with 1 μM of indicated agonists (S1P, trace 2, lower panel or FTY720-phosphate, trace 2 upper panel). Negative controls are untreated myc-S1P₁ transfected HeLa cells (traces 1).

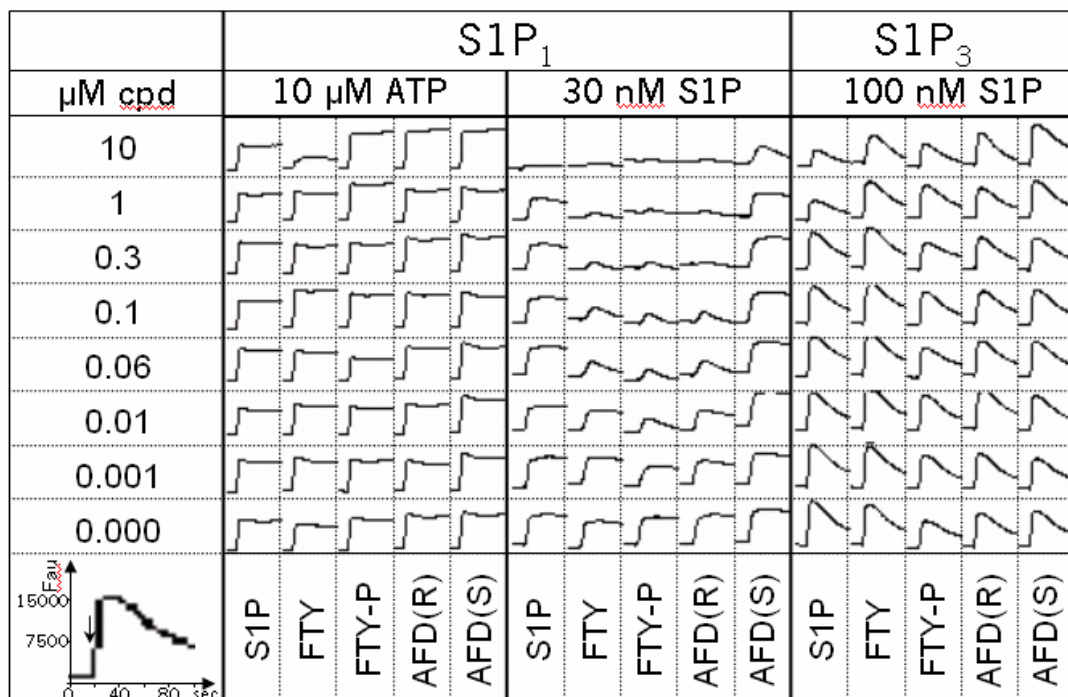
(B) Summary of the results of the flow cytometry analysis. The amount of internalized receptors was calculated after 1 hour treatment with agonist by determining the height of the peaks observed in FCM experiments. The following equation was used: $(1 - (F_{cp} - F_{iso}) / (F_o - F_{iso})) \cdot 100$; F_{cp} =fluorescence peak after the addition of agonist, F_{iso} = fluorescence peak obtained with isotype control antibodies, F_o =fluorescence prior to the addition of compound. Complete (100%) internalization is reached when the fluorescence detected by FCM is equal to that obtained with isotype controls. S= sphingosine. Means ± S.D. (n>3).



Supplementary Figure 3. Agonist-induced myc-S1P₄ internalization on CHO cells, analyzed by flow cytometry.

(A) Effect of agonists on the surface expression of myc-mouse S1P₄: Cells were incubated for one hour with 1 μ M of S1P, FTY720-phosphate, AFD(R) or medium alone prior to analyzing them by FCM.

(B) Summary of the effects of agonists on mS1P₄ as measured by FCM. The extent of internalization (as defined in the legend of Fig.2) is shown for myc-mouseS1P₄ after 1 hour treatment with. Means \pm S.D (n>2).



Supplementary Figure 4. The effect of the pre-incubation of agonist on the activity of S1P receptors expressing CHO cells.

CHO cells expressing S1P₁ and S1P₃ were preincubated with S1P, FTY720-P, AFD(R) and AFD(S) at different concentrations. The cells were washed after 3h incubation and further incubated for 3 h (without agonist) prior to measuring of agonists induced Ca²⁺ transient by FLIPR. 30 nM S1P for S1P₁ and to 100 nM for S1P₃ were used.

(A) Traces of single Ca²⁺ transients at the S1P₁ and S1P₃ receptors. In the case of S1P₁ the response to 10 $\mu\text{M ATP}$ was collected on the same cells prior to the addition of the S1P and after the pre-treatment and 3 h wash-out. At the left bottom edge of the panel details of the graphs are given: Fau= Fluorescence arbitrary units, the arrow indicate the addition of the agonist.