The rainbow trout glucocorticoid receptors - a mechanistic and toxicological study

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Abbreviations

AF-1	activation function 1
AF-2	activation function 2
AhR	arylhydrocarbon receptor
ald	aldosterone
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BaP	benzo(a)pyrene
C	cytoplasm
cAMP	cyclic adenosine monophosphate
Cd	cadmium
cDNA	complementary DNA
CO_2	carbon dioxide
CR	corticosteroid receptor
CREB	cAMP response element binding protein
Cu	copper
Da	dalton
DBD	DNA binding domain
dex	dexamethasone (synthetic glucocorticoid)
DMEM	Dulbecco Modified Eagle's Minimal Essential Medium
DNA	deoxyribonucleic acid
ED50	effective dose that causes 50 % of the observed effect
ER	estrogen receptor

FCS	fetal calf serum
GFP	green fluorescent protein
GFP-rtGR1	GFP tagged rtGR1
GFP-rtGR2	GFP tagged rtGR2
GR	glucocorticoid receptor
GRE	glucocorticoid response element
H_2O_2	hydrogen peroxide
hop	heat shock protein organising protein
hsp	heat shock protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LBD	ligand binding domain
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
N	nuclear
NES	nuclear export signal
NL1	nuclear localisation signal 1
NL2	nuclear localisation signal 2
NLS	nuclear localisation signal
NRS	nuclear retention signal
PBS	phosphate buffered saline
PR	progesterone receptor
pro	progesterone
RAP	receptor associated protein
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RTG-2	rainbow trout gonad 2 (fish cell line)
rtGR1	rainbow trout glucocorticoid receptor 1
rtGR2	rainbow trout glucocorticoid receptor 2
RU486	Mifegyne (synthetic antagonist of mammalian GR)
SOC	a bacterial growth medium
SRC-1	steroid receptor coactivator 1
$t_{1/2}$	half-life period
TBP	TATA box binding protein
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin
TFIID	transcription factor II D
X-Gal	5-bromo-4-chloro-3-hydroxyindole

Summary

Glucocorticoids play an essential role in the regulation of key physiological processes, including immune response, brain function, energy metabolism, electrolyte balance, blood pressure and stress response (Vijayan et al. 2005). The action of glucocorticoids is mediated by the glucocorticoid receptor (GR). Phylogenetically diverse teleost fishes have been shown to possess two GRs that arose following a whole genome duplication event that occurred early in the teleost lineage (Greenwood et al. 2003, Jaillon et al. 2004, Stolte et al. 2006, Bury and Sturm, 2007). The two rainbow trout glucocorticoid receptors, termed rtGR1 and rtGR2, display a marked difference in their hormone sensitivity in transactivation and transrepression assays, with rtGR2 exerting its activity at far lower concentrations of cortisol compared to rtGR1 (Bury et al. 2003, Bury and Sturm 2007). Glucocorticoid signal transduction is regulated by the subcellular distribution of the GRs (Savory et al. 1999; Shank and Pascal 2005), and the first purpose of the present work was to explore determinants of subcellular localisation and nuclear translocation of rtGR1 and rtGR2 using GFP-tagged recombinant receptor proteins.

I observed that trafficking to the nucleus showed the same differences in hormonal sensitivity as those previously found in transactivation or transrepression studies. rtGR2 nuclear transfer was more sensitive to cortisol than rtGR1 nuclear translocation. However, to my surprise I observed that the majority of the naïve trout GRs were constitutively localised in the nucleus. This is in strong contrast to the cytoplasmic localisation reported for mammalian GRs. Further studies using domain swap mutants showed that this unusual cellular distribution is due to an undefined component of the A/B-domain. To date, nuclear localisation signals have been identified only in the C-, D- and E-domain of the mammalian GR protein (Picard and Yammoto, 1987).

Only few studies have so far addressed the impact of contaminants on mammlian GR action and even fewer studies have investigated the impact of environmental contaminants on teleost rtGR1. No study ever has been conducted about the impairment of rtGR2 by contaminants. I therefore for the first time describe the impact of a set of contaminants on the action of both trout receptors on the level of the nuclear import

of the receptor and transactivation of target genes. Nonylphenol, TCDD, PCB, heavy metals, and pharmaceuticals were investigated. Of all substances tested, the heavy metals copper and cadmium, the non steroidal anti-inflammatory drug diclofenac, and the alkylphenol nonylphenol had the greatest impact on trout GR action. Cadmium was a potent inhibitor of nuclear transfer and of transactivation activity of both rtGR1 and rtGR2. Diclofenac impaired nuclear transfer and transactivation activity of both rtGRs. Nonylphenol was able to increase nuclear transfer rates and transactivation of target genes.

In summary, this work revealed that both rainbow trout GRs in the absence of specific ligand were already located partially within the nucleus. This contrasts the behaviour of mammalian GR, in which the naïve GR displays an exclusively cytoplasmic localisation. The A/B domain of the two trout GRs was identified to be at least partly responsible for the nuclear localisation in the absence of ligand. This work moreover shed light on the impact of environmental compounds on the function of both trout GRs. Copper, cadmium, diclofenac, and nonylphenol influenced the action of rainbow trout GRs on the level of nuclear translocation and/or transactivation of target genes.

Zusammenfassung

Glukokortikoide übernehmen eine wesentliche Rolle während der Regulation von vielfältigen physiologischen Prozessen im Organismus. Beispielsweise greifen sie regulierend in die Immunantwort, in Abläufe im Gehirn und in den Energiemetabolismus ein. Glukokortikoide regulieren auch den Elektrolythaushalt, den Blutdruck und die Stressantwort (Vijayan et al., 2005). Die Antwort der Glukokortikoide wird durch Glukokortikoid-Rezeptoren (GR) vermittelt. Interessanterweise, konnten in verschiedenen Vertretern der Knochenfische zwei Glukokortikoid-Rezeptorgene beschrieben werden, welche das Ergebnise einer frühen Genomduplikation in der Linie der Knochenfische ist (Greenwood et al., 2003; Jaillon et al., 2004; Stolte et al., 2006; Bury and Sturm, 2007). Die exprimierten Glukokortikoid-Rezeptoren der Regenbogenforelle, welche mit rtGR1 und rtGR2 bezeichnet wurden, weisen interessante unterschiedliche Charakteristika auf, wenn ihre Hormonsensitivität in Transaktivierungs- und Transrepressionsstudien beleuchtet wird. Der rtGR2 übt seine Aktivität bei deutlich niedrigeren Konzentrationen von Cortisol aus, verglichen mit dem rtGR1 (Bury et al., 2003; Bury and Sturm, 2007). Es gibt Überlegungen, dass die Signalweitergabe der Glukokortikoide durch die subzellulare Verteilung der entsprechenden Rezeptoren beeinflusst wird (Shank and Paschal et al., 2005; Savory et al., 1999). Das erste Ziel der hier präsentierten Arbeit war es deshalb die Faktoren zu bestimmen, welche die zellulare Lokalisation sowie den Transport der Rezeptoren in den Zellkern beeinflussen. Für die Visualisierung der Rezeptoren wurden diese mit Grün Fluoreszierendem Protein gekoppelt.

Der Transfer der Rezeptoren in den Zellkern folgte denselben hormonellen Unterschieden, welche bereits in Transaktivierungs- und Transrepressionsstudien bekannt wurden. Der Import von rtGR2 in den Zellkern wurde bei niedrigeren Konzentrationen verglichen mit dem Import von rtGR1 ausgelöst. Überraschender Weise konnte beobachtet werden, dass ein Großteil der unstimulierten Regenbogenforellen-Rezeptoren konstitutiv im Zellkern nachzuweisen war. Dieses Ergebnis steht im starken Kontrast zu der bisher in Säugetieren beschriebenen Situation. In Abwesenheit des spezifischen

Liganden weisen die Glukokortikoid-Rezeptoren der Säugetiere eine ausschließlich cytoplasmatische Lokalisation auf. In weiterführenden Studien im Rahmen der vorliegenden Arbeit konnten definierte Abschnitte in den Proteinen des rtGR1 und rtGR2 identifiziert werden, welche verantwortlich für diese ungewöhnliche subzelluläre Lokalisation, in Abwesenheit von spezifischem Hormon, sind. Die Proteindomäne A/B beinhaltet eine bisher für Glukokortikoid-Rezeptoren unbekannte Komponente, welche den Rezeptor, auch in Abwesenheit des Liganden, in den Zellkern verschiebt. Bis heute wurden Signale, welche den Import in den Zellkern steuern, nur in den C-, D- und E-Domänen des GR beschrieben (Picard and Yammoto, 1987).

Nur wenige Studien beschreiben bisher den Einfluss von Umweltschadstoffen auf die Funktion des GR in Säugetieren und nur vereinzelt wurden toxikologische Studien des rtGR1 durchgeführt. Keine bisher publizierte Studie beschreibt den Einfluss von Schadstoffen auf die Funktionalität des rtGR2. In der vorgestellten Arbeit wird somit zum ersten Mal das Eingreifen von Schadstoffen in den ungestörten Ablauf der Antwort beider Regenbogenforellen-GR beleuchtet. Untersucht wurden in diesem Zusammenhang der Einfluss von Nonylphenol, TCDD, PCB, Schwermetallen und verschiedenen Medikamenten auf die Translokation der Rezeptoren in den Zellkern sowie auf die nachgeordnete Aktivierung der Transkription von Zielgenen. Der größte Einfluss auf die Funktionen des rtGR1 und rtGR2 konnte in Gegenwart der beiden Schwermetalle Cadmium und Kupfer, sowie dem entzündungshemmenden Medikament Diclofenac und dem Alkylphenol Nonylphenol beschrieben werden. Cadmium hemmte wirkungsvoll den Transport der Rezeptoren in den Zellkern, sowie die nachgeschaltete Aktivierung von Zielgenen. Diclofenac griff ebenfalls hemmend in den Transport der Rezeptoren in den Zellkern und die Aktivierung von Zielgenen ein. Nonylphenol hingegen erhöhte die Geschwindigkeit des Transportes in den Zellkern und verstärkte die Aktivierung von Zielgenen.

Zusammenfassend zeigte die vorliegende Studie, dass beide Regenbogenforellen-Rezeptoren, auch in Abwesenheit ihres spezifischen Liganden, bereits im Zellkern vorliegen. Dieses Ergebnis steht im einem deutlichen Kontrast zu der für Säugetiere beschriebenen Situation. Verantwortlich für diese aussergewöhnliche zelluläre Lokalisation der unstimulierten Rezeptoren sind bisher unbekannte Komponenten in der A/B-Domäne beider Rezeptoren. Die vorliegende Arbeit beschäftigte sich weiterhin mit dem Einfluss von Umweltschadstoffen auf die Funktionsweise der Rezeptoren. Es wurde gezeigt, dass Kupfer, Cadmium, Diclofenac und Nonylphenol die Aktivität der Regenbogenforellen-Rezeptoren beeinflussen. Diese Störung konnte auf Ebene des Transportes der Rezeptoren vom Zytoplasma in den Zellkern und/oder auf Ebene der nachgeschaltete Aktivierung der Transkription von Zielgenen nachgewiesen werden.

Chapter 1

Introduction

A considerable body of evidences indicates that many classes of environmental contaminants have the ability to interfere with normal steroid hormone action (McLachlan, 1993; Fent et al., 2005). Steroid hormones exert their physiological effects via interactions with specific steroid receptor proteins. These steroid receptor proteins act as ligand-dependent transcription factors mediating activation or repression of target genes via binding to control elements in the promoter region upstream of target genes (Escriva et al., 2003) or via direct protein-protein interaction with other transcription factors (Schoneveld et al., 2004). The steroid receptor proteins are a subgroup of the nuclear receptor family and all members of this receptor subgroup share a common evolutionary history as indicated by the presence of a conserved structure and a high degree of sequence conservation. The steroid receptors encompass the estrogen receptor (ER), the progesterone receptor (PR), the vitamin D receptor, the thyroid receptor, and the corticosteroid receptors (CR). The corticosteroid receptors, in particular, include the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Escriva et al., 2004). Contaminants have been reported to act through corticosteroid receptor proteins as well as other steroid hormone receptor proteins. Especially aquatic contaminants are of interest because they can affect a wide range of organisms that are continuously exposed throughout their lives. One important group of aquatic organisms are fish, in particular the teleost fishes.

1.1 Overview of the corticosteroid receptor system in fish

Corticosteroids in teleost fishes are involved in the regulation of a plethora of physiological functions, such as osmoregulation, respiration, immune responses, reproduction, growth, metabolism, and stress response (Mommsen et al., 1999, Charmandari et al.,

2005). Corticosteroids are secreted from the interrenal tissue, which is the analogous to the adrenal cortex of mammals and is located in the head kidney (Henderson and Kime, 1987). The most remarkable difference between teleost and mammalian secretion of corticosteroids is the absence of a significant production of the mineralocorticoid hormone aldosterone (Bern 1967). Instead, the major corticosteroid released from the teleost interrenal tissue is cortisol. Accordingly, many studies have shown that cortisol is the key hormone in fish for osmoregulation during seawater (Foskett et al., 1983; McCormick, 2001) and freshwater adaptation (Flik and Perry, 1989; Laurent and Perry, 1990).

The first evidence for CR in teleost fishes came from binding studies using radiolabeled ligand (Mommsen et al., 1999). These studies indicated the existence of a single class of high affinity, low-capacity binding sites in the cytosol from various tissues (Mommsen et al., 1999, Prunet et al., 2006). The same reports also showed that the CR concentration and CR affinity can be altered by experimental manipulations, such as hormonal treatments, application of stressors, and seawater transfer or by smoltification in salmonid species (Dean et al., 2003; Maule and Schreck, 1991; Pottinger et al., 1994; Shrimpton et al., 1995; Shrimpton and McCormick, 1999).

The first molecular characterization of a fish CR was achieved by the cloning of the rainbow trout GR in 1995 (Ducouret et al., 1995), and later on in tilapia (Tagawa et al., 1997) and flounder (Tokuda et al., 2005). Recent studies also demonstrated the presence of a second GR isoform in trout (Bury et al., 2003) and Burton's mouthbrooder (Greenwood et al., 2003). This second GR isoform is a product of a distinct GR gene, which constitutes a second striking difference to the mammalian system where only one GR gene is present. At about the same time, the MR was identified and characterized in trout (Colombe et al., 2000; Sturm et al., 2005) and in Burton's mouthbrooder (Greenwood et al., 2003). In the context of the absence of significant levels of the mineralocorticoid hormone aldosterone in fish and cortisol acting through a single class of high affinity binding sites, this finding was rather unexpected.

Cortisol was the most effective steroid in enhancing GR transactivation when tested in trout, a result which is very similar to what was observed with rat GR (Bury et al., 2003; Ducouret et al., 1995). Analysis of MR transactivation activity showed that aldosterone and deoxycorticosterone were the most effective stimulants in trout (Sturm et al., 2005). In light of the absence of a significant production of the mineralocorticoid hormone aldosterone (Bern, 1967) deoxycorticosterone was suggested to constitute a physiological ligand of MR, similar to aldosterone in mammals (Sturm et al., 2005). Nevertheless, cortisol is known as the key regulator in fish hydromineral regulation. Interestingly, the trout MR also appeared sensitive to very low levels of cortisol, being even much more sensitive to this steroid than the GR (10 to 100 times). Such a situation has also been observed in mammals (Rashid and Lewis, 2005). In mammals, the interaction of aldosterone with the MR requires factors that prevent the more abundant ligand cortisol from accessing the receptor. In this context, the enzyme 11β-hydroxysteroid dehydrogenase type 2 plays a key-role in oxidizing cortisol to cortisone, an inactive steroid, and then protecting aldosterone selectivity *in vivo* (Farman

and Rafestin-Oblin, 2001). Intriguingly, this enzyme has recently been isolated and characterized in fish (Jiang et al., 2003; Kusakabe et al., 2003).

Despite the unexpected complexity of the teleost corticosteroid system, only few mechanistic studies have been conducted so far on fish CR (Ducouret et al., 1995; Bury et al., 2003; Greenwood et al., 2003; Stolte et al., 2006, Colombe et al., 2002; Sturm et al., 2005). The presented work therefore set out to characterize the glucocorticoid receptors of the teleostean corticosteroid system, with specific focus on rainbow trout *(Oncorhynchus mykiss)*. As stated above, the glucocorticoid receptor system in teleost fishes is more complex than that of mammalians. In mammals multiple GR isoforms are generated by alternative splicing from one single GR gene (Lu and Cidlowski, 2004) whereas a whole genome duplication event occurred in teleost fish (Jaillon et al., 2004), leading to two distinct GR genes (Bury et al., 2003, Ducouret et al., 1995). Already two isoforms of the GR1 gene generated by alternative splicing and one GR2 gene product have been desribed (Ducouret et al., 1995, Takeo et al., 1996, Bury et al., 2003). The two major GR isoforms in rainbow trout, termed rtGR1 and rtGR2, display a marked difference in their hormone sensitivity in transactivation and transrepression assays. The rtGR2 exerts its action at far lower concentrations of cortisol than rtGR1 (Bury et al., 2003, Bury and Sturm 2007). One basic idea which could explain this phenomenon is that the transcriptional potential of nuclear receptors is influenced by the extent of nuclear translocation following hormone treatment (Savory et al., 1999, Shank and Pascal, 2005). Therefore the first part of the presented study addressed the comparison of the nuclear transfer of the rainbow trout glucocorticoid receptors, rtGR1 and rtGR2, by the use of green fluorescent protein (GFP) tagged fusion proteins.

In the following I will first describe the known mechanisms important for cellular movement of the mammalian GR. This will be followed by a summary of mechanistic properties of fish GR. After providing the background on the mechanistic properties, I will provide an outline of endocrine disruptors of mammalian and/or fish GR. Only few studies addressed the impact of environmental contaminants on mammalian GR and even fewer studies were conducted on the impact of pollutants on fish GR action. In the second part of this thesis I thus investigated the impact of a set of contaminants found in the aquatic environment on the action of rainbow trout rtGR1 and for the first time rtGR2.

1.2 Mechanistic properties of mammalian glucocorticoid receptors

1.2.1 Structure of the glucocorticoid receptor

The GR is a modular protein consisting of four domains attributed with distinct functions (Figure 1.1). The amino-terminal A/B domain is important for transcriptional activity, the C-domain or DNA-binding domain (DBD) is responsible for DNA binding

and receptor dimerization, the D domain is involved in conformational changes, and the E-domain or ligand-binding domain (LBD) mediates hormone binding (Kumar and Thompson, 2005).

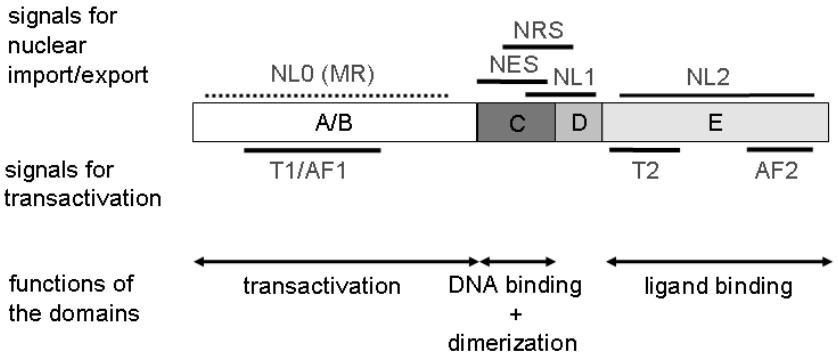


Figure 1.1: Structural and functional features of mammalian GR. GR is a modular protein consisting of 4 distinct domains - the A/B domain important for tansactivaiton, the C-domain responsible for DNA-binding and receptor dimerization, the D-domain mediating structural changes within the receptor and the E-domain important for ligand binding. Additionally, signals important for both nuclear import and nuclear export as well as for transactivation are presented. *NL0, nuclear localisationsignal 0 - only detected in MR; NRS, nuclear retention signal; NES, nuclear export signal; NL1, nuclear localisation signal 1; NL2, nuclear localisation signal 2; τ1/AF1, τ2, AF2, transactivation domains.*

A/B domain (transactivation domain: The A/B domain is involved in transactivation of downstream genes and varies greatly among different species (Giguere et al., 1986). Within this domain is a powerful, glucocorticoid dependent, activation region, called activation function 1 (τ1/AF1) (Dieken and Miesfeld, 1992). AF-1 has been shown to interact with the TATA-box binding protein (TBP) of the largest of the general transcription factors TFIID of the basic transcription machinery (Ford et al., 1997). Under physiological conditions, AF-1 exists as a partially folded structure and only ligand binding triggers the final folding of AF-1 (Kumar and Thompson, 2005).

C-domain (DNA binding domain, DBD): The 3D structure of the DNA-binding region shows two sub-domains (CI and CII), each consisting of one zinc finger (Hard et al., 1990). The N-terminal zinc-finger is involved in DNA-binding and in particular required for recognition of glucocorticoid response elements (GREs) on the DNA (Green et al., 1988). The site-specific DNA recognition depends on certain amino acids in the proximal P-box which bind the hormone responsive element of target genes in the major groove of the DNA (Umesono and Evans 1989; Luisi et al., 1991). The C-terminal zinc-finger is involved in receptor homodimerization at the GRE and stabilises binding of the GR to GRE, which is mediated by distinct amino acids in the distal D-box (Luisi et al., 1991; Kumar and Thompson 1999, 2005; Dahlman-Wright et al., 1991).

E-domain (Ligand binding domain, LBD): The ligand binding domain is composed of 12 helices forming a hydrophobic pocket that binds the ligand; plus an additional side

pocket which is thought to allow selective binding of glucocorticoids that have larger substituents (Bledsoe et al., 2002, Kauppi 2003). The LBD, in addition to being the site for ligand binding, also plays an important role in ligand induced transactivation with two regions being of significance, the tau2 in the N-terminal part of the LBD and the hormone in-sensitive AF-2 in the C-terminus of the LBD (Danielian et al., 1992; Boruk et al., 1998; Milhon et al., 1997).

1.2.2 Glucocorticoid receptor functioning

In addition to being structurally related, steroid receptors also share a common mechanism of action. All steroid receptors act as ligand-inducible transcription factors. In the ligand-bound form, the subcellular localisation of all steroid receptor is nuclear. In the absence of ligand, subcellular localisation has been described to differ between steroid receptors; unliganded GR resides in the cytoplasm, naïve MR is found in the cytoplasm as well as the nucleus and ER and PR are predominantly nuclear even in the absence of hormone (Evans et al., 1988).

The action of the mammalian GR has been extensively described. The receptor resides in the cytoplasm as a large heteromeric complex involving, among others, Heat Shock protein 90 (hsp90), hsp70, hop (hsp70/hsp90 organising protein) and different immunophilins (Tissing et al., 2005; Ogawa et al., 1995; Htun et al., 1995; Sackey et al., 1996; Walker et al., 1999). All of them maintain the receptor in a high ligand-affinity conformation. Hsp90 and hsp70 are the most important chaperones for the formation of the high affinity ligand-binding state, and are responsible for the opening of the hydrophobic pocket in an ATP-dependent process (Pratt and Toft 1997, 2003; Pratt et al., 2006).

The lipophilic hormone enters the cells by passive diffusion through the lipid membrane. As soon as the steroid-binding pocket is opened, cortisol enters and binds in the pocket. Subsequently the chaperone complex dissociates (Pratt and Toft, 2003; Kumar et al., 2006). The LBD undergoes a conformational change, thereby closing the pocket (Bledsoe et al., 2002). This ligand bound form can now move to the nucleus and exert its transcriptional activity.

1.2.3 Nuclear localisation, trafficking and shuttling of GR

Within minutes of glucocorticoid binding ($t_{1/2} \approx 4\,\text{min}$), the GR enters the nucleus to activate or repress target gene transcription (Ogawa et al., 1995; Htun et al., 1995; Sackey et al., 1996; Walker et al., 1999). The GR is believed to move along the microtubules and dynein is the motor responsible for the movement (Pratt et al., 2006). When the GR arrives at the nuclear membrane, it has to translocate across the nuclear pore, a 125-MDa complex in the nuclear envelope (Stoffler et al., 1999). Small molecules readily diffuse through the nuclear pore, whereas molecules greater than 40 kDa require an import and export machinery for transport (Stoffler et al., 1999; Gorlich and Kutay,

1999; Macara, 2001). The GR is known to continuously shuttle back and forth between the nuclear and cytosolic compartments (Madan and Defranco 1993) and the steady state of nucleocytoplasmic localisation is the consequence of a fine balance between operational strengths of nuclear import signals (NLS), nuclear export signals (NES), nuclear retention signals (NRS) and possibly cytoplasmic retention signals (Figure 1). In the mammalian GR, two NLS, termed NL1 and NL2, have been identified (Picard and Yamamoto 1987, Cadepond et al., 1992; Tang et al., 1997) as well as one nuclear export signal within the DBD (Black et al., 2001) and one nuclear retention signal (Carrigan et al., 2007; Figure 1).

The best known nuclear import process is mediated by recognition of importins of one or more clusters of basic amino acids in the NLS. This is followed by nuclear pore docking, translocation through the pore and cargo release from the inner side of the pore (Macara, 2001; Weis, 2003). The NLS of the GR first discovered was NL1 (Picard and Yamamoto 1987), which overlaps with and extends C-terminally from the receptor DBD (LaCasse and Lefebvre, 1995). NL1 is comprised of three partitions (Tang et al., 1997). The close localisation of NL1 and the DNA binding region (Luise et al., 1991) is an intriguing feature common to nuclear receptors (LaCasse and Lefebvre 1995) and is a result of co-evolutionary selective pressure to assure that proteins which bind to the DNA are also transported into the nucleus. One core basic sequence adjacent to the DBD is required for NLS functioning. Moreover two smaller clusters of basic amino acids at the C-terminus of the DBD appear to contribute to increasing the strength of NLS, and thus the efficiency, with which these receptors are imported into the nucleus (Tang et al., 1997). NL1 is bound by importin-α (Savory et al., 1999), which together with importin-β confers nuclear localisation (Freedman and Yamamoto 2004). Importin-β thereby potentiates the binding of importin-α (Freedman and Yamamoto 2004). NL2 is strictly steroid-dependent, and both the sequence within the LBD that includes NL2 and the karyopherins that determine NL2-mediated nuclear import remain to be identified. It has been proposed that NL2 only assembles upon ligand binding (Picard and Yamamoto 1987). Using a mutant that lacks NL1 it has been demonstrated that NL2 is an agonist-specific NLS that mediates the incomplete localisation of GR to the nucleus in cells treated with cortisol or the synthetic steroid dexamethasone (Savory et al., 1999).

Upon hormone withdrawal, GR reassociates rapidly into a cochaperone complex, but only slowly redistributes to the cytoplasm over periods that can extend from 12 - 24 h (Sackey et al., 1996, Haché et al., 1999). Recently, one nuclear retention signal (NRS) has been defined in the hinge region of the GR that actively opposes the nuclear export of the GR (Carrigan et al., 2007). The GR NRS overlaps closely with the basic NL1 but can be distinguished from NL1 by targeted mutagenesis. Such an active retention of the GR in the nucleus inhibits nuclear export and appears to play an important role in determining the transcriptional potential of the receptor (Carrigan et al., 2007).

The nuclear export signal of the GR has been defined to 15 amino acids in the linker region between the zinc-fingers of the GR DBD (Black et al., 2001). Nuclear export of the GR is suggested to be accomplished by both Crm-1-dependent and -independent

mechanisms (Liu and DeFranco 2000). Export of steroid-withdrawn GR from the nucleus has been found to be sensitive to leptomycin B treatment and thus likely involves Crm-1, whereas export of liganded GR appears to occur independently of Crm-1 (Savory et al., 1999). More recently Holaska (2001) reported calreticulin to be the receptor for nuclear export.

Significant advances in the understanding of subcellular localisation of the GR were gained by tagging GR with green fluorescent protein (GFP), a 27 kDa protein from jellyfish *Aequorea victoria* (Chalfie et al., 1994). GFP provides an excellent tool to monitor protein localisation and translocation of proteins. The GFP tag eliminates the use of fixation, cell permeabilization and antibody incubation for immunohistochemistry, and permits the dynamic study of localisation and/or trafficking of the protein of interest. Due to the resistance of enhanced GFP to photobleaching, the translocation process can be studied by time-laps video microscopy. In addition, multicolour variants of jellyfish GFP and sea anemone red fluorescent protein are available (Chalfie et al., 1994; Kawata et al., 2001). These allow to investigate the multi-imaging of at least two different molecules in a single cell in real time. Although GFP fusion has many advantages, it also has a disadvantage due to its large molecular size. Tagging of proteins with the 27 kDa GFP molecule and their over-expression may change the original function and localisation.

1.2.4 Activation and repression of genes by the GR

The DNA recognition functions via the DBD which possesses two zinc fingers separated by a short sequence of amino acids (Hollenberg et al., 1985). The P-box of the first zinc finger is of importance in recognizing the glucocorticoid response element (GRE) upstream of target genes (Eriksson and Nilson, 1998). The classical GRE is an imperfect palindrome GGTACAnnnTGTTCT that the PR, MR and GR recognise. The 3 basepair spacer between the two halves of the GRE facilitates cooperative binding of two GR monomers to form the heterodimer complex. Dimerization of GR receptor proteins involves interaction of the DBD of both receptors at the GRE. The GRE acts as an allosteric activator by providing a scaffold to bind the GR in the correct position for dimerization. Binding of the first GR monomer favours binding of the second GR (Luisi et al., 1991). The GR dimer complex surface forms bridges with regulators, coactivators and recruits other transcription factors which finally lead to the induction of gene transcription (Glass and Rosenfeld, 2000). Factors recruited include SRC-1, cyclic AMP-response element-binding protein (CREB), CREB-binding proteins, and chromatin modifiers (SWI/SNF) and RNA polymerase II, which together induce histone modifications, including acetylation and chromatin remodelling, and subsequent production of mRNAs (Ito et al., 2006).

The GR mediates its regulatory action in the organism not only by activation of gene transcription but also by repression of target gene expression. Repression of transcription by GR is mostly achieved by antagonising the transactivating properties of other

transcription factors (Schoneveld et al., 2004). In a competition model, liganded GR binds to a negative GRE and competes with other transcription factors for binding to their response element. This is accomplished by positioning the negative GRE across the binding site of another transcription factor (Ito et al., 2006). GR can on the other hand act as a ligand-inducible co-regulator itself and be tethered to other transcription factors and thereby repress gene expression (Schoneveld et al., 2004).

After dissociation of hormone, the receptor is released from the high affinity chromatin binding sites, which is a chaperone dependent process (Yang et al., 1997, Freedman and Yamamoto 2002). GR released from chromatin can recycle to chromatin upon rebinding hormone without exiting the nucleus (Yang et al., 1997). Photobleaching experiments showed that the hormone-bound GFP-GR exchanges rapidly at the chromosomal regulatory sites, with a half maximal time of approximately 5 s (Pratt et al., 2006).

1.3 Mechanistic properties of fish glucocorticoid receptors

1.3.1 Molecular characterization of glucocorticoid receptors in fish

The first molecular characterization of a fish GR was achieved by the cloning of a rainbow trout (*Oncorhynchus mykiss*) GR in 1995 (Ducouret et al., 1995). This was followed by the isolation of a partial GR cDNA in tilapia (*Oreochromis mossambicus*) (Tagawa et al., 1997) and flounder (*Paralichthys olivaceus*) (Tokuda et al., 2005). Recent studies demonstrated the presence of a second GR isoform in trout (Bury et al., 2003) and Burton's mouthbrooder (*Haplochromis burtoni*) (Greenwood et al., 2003). The existence of two GR genes in fish was later confirmed by analyses of the tetraodon and fugu genomes (Stolte et al., 2006). Additionally, splicing variants of rainbow trout, tetraodon and fugu GR1 (Takeo et al., 1996, Stolte et al., 2006) and GR2 of Burton's mouthbrooder (Greenwood et al., 2003) have been described.

The duplicated GR genes in teleostean fishes appear to be a result of a genome duplication that occurred in the evolution of actinopterygii, before the radiation of the teleost and after the divergence of tetrapods from the fish lineages (Bury and Sturm 2007). The retention of two GR isoforms in the teleost lineage that diverge some 335 million years ago would suggest neofunctionalisation. It is feasible that a duplication of the GR would provide a greater plasticity in the ability of a fish to respond to a stressor and control homeostasis at a multitude of physiological, immunological, and developmental axes (Bury and Sturm 2007). A further level of control may also be offered by the presence of splice variants of one of the GR isoforms (Bury and Sturm 2007). However, the full details of functional differences of trout GR isoforms are complex and have yet to be fully determined.

1.3.2 The DNA binding region in fish glucocorticoid receptors

The DBD in fish, despite being the best-conserved region of the GR protein, contains an additional in-frame amino acid insertion in the interfinger region between the two zinc fingers. In detail, nine additional amino acids in the DBD of rtGR1 (Ducouret et al., 1995) and five additional amino acids in the DBD of rtGR2 (Bury et al., 2003) have been described. The interfinger region of GR DBDs seems to be flexible and can cope with insertion of up to 9 amino acids. Such insertions have also been described for rare splice variants of GR of marmoset and MR of human, rat and *Xenopus laevis* (Wickert and Selbig, 2002).

The nine amino acid insertion WRARQNTDG between the two zinc fingers in the DBD of rtGR1 is remarkably conserved among telostean species. It has thus far been described in rainbow trout (Docouret et al., 1995, Takeo et al., 1996), Japanese eel (Todo and Nagahama, 1998), Japanese flounder (Tokuda et al., 1999), Burtons' mouthbrooder (Greenwood et al., 2003), brown trout (Stolte et al., 2006), European sea bass (Stolte et al., 2006) and fugu (Stolte et al., 2006). The insertion appears to be a result of alternative splicing and is encoded by an extra exon (Stolte et al., 2006, Lethimonier et al., 2002). This additional sequence in the rtGR1 confers a better binding affinity of the receptor to a single GRE. This better binding to single GRE is correlated with a higher constitutive transcriptional activity of the receptor on a reporter driven by a single GRE. However, ligand-induced transcriptional activity is not affected (Lethimonier et al., 2002). Nevertheless, on a double GRE the amino acid insertion does not influence constitutive or ligand-induced transcriptional activity (Lethimonier et al., 2002).

Rainbow trout GR2 possesses the five additional amino acids, GTGAR, (Bury et al., 2003). One of the two GR isoforms of H. burtoni (HbBR2a) does not present any additional residues within the DBD (Takeo et al., 1996, Greenwood et al., 2003). To date no work has addressed the function of the amino acid insertion in rtGR2 DBD.

1.3.3 Transactivational capacities of rainbow trout glucocorticoid receptors

The startling difference between activity of the two trout rtGR isoforms is their transactivational sensitivity. Analyses of transactivational properties of rainbow trout GR isoforms have been carried out in transiently co-transfected mammalian cells, using an expression construct containing the receptor cDNA and a reporter plasmid in which luciferase expression is under the control of a cortisol-responsive promoter containing several classical GREs. It was found that rtGR2 is more sensitive than rtGR1 (Bury et al., 2003). The difference in sensitivity is not restricted to cell type or reporter plasmid and is also seen in transrepression studies (Bury et al., 2003, Bury & Sturm 2007). In this context it is of interest to compare cortisol effective dose 50 % (ED_{50}) for trout GR isoforms in transfected cells (Bury et al., 2003) and circulating cortisol concentrations in fish (Wendelaar Bonga, 1997). Whereas plasma cortisol levels in stressed

fish are above ED_{50} values for both rtGR1 and rtGR2, plasma cortisol levels in non-stressed fish appear below cortisol ED_{50} for rtGR1 and above ED_{50} for rtGR2 (Figure 1.2). Although *in vivo/in vitro* extrapolations are always difficult, this would indicate that cortisol would not activate the rtGR1 receptor in non-stressed or under mildly stressful conditions and that both receptors are mobilized under stressful conditions.

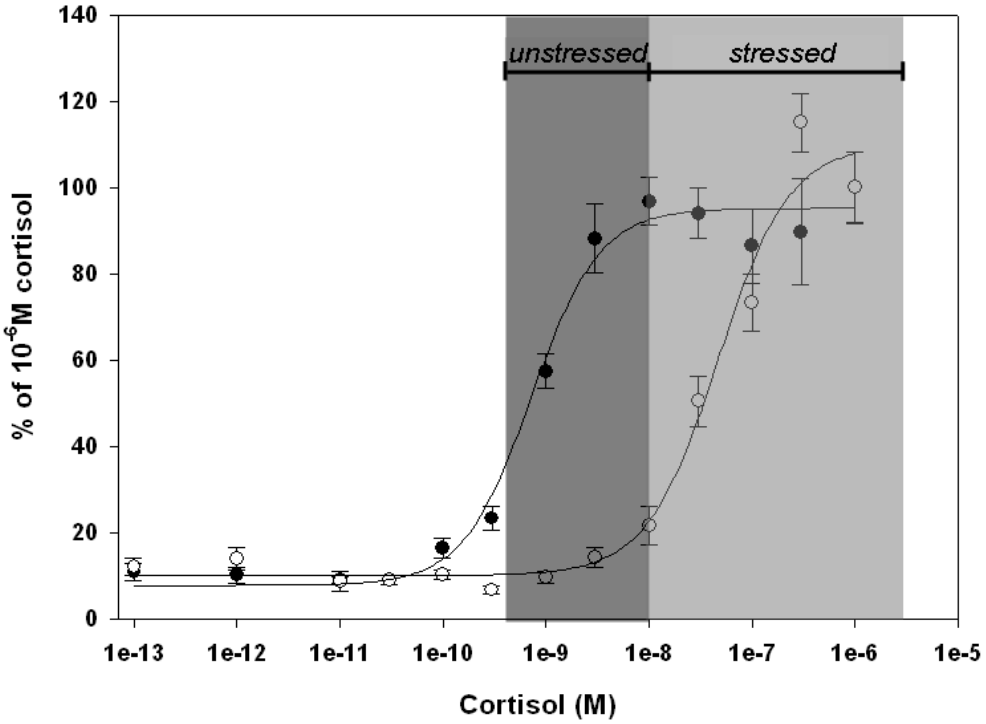


Figure 1.2: Transactivation activity of rtGR1 and rtGR2 of CHO cells co-transfected with either rtGR1 (open circle) or rtGR2 (filled circle) expression vectors, as well as the reporter plasmid pFC31Luc, and the pSVβ plasmid which expresses β-galactosidase. After transfection, cells were treated with varying concentrations of cortisol. Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control, and expressed as a percent of GR1 or GR2 construct activities measured in the presence of 10^{-6} M cortisol. Values represent means +- S.E.M. of three separate experiments, with each experiment performed in triplicate (modified after Bury et al., 2003). Shades mark plasma cortisol concentrations in unstressed (light grey shade) and stressed fishes (dark grey shade).

1.4 Toxicological analysis of fish glucocorticoid receptors

The multiple effects mediated by GRs, the complex involvement of distinct functional regions within the receptor proteins, and the tightly regulated network of associated proteins and co-factors required for transcriptional regulation by GR represent

a plethora of possible points of toxicant attack. Environmental compounds that disrupt the endocrine control of steroid hormones can produce their effect either via direct or indirect interaction with the specific steroid receptor proteins (Bradley et al., 1997). Interestingly, if the scientific community is speaking about endocrine disruption of natural hormone action, the discussion is usually focused on the disturbance of sex steroid hormone receptors, and in particular the estrogen receptor (Iguchi et al., 2006; Charles et al., 2004; Rotchell and Ostrand 2003).

Perhaps the focus has been on the reproductive axis mainly because of the ease of quantifying the response elicited by xenoestrogens. However, the disturbance of ER-mediated processes alone can not explain the complex effects elicited by most of the compounds disrupting endocrine regulation, and disturbance of the function of other proteins is likely to occur (Odermatt et al., 2006; Vijayan et al., 2005). For the detection of xenoestrogens a whole test battery has been developed covering all actions of the ER. This battery includes measuring competitive ligand binding (Korach, 1979), evaluating the binding to response elements on the DNA (Nikov et al., 2000), investigating transactivation of downstream genes in mammalian cell systems as well as in yeast (Legler et al., 1999; Arnold et al., 1996), and measurement of cell proliferation (Soto et al., 1994). In contrast, only few studies exist describing the impact of compounds disrupting GR mediating pathways.

1.4.1 Direct interaction of contaminants with GR

Several chemicals, including methyl-PCBs and metals, are known to directly compete with the endogenous ligand for the GR-binding sites (Johansson et al., 2002). With heavy metals, major concerns are the interference with sulfhydryl groups leading to denatured and aggregated proteins (Dundjerski et al., 2000; Elez et al., 2001; Li et al., 1998; Richards and Playle 1999, Mazon et al., 2004; Manzl et al., 2003; Block and Pärt 1992). Arsenic e.g. is known to impair promoter binding with an interesting biphasic effect. At low concentrations, transactivational activity is stimulated while higher arsenic concentrations repress the GR-mediated transactivation of target genes (Kaltreider et al., 2001). Knudsen and Pottinger (1999) examined the interaction of representative alkylphenols and phthalates with steroid receptors and showed that these compounds displace estrogen from the ER, but not cortisol from the GR (Knudsen and Pottinger, 1999). However, despite the absence of direct binding to the receptor, one cannot rule out the possibility that chemicals may affect corticosteroid signalling indirectly.

1.4.2 Indirect impact of contaminants on GR

GR is assembled with a plethora of receptor associated proteins (RAPs) important for all steps of GR action. RAPs facilitate the high ligand-affinity conformation of the receptor, mediate nuclear translocation, DNA-binding and nuclear export. Such

RAPs include chaperones, co-chaperones, immunophilines, coactivators, corepressors, and stress activated kinases (Vijayan et al., 2005). The immunosuppressants FK506 and cyclosporine, for example, have been shown to bind rat GR immunophilins and enhance sub-maximal GR transactivation activity (Yang-Min et al., 1992; Jack-Michel et al., 1995; Prima at al. 2000). On the other hand RAPs can be altered in their availability and thereby affecting GR action. For example, the response to contaminants is often mediated by the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR). AhR and GR have very similar biochemical properties (Cuthill et al., 1988) and share a similar complement of accessory proteins for their signalling. It is possible that high traffic through one pathway could occupy and deplete the free pool of accessory proteins for the other pathway.

1.5 Specific objectives of this thesis

The objectives of this study were:

- the determination of subcellular localisation and nuclear trafficking of both rainbow trout glucocorticoid receptors rtGR1 and rtGR2, and
- the evaluation of the impact of environmental contaminants on the subcellular localisation, nuclear trafficking and transactivation of target genes by rtGR1 and rtGR2.

In order to achieve these goals, expression vector constructs were generated linking the coding region for rtGR1 and rtGR2, respectively, with the coding region for green fluorescent protein (GFP). The GFP-tagged fusion proteins were then expressed in the mammalian cell line COS-7 as well as the rainbow trout cell line RTG-2. Specific fluorescence was determined using confocal laser scanning or fluorescence microscopy.

The impact of environmental contaminants on rtGR1 and rtGR2 functioning was determined by the use of these GFP-tagged fusion protein expressed in COS-7 cells. Moreover the transactivation potential of rtGR1 and rtGR2 was evaluated in the presence of pollutants in COS-7 cells.

Chapter 2

Material and Methods

2.1 Screening for a fish cell model and method development

So far, both rainbow trout glucocorticoid receptors have been studied in mammalian cell systems only. Hence, the first aim of this study was to introduce the use of fish cell lines. Ideally, a rainbow trout cell line would be needed that does not express either or both trout GRs. For this purpose I started screening seven different rainbow trout cell lines for the presence of mRNA of rtGR1 or rtGR2 by conventional reverse transcriptase PCR. I will first start describing the routine handling of all cell lines used in the study, including the mammalian cell line COS-7. This will be followed by a description of the methodology for the conventional PCR.

2.1.1 Routine growth of cell lines

Stock cultures: Seven fish cell lines, derived from different organs of rainbow trout, and one mammalian cell line, COS-7, derived from African green monkey, were used in this study (Table 2.1).

Table 2.1: Cell lines used for studying the glucocorticoid receptors

Cell line	Origin	Medium	Supplements	Temperature	Reference/source
RTL-W1	Rainbow trout liver	L15 without phenol red	5 % calf serum, 100 IU penicillin 100 μg/ml streptomycin	19°C	Lee et al., 1986
RTH-149	Rainbow trout hepatoma	L15 without phenol red	10 % calf serum, 100 IU penicillin 100 μg/ml streptomycin	19°C	ATCC CCL-1710
RTgill-W1	Rainbow trout gill	L15 without phenol red	10 % calf serum, 100 IU penicillin 100 μg/ml streptomycin	19°C	ATCC CRL 2523
R1	Rainbow trout liver	L15 without phenol red	5 % calf serum, 100 IU penicillin 100 μg/ml streptomycin	19°C	Ahne, 1985
RTgut	Rainbow trout	L15 without phenol red	5 % calf serum, 100 IU penicillin	19°C	Developed by Schirmer; characterization not yet published
RTG-2 (epithelial)	Rainbow trout gonads	L15 without phenol red	10 % calf serum, 100 IU penicillin 100 μg/ml streptomycin	21°C	ATCC CCL-55
RTbrain	Rainbow trout	L15 without phenol red	10 % calf serum, 100 IU penicillin	19°C	Developed by Bols; characterzation not yet published
COS-7	CV-1 (African green monkey)	DMEM 4.5 g/l glucose 110 mg/l pyruvate	10 % calf serum, 100 IU penicillin 100 μg/ml streptomycin 2 mM glutamine	37°C	ATCC CCL-1651

Cell culture: Fish cells were maintained in Leibovitz L15 medium (Invitrogen, Karlsruhe, Germany) without phenol red, supplemented with penicillin, streptomycin and calf serum (see Table 2.1 for respective amounts of each supplement). All fish cell lines were maintained at $19 \pm 1°C$, except RTG-2, which were grown at $21 \pm 1°C$. The mammalian cell line, COS-7, was maintained at 37°C in a CO_2 enriched environment (5 %) in DMEM (Invitrogen, Karlsruhe, Germany) containing 4.5 g/l glucose ad 110 mg/l pyruvate (Invitrogen, Karlsruhe, Germany) plus supplements (Table 2.1). Cells were cultured until confluent. Confluent flasks were subcultured or used for the preparation of experiments. All fish cell lines except RTG-2 needed about 1-2 weeks to reach confluency and were subcultured at a ratio of 1 : 1. RTG-2 cells were subcultured twice a week at a ratio of 1 : 1. The COS-7 cells were subcultured every three days at a ratio of 1 : 10.

Cells were subcultured by a routine procedure. Confluent cell monolayers were washed twice with Versene (Sigma-Aldrich, Munich, Germany) and then trypsin (Sigma-Aldrich, Munich, Germany) was added. After 5-10 min incubation, trypsin digestion was stopped by the addition of fresh, serum containing medium and cell suspensions were divided into new flasks. For initiation of experiments cell number was determined by counting with a Hemacytometer Slide (Neubauer Chamber).

2.1.2 RT-PCR of rtGR1 and rtGR2 mRNA

Sample preparation: Cells were seeded at a density of 1×10^5 cells/cm^2, in standard growth medium (Table 2.1). Cells were grown for 48 h until sample collection. Monolayers were washed once with PBS, and cells were detached using a cell scraper. Cell suspensions were centrifuged and dry cell pellets were frozen in liquid nitrogen.

RNA Isolation: Frozen cell pellets were thawed in 1 ml Trizol-reagent (Invitrogen, Karlsruhe, Germany), and incubated for 5 min at room temperature on a thermomixer at 1400 rpm. A volume of 0.2 ml chloroform was added to the test tube and mixed vigorously for 15 sec on a vortexer. The reaction was incubated at room temperature for 3 min, and centrifuged for 15 min at 12000 x g at room temperature. The aqueous, upper phase was transferred into a new tube and the RNA precipitated with 0.5 μl isopropyl alcohol for 10 min at room temperature. Samples were centrifuged at 12000 x g at 4°C. The RNA-pellets were washed with 0.5 ml 70 % ethanol by centrifugation at 12000 x g at 4°C for 10 min. The pellet was air dried for 30 min and dissolved in RNase free water at 55°C for 10 min. The amount of RNA was measured photometrically at 260 nm and the quality of the RNA was analysed by agarose gel electrophoresis and ethidium bromide staining. Contamination of genomic DNA was removed by treatment with DNase (Roche, Grenzach, Germany) for 15 min at 25°C.

cDNA Synthesis: cDNA was synthesized from 2 μg of total RNA using the RevAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) and random hexamer primers (Invitrogen, Karlsruhe, Germany) according to the manufacturers instructions.

PCR: Both GRs and the housekeeping gene 18S were amplified from 1 μl of cDNA using 1 unit of *Taq* Polymerase (Promega, Mannheim, Germany), 50 mM TRIS-HCl, pH 9.0, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 0.1 % (v/v) Triton-X 100, 200 μM dNTP mix, 500 nM of each primer in a 50 μl reaction volume. The number of cycles was 18 cycles for amplification of 18S ribosomal RNA and 30 cycles for amplification of rtGR1 and rtGR2. The PCR-fragments were analysed by agarose gel electrophoresis and ethidium bromide staining. All primers were designed using the programme primer3 (http://frodo.wi.mit.edu/). Primer sequence and amplified fragment length is summarized in Table 2.2.

Table 2.2: Primer sequences used for RT-PCR

Primer	Sequence	Fragment length	Genbank ACC #
rtGR1 forward	tcagcagtccaaaggcaagac	377 bp	Z54210
reverse	acgacgatggagccgaac		
rtGR2 forward	caccttggcttcca	565 bp	AY495372
reverse	cgtccactcccagaggcc		
18S forward	gagcctgagaaacggctac	429 bp	AF308735
reverse	cgcagctaggaataatggg		

2.1.3 Method development for the transfection of RTG-2

After choosing the fish cell line RTG-2, an appropriate transfection technique needed to be identified. Only few studies have addressed the transfection of fish cell lines so far. E.g. RTH-149 cells were transfected using modified calcium phosphate coprecipitation methods (Zarafullah et al., 1988), FuGene (LaRoche, Basel, Switzerland; Hornung and Ankley, 2002) or the polybrene/DMSO method (Richter et al., 1996). In the presented study, three different methodologies were compared to transiently transfect RTG-2 cells: calcium phosphate precipitation, liposome mediated transfection by Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) and electroporation. Cells were transfected with pEGFP-C1. Applying fluorescence microscopy, the transfection efficieny, the number of cell which express GFP compared to total amount of cells, was determined. The transfection techniques were also evaluated on COS-7 cells to ensure that both cell lines could be transfected with the same method.

Calcium phosphate transfection relies on the formation of co-precipitates of plasmid DNA due to interaction with calcium ions. The DNA precipitates are thought to enter the cells by endocytosis. The calcium phophat coprecipitation method is a very inexpensive and simple technique to perform. Plasmid DNA is mixed in a solution of calcium chloride, and then added to a phosphate-buffered solution. A fine precipitate forms in the solution, which is then added directly to the cells in culture. Transfection

efficiency is usually quite limited. Very seldotm it reaches levels greater the 10 % (Sambrook and Russel 2001). Usually, the transfection efficiency is less than 1 %. Transfection efficiencies can be improved in some cell lines by shocking the cells with DMSO or glycerol.

Method according to Sambrook and Russel (2001): RTG-2 cells, being in the logarithmic growth phase, were plated the evening before transfection at a density of 2.4×10^6 cells per well of a 6-well plate. Two hours prior transfection the medium was removed, cells washed once with PBS, and the medium replaced with DMEM nutrient mix F-12 Ham supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 3.7 g/l $NaHCO_3$ containing 2.5 % charcoal-dextran treated fetal calf serum (FCS). Cells were co-transfected with 1 μg/6-well plate pEGFP-C1 and 19 μg/6-well plate pBluescript SK(+). Image acquisition was carried out after 36 h using fluorescence microscopy.

Method with glycerol shock (Zarafullah, 1988): RTG-2 cells were initially treated as described for calcium phosphate precipitation. Four hours after addition of the precipitate the cells were shocked for 1 min with 15 % glycerol, washed three times with DMEM nutrient mix F-12 Ham without supplements and then DMEM nutrient mix F-12 Ham supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 3.7 g/l $NaHCO_3$ containing 2.5 % charcoal-dextran FCS was added to the cells. Cells were allowed to recover for 48 hours and express GFP.

Electroporation is the application of high voltage to a mixture of DNA and cells in suspension. The cell-DNA suspension is placed between two electrodes and subjected to an electrical pulse. For efficient gene transfer, electroporation depends upon the nature of the electrical pulse, the distance between the electrodes, the ionic strength of the suspension buffer, and the nature of the cells.

Method: Electroporation was carried out with BioRad Gene Pulser Cuvettes with an electrode gap of 4 mm, to which 400 μl of COS-7 or RTG-2 cells with a cell density of 10 x 10^6/ml had been aliquoted with 5 g of receptor plasmid/ml and 45 μg pBluescript SK(+). The settings applied on the BioRad Electroporator II were 260 V, 1000 F and an infinite internal resistance. The cells were left in the electroporator for 1 - 2 min, and were then placed on ice for another 2 min. The cells were subsequently diluted with 12 ml of fresh DMEM nutrient mix F-12 Ham supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 3.7 g/l $NaHCO_3$ containing 2.5 % charcoal-dextran treated fetal calf serum (FCS) and plated in a 6-well plate. Cells were allowed to grow for 36 h before image acquisition using fluorescence microscopy.

Lipofectamine 2000 is a cationic liposome-based reagent that provides high transfection efficiency and high levels of transgene expression in a range of mammalian cell types using a simple protocol.

Method - over night incubation: RTG-2 and COS-7 cells, being in logarithmic growth phase, were plated the evening before transfection, at a density of 4.2×10^6 cells per well of a 6-well plate for RTG-2 and 3×10^6 cells per well of a 6-well plate for COS-7. Two hours prior transfection the medium was removed, cells washed twice with PBS, and

DMEM nutrient mix F-12 Ham without supplements was added. Cells were transfected using Lipofectamine 2000 according the manufactures protocol with 1 μg/6-well plate pEGFP-C1 and 19 μg/6-well plate pBluescript SK(+) and incubated over night with the Lipofectamine/DNA mixture and medium changed the next morning. 36 h after transfection, efficiency was measured using fluorescence microscopy.

Method - 4 h incubation: RTG-2 and COS-7 cells were treated as described above for transfection using Lipofectamine 2000 with over night incubation. However, incubation time with Lipofectime/DNA mixture was reduced to 4 h. 36 h after transfection, efficiency was measured using fluorescence microcopy.

2.2 Mechanistic studies of fish glucocorticoid receptors

In the next step I determined the subcellular localisation of both rainbow trout receptors. For that purpose GFP-tagged fusion proteins were constructed. GFP tagged-rtGR1 and rtGR2 were generated by inserting the cDNAs encoding for rtGR1 and rtGR2 in-frame into the expression plasmid pEGFP-C1 (Clontech). The GFP was linked N-terminally to the receptor cDNAs of rtGR1 and rtGR2. To insert the cDNAs into the GFP expression vector pEGFP-C1, two restriction sites were introduced, flanking the sequence of the receptorc DNAs, by amplification of the cDNA with specific primers, elongated with sequences of restriction sites. The produced PCR fragment was first ligated into pGEM-Teasy and second cloned into the pEGFP-C1. In the upcoming paragraphs I describe the methods needed for the cloning process. This includes first the description of all plasmids used, the primer design, introduction of the restriction site by PCR, ligation with the target plasmid, transformation into competent bacteria and sequencing of the cloning product. All constructed plasmids were sequenced to determine fidelity.

As a control, I also studied the localisation of the previously well characterised GPF-tagged rat GR mutant C565G (termed rGR$_{C656G}$, Htun et al., 1996). The plasmid pcI-nGFP-C656G (Htun et al., 1996, PubMed accession number U53602, kindly provided by Prof. Hager) expresses the rat GR with the C656G mutation from the cytomegalovirus promoter/enhancer, and the GFP cDNA fragment with the S65T mutation. This mutation disrupts the AF2 site causing a receptor that in transcription assays shows a 500 fold higher affinity for dexamethasone than the wild-type rGR (Kucera et al., 2002).

2.2.1 Cloning

Plasmids: The plasmids pCMrtGR1 (Ducouret, 1995) and pCMrtGR2 (Bury 2003) are pMV5 backbone based vectors, containing receptor cDNA for rtGR1 (PubMed

accession number Z54210) and rtGR2 (PubMed accession number AY495372), respectively, and an ampicillin resistance. The plasmid pcI-nGFP-C656G (Htun et al., 1996, PubMed accession number U53602) expresses rat GR with the C656G mutation. The expression vector pEGFP-C1 is commercially available from Biotech/Clontech and encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells.

Primer design: Primers were designed using primer3 (http://frodo.wi.mit.edu/). All primers were elongated by specific restriction enzyme recognition sites plus 4 - 6 additional amino acids to faciliate restriction enzyme binding (Table 2.3 and 2.4). It was important to assure that the correct open reading frame of GFP and receptor genes remained intact. Left primer was designed upstream of the start codon ATG and the right primer annealed on the DNA upstream of the stop codon.

Table 2.3: Strategies for cloning rainbow trout GRs into pEGFP-C1

Gene	Source	Target vector	Primer	Restriction enzymes	Created constructs
rtGR1	pcMV-rtGR1	pEGFP-C1	GR1EcoF GR1EcoR	EcoRI EcoRI	pEGFP-rtGR1
rtGR2	pcMV-rtGR2	pEGFP-C1	GR2EcoF GR2KpnR	EcoRI KpnI	pEGFP-rtGR2
rGR_{C656G}	pcI-nGFP-C656G	pEGFP-C1	rGRiniRER rGRstopREL	BamHI KpnI	pEGFP-rGR_{C656G}

Table 2.4: Primers used for cloning

Primer	sequence
GR1EcoF	ccggaattcaggtggactgaaacacagc
GR1EcoR	cggaattccttaaggcattgtgtcatggtttaag
GR2EcoF	ccggaattccctaatcggggactcagaccc
GR2KpnR	cggggtaccacacagtcatttctggtggaagag
rGRiniRER	ggggatcctcatttttgatgaaacagaagc
rGRstopREL	ggggtaccctggaatccaaagaatcctt

Underlined letters represent the introduced restriction sites

Introduction of restriction sites: For PCR, proof reading polymerase PfuTurbo® (Stratagene) with a low error rate of 1.3×10^{-6} was used. For each reaction, 1.25 U PfuTurbo® (Stratagene), 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$,

1 % Triton X-100, 0.1 mg/ml BSA, 200 μM dNTP mix, 200 nM primer and 500 pg plasmid were mixed in a final volume of 25 μl and incubated at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min for 16 cycles. For specific primer combination see Table 2.3 and 2.4. PCR fragments were analysed by agarose gel electrophoresis and ethidium bromide staining. Single bands, representing the amplified cDNA were cut out, and the DNA eluted from the gel using the GFX PCR DNA and Gel Purification Kit (Amersham Biosciences).

Poly-A overhangs and Cloning into pGEM-Teasy: To insert the PCR fragment into pGEM®-Teasy Vector (Promega, Mannheim, Germany), poly-adenosine overhangs were added. For each reaction 2.5 U Taq Polymerase, 10 mM TRIS-HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.1 % (v/v) Triton-X 100, 400 μM dATP were mixed in a final volume of 12.5 μl and incubated for 15 min at 72°C. PCR fragments with poly-A overhangs were ligated with pGEM®-Teasy Vector system (Promega, Mannheim, Germany), according to manufactures protocol.

Transformation: Chemo-competent cells (E.coli DH5α) were thawed on ice and 10 μl of the ligation reaction was added, reaction mixed and incubated for 60 - 180 min on ice. Bacteria were heat shocked in a 42°C water bath for 90 sec and chilled on ice for 2 min. 1 ml of room temperature bacterial culture medium SOC was added and was incubated for 1 h in at 37°C and agitation at 220 rpm. The transformed bacteria were then pelleted at 6500 rpm for 5 min and resuspended in 600 μl of SOC. Aliquots of 2 x 200 μl were spread on prewarmed Luria Bertani (LB) plates containing 60 μg/ml ampicillin, 20 μl X-Gal (Promega, Mannheim, Germany) and 200 μl 0.1 M IPTG. The plates were incubated over night at 37°C. The next morning, bacteria colonies were selected by blue-white selection.

Colony PCR: White colonies were inoculated into an micro culture of 200 μl LB containing 30 μg/ml ampicillin and incubated for 4 h at 37° and 220 rpm. For the colony PCR, 0.4 U Taq Polymerase, 2 mM $MgCl_2$, 10 mM TRIS-HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.1 % (v/v) Triton-X 100, 160 μM of each dNTP, 300 nM primer and 1 μl of bacterial culture were mixed. The PCR programme was run with an elongated denaturing period of 94°C for 2 min before the cycling started; cycle conditions were 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min for 30 cycles. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Plasmid preparation: If the colony-PCR proofed that the bacterial clones carried the pGEM-Teasy vector with the inserted glucocorticoid receptor cDNA appropiate micro cultures were used to inoculate 5 ml LB medium containing 30 μg/ml ampicillin. Bacteria were incubated over night at 37°C and 220 rpm. Plasmids were isolated by Promega Wizard Plus SV Minipreps DNA Purification System (Promega, Mannheim, Germany). Plasmid concentration was determined by photometrical measurement at 260 nm.

Digestion: Inserted glucocorticoid genes were excised from pGEM-Teasy by digestion with specific restriction enzymes (Table 2.3). Further target vectors were prepared for insertion of glucocorticoid genes by digestion with specific restriction enzymes (Table

2.3). If genes were inserted by a bidirectional cloning, different digestions were either carried out individually over night in the specific restriction enzyme buffer, or as double digestion using the recommended buffer. Reactions were cleaned between steps with GFX PCR DNA and Gel Purification Kit (Amersham Biosciences). Digestions were analysed by agarose gel electrophoresis and ethidium bromide staining. DNA bands representing the insert or digested target vectors were cut out and eluted from the gel.

Ligation into the target plasmid: The ligation of the digested inserts into the prepared target vectors was carried out using T4 DNA Ligase from Promega (Mannheim, Germany) according to manufactures protocol. Vector:insert DNA molar ratios of 1 : 1, 1 : 3 or 3 : 1 were used for ligation reactions.

Ligated expression vectors were inserted into chemo-competent bacterial cells as described above. Bacteria transformed with pEGFP-rtGR1, pEGFP-rtGR2 and pEGFP-rGR$_{C656G}$ were grown on LB supplanted with kanamycine. Plasmids were isolated by plasmid mini preparation (as described above) and tested by asymmetric restriction analysis.

Sequencing: The inserts of GFP-receptor constructs were sequenced by a commercial supplier (MWG DNA Sequencing) to confirm correct insertion and sequence.

The newly constructed expression plasmids coding for one of both trout GRs and the rat GR gene containing the mutation C656G, linked to the gene for green fluorescent protein, were transfected into COS-7 as well as RTG-2 cells. Using either laser scanning microscopy or fluorescence microscopy the subcellular localisation and nuclear transfer of the three receptors were investigated.

2.2.2 Nuclear import studies using COS-7 and RTG-2

Transfection: The day before transfection, cells were seeded into Lab-Tek™ Chamber Slides (Nunc) with 8 chambers with a culture area of 0.8 cm^2 per chamber. COS-7 cells were seeded at an initial plating density of 1×10^5 cells/chamber and RTG-2 cells were seeded at 3×10^5 cells/chamber in 400 µl DMEM containing 4.5 g/l glucose ad 110 mg/l pyruvate supplemented with 2 mM glutamine and 10 % calf serum and cultivated at 37°C in the presence of 5 % CO_2. Two hours prior transfection, the cell layer was washed twice with 400 µl PBS. The cells were then cultured in serum-free and antibiotics-free DMEM nutrient mix F-12 Ham (Sigma-Alrich, Munich Germany), supplemented with 2 mM glutamine and 3.7 g/l $NaHCO_3$. Plasmid DNA (60 ng pEGFP-rtGR1, pEGFPrtGR2 or pEGFP-rGR$_{C656G}$ + 750 ng pBluescript SK(+)/chamber) was transiently transfected into the cells using Lipofectamine 2000. Cells were incubated with the Lipofectamine/DNA mixture for 4 h. After the incubation, 200 µl fresh serum- and antibiotics-free DMEM nutrient mix F-12 Ham was added and cells were cultured for another 16 h. Hormonal withdrawal for 16 h upon transient transfection is known to synchronize cells to G_0-phase allowing for the study of a stably maintained GR pool (Haché et al., 1996; Savory et al., 1999).

Image acquisition: The living cell image acquisition of transiently transfected COS-7 cells was performed in a temperature-controlled chamber at 37°C. The nuclear transfer studies in the fish cell line RTG-2 were carried out at room temperature. For identification of the nuclear position, the chromatin was stained with 100 ng/ml Hoechst 33342 (Sigma-Aldrich (Munich, Germany)). Cells were examined for 30 min with a Leica TCS SP2 confocal microscope using the x20 objective. GFP fluorescence was excited at 488 nm using an argon laser and fluorescence emission was collected between 500 to 560 nm. Of each picture taken, about 20 - 30 cells were analysed and classified into distinct categories. I employed a localisation scoring protocol that was described previously (Sackey et al., 1999, Haché et al., 1999, Savory et al., 1999): C > N for cells showing the fluorescence signal predominantly in the cytoplasm; N = C for cells having equal distribution of fluorescence signal in the cytoplasmic and nuclear compartment; N > C showing the receptor with nuclear-dominant localisation; and N for cells showing the receptor with exclusive nuclear localisation.

2.3 Toxicological analysis of fish glucocorticoid receptors

Because the main function of the glucocorticoid receptor is the regulation of target gene expression, the final method used in this study analysis the ability of both rainbow trout receptors to activate gene transcription of a reporter gene under control of a GR driven promoter.

2.3.1 Transactivation assay using COS-7

COS-7 cells were transiently transfected using the calcium phosphate precipitation method which has been described earlier (Sambrook and Russell 2001, see also 2.3.1). All treatments were performed in duplicates (i.a. two separate wells) and repeated at least three times.

Plasmids: Plasmids used for transactivation assays were the reporter plasmid Luc, which contains the GRE containing mouse mammary tumour virus promoter driving the luciferase gene (MMTV-LUC), and pSVβ (Clontech), a second reporter plasmid containing the β-galactosidase gene under the control of the SV40 promoter and pBluescript SK(+), the plasmids pCMrtGR1 (Ducouret, 1995) and pCMrtGR2 (Bury 2003) are pCMV5 backbone based vectors, containing receptor cDNA for rtGR1 (PubMed accession number Z54210) and rtGR2 (PubMed accession number AY495372).

Transfection: COS-7 cells, being in logarithmic growth phase, were plated the evening before transfection at a density of 25.000 cells per well of a 24-well plate in DMEM containing 4.5 g/l glucose ad 110 mg/l pyruvate supplemented with 10 % FCS, 100 IU penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Two hours prior transfection the medium was removed, cells were washed once with PBS, and the medium

replaced with DMEM nutrient mix F-12 Ham supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 3.7 g/l $NaHCO_3$ containing 2.5 % charcoal stripped FCS. Cells were co-transfected with the following plasmids: 1 μg/24-well plate pCMrtGR1 or pCMrtGR2, respectively, 10 μg/24-well plate pFC31Luc and 2 μg/24-well plate pSVβ. In total, 20 μg plasmid per 24-well plate were used and pBluescript SK(+) was used to fill up the amount of plasmid needed. Sixteen hours after transfection, the cells were washed with PBS and the medium was replaced with medium containing cortisol alone or in the presence of pollutants. The cells were incubated for further 36 h, and then harvested using 75 μl reporter lysis buffer (Promega, Mannheim, Germany) per well of a 24-well plate following the manufacture's instructions.

Reporter Assays: Luciferase activities were determined using 10 μl cell lysate and 50 μl luciferase assay substrate (Promega, Mannheim, Germany) in a micoplate reader (TECAN, Genios Plus). For the measurement of β-galactosidase activity, 100 μl substrate solution (3 mM o-nitrophenyl β-D-galactopyranoside, 0.56 M potassium phosphate, 1 mM $MgCl_2$, 44 mM β-mercaptoethanol) were added to 30 μl cell lysate in a 96-well microplate and incubated for 30 - 60 min at 37°C before absorbance at 405 nm was measured. Luciferase activity was corrected for well-specific transfection efficiencies (as determined by β-galactosidase activity) and then expressed as a percentage of the luciferase activity observed in cells incubated with the highest concentration of cortisol used.

2.3.2 Exposure of COS-7 and RTG-2 cells with pollutants

I studied the impact of a broad set of environmental pollutants, present in the aquatic environment, on different levels of function of rtGR1 and rtGR2. Using the previously described reporter gene assay for testing transactivation of target genes by rtGR1 and rtGR2 (Bury et al., 2003), I used for the first time this experimental setup to investigate pollutants and their impact on transactivation activity of both trout GRs. To select substances which impair clearly the trout GR function I first used one high concentration of all compounds listed in Table 2.5. Table 2.5 gives a short comment on the application or the origin of the particular contaminants, the chemical name and structure and summarized the high and low concentrations used in the experiments. Compounds were dissolved in either DMSO (Merck, Darmstadt) or methanol (Merck, Darmstadt) as stated in Table 2.5). Final concentration of the solvent accounted for 0.1 % (v/v) for all experiments.

Table 2.5: Environmental contaminants tested for impact on rtGR1 and rtGR2 functioning

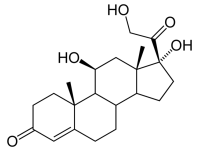
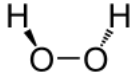
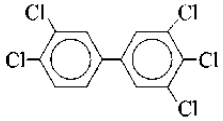
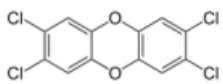
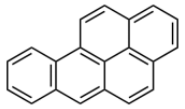
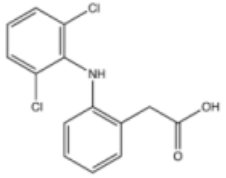
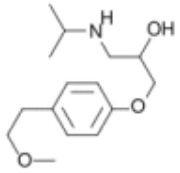
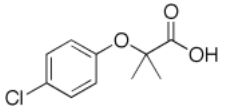
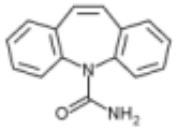
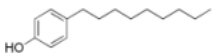
Compound	Scientific name	Chemical structure	comment	High concentration	Low concentration	Solvent	Source
Cortisol	11,17,21-trihydroxy-(11beta)-pregn-4-ene-3,20-dione		Natural ligand of rtGR1 and rtGR2				
H_2O_2	hydrogen peroxide		Positive control	0.5 μM	-	water	Merck (Darmstadt, Germany)
PCB126	3,3',4,4',5-pentachlor-biphenyle		Fire-retardant, plasticiser	100 μM	-	DMSO	
TCDD	2,3,7,8-Tetrachlor-dibenzo-paradioxin		Byproduct of synthesis of organic chlorines	10 nM	-	DMSO	Promochem (Wesel, Germany)
BaP	Benzo[a]pyrene		Product of incomplete combustion	10 nM	-	DMSO	Sigma-Aldrich (Munich, Germany)
Copper			Heavy metal	100 μM	100 nM	water	Sigma-Aldrich (Munich, Germany)
Cadmium			Heavy metal	100 nM	-	water	Sigma-Aldrich (Munich, Germany)

Table 2.5: Environmental contaminants tested for impact on rtGR1 and rtGR2 functioning

Compound	Scientific name	Chemical structure	comment	High concentration	Low concentration	Solvent	Source
Nickel			Heavy metal	100 μM	-	water	Sigma-Aldrich (Munich, Germany)
Diclofenac	2-[2-(2,6-dichlorophenyl) aminophenyl]-ethanoic acid		Analgesics, antipyretic and anti-inflammatory drug	10 μM	100 pM	methanol	ICN (Costa Mesa, USA)
Metoprolol	1-[4-(2-methoxyethyl)-phenoxy]-3-propan-2-ylamino-propan-2-ol		Beta-Blocker	10 μM	-	methanol	ICN (Costa Mesa, USA)
Clofibric acid	2-(4-Chlorophenoxy)-2-methyl-propanoic acid		Blood lipid lowering agent	10 μM	-	methanol	Acros (Geel, Belgium)
Carbamazepin	5H-dibenz-[b,f]azepine-5-carboxamide		Neuroactive drug	10 μM	-	methanol	Acros (Geel, Belgium)
Technical nonylphenol	Mixture of different nonylphenol isomers		Surfactant, detergent and pesticide	20 nM	2 pM	ethanol	Sigma-Aldrich (Munich, Germany)

2.3.3 Statistics

Statistical analysis of data was performed using Statistika (StatSoft). To evaluate the impact of environmental contaminants on the nuclear transfer of rtGR1 and rtGR2 the Kolmogorov-Smirnov test was used. Changes in subcellular localisation in the absence of cortisol of both GFP-tagged trout GRs due to incubation with seleceted contaminants were evaluated using the Chi-square test. Significant differences of transactivation activity of rtGR1 and rtGR2 after incubation of the cells with contaminants and different concentrations of cortisol were calculated using Student t-test. Significant aberrations are marked with asterisks in the graphs or the legends.

Chapter 3

Results

3.1 Screening for a fish cell model and method development

3.1.1 Screening of fish cell lines

Up to date, all data about the action of the two rainbow trout GRs (rtGR1 and rtGR2) were obtained in artificial mammalian cell lines COS-7 and CHO. These cell lines are designed to be easily transfected and, most importantly, do not express endogenous receptors.

One goal of this thesis was to use a rainbow trout cell line to study the rainbow trout GRs in a system that originated from this species. For this purpose, the first objective was to establish a rainbow trout fish cell line model. An ideal cell line would express none or only one of both rainbow trout GRs. Therefore, eight rainbow trout cell lines, derived from various organs, were tested for the presence of both rtGR1 and rtGR2 mRNAs. Results of the screening are summarized in Table 3.1

The general description of the eight fish cell lines showed that all cell lines tested expressed mRNA of both rtGR1 and rtGR2 (Table 3.1). Only the cell line RTG-2 has been described before to possess cortisol binding sites (Lee and Bols, 1989) and the presence of mRNA is a confirmation of this. Because none of the cell lines lacked one or both of rainbow trout GR mRNAs, none was perfect for the transfection studies. To carry out transfection studies with many different treatments a lot of cells would be needed. Therefore I focused on growth rats as the second attribute of choise. Fish cells are cultured at low temperatures of 19°C and flasks are confluent within a week if split at ratios of 1 : 1. Among all fish cell lines tested, RTG-2 possessed the highest growth rate and flasks were confluent within 3 days when split at a ratio of 1 : 1. This

Table 3.1: Presence of rtGR1 and rtGR2 mRNA in diverse fish cell lines

Cell line	origin	rtGR1 mRNA	rtGR2 mRNA
RTL-W1	liver	+	+
RI	liver	+	+
RTH-149	hepatoma	+	+
RTgill	gill	+	+
RTgut	gut	+	+
RTG-2	gonads	+	+
RTbrain	brain	+	+

(+) mRNA was easily discernable by conventional rtPCR and subsequent gel electrophoresis

high growth rate was found as an advantage of the RTG-2 cell line and RTG-2 was finally chosen as the fish cell model for this study.

3.1.2 Development of a transfection technique for RTG-2 and COS-7

After selection of the fish cell line, the transfection method of choice needed to be developed. Two issues were important. First, the selected technique had to achieve a high transfection efficiency in RTG-2. Second, the same technique had to also be suitable for transfection of COS-7. This was particularly important as some transfection techniques can change the outcome of results and comparing data obtained with different cell lines would be questionably if different transfection techniques would be used. Three different transfection techniques were tested; the calcium phosphate precipitation, the electroporation and the liposome based transfection using Lipofectamine 2000.

First, the calcium phosphate precipitation (Sambrook and Russell 2000, Molecular Cloning) was the standard transfection technique used for COS-7. This method did not turn out suitable for transfection of RTG-2 because transfection efficiency was $< 1\,\%$ and salt crystals formed, indicating the reaction of medium compounds with precipitation products while incubating over night. I further adapted the protocol of the calcium phosphate precipitation after Zarafullah et al., (1988). This methodology is performed similar to calcium phosphate precipitation but after 4 h incubation of the cells with the DNA-precipitate cells were shocked with 15 % glycerol. This technique has been described for transient transfection of the fish cell line RTH-149 (Zarafullah et al., 1988). However, transfection efficiency remained low and only 2 % of the total cells were transfected.

I then tested electroporation for transfection of RTG-2 cells. The transfection efficiency was about 30 % and no cytotoxic effects of the treatment were observed for RTG-2 cells.

In contrast, using electroporation for the transfection of COS-7 cells turned out to be cytotoxic to COS-7 cells. For that reason electroporation was not used for transfection in the presented study.

I continued testing the commercially available liposome based transfection reagent Lipofectamine 2000. If cells were incubated over night with Lipofectamine 2000, as stated in the manufactures protocol, RTG-2 cells had dislodged from the growth surface. I then tested the incubation of the cells with the Lipofectamine/DNA complex for 4 h. None of the cells dislodged after the treatment and about 30 % of the cells were transfected. In addition COS-7 cells are easy to transfect using Lipofectamine 2000. About 30 % of the cells were transfected and no cytotoxic effects were observed, using the same 4 h incubation period.

3.2 Mechanistic studies of fish glucocorticoid receptors

3.2.1 Cellular localisation of GFP-rtGR1 and GFP-rtGR2

To examine the subcellular localisation and dynamics of nuclear translocation of the rainbow trout glucocorticoid receptors, rtGR1 and rtGR2, GFP-tagged versions of these receptors were constructed. Before using these to study nuclear import, the expression and functional integrity of the GFP-fusion proteins were examined. First, the expression levels of GFP-tagged rainbow trout GRs in transiently transfected COS-7 cells were compared. Immunoblots showed single bands at expected sizes (Fig 3.1A) and GFP-tagged proteins were expressed at similar levels. Second, the functional integrity of both trout glucocorticoid receptor GFP fusion proteins were examined using transactivation assays. Both trout GRs induced transactivation of MMTV-Luc reporter gene and the two chimeras possessed the same sensitivity and followed the same kinetics of induction than known for the wild type receptors (Figure 3.1 B).

After verifying the proper function of both trout GRs, I quantified trout GR localisation by confocal laser scanning microscopy. I observed that the majority of COS-7 cells transfected with GFP-rGR$_{C656G}$ and without hormone treatment (termed naïve) showed a predominantly cytoplasmic receptor distribution (C > N 64 % ± 7 %) with the remaining cells showing an about equal distribution of fluorescence between the cytoplasm and nucleus (N = C 36 % ± 7 %; Figure 3.2 A). A comparable distribution pattern for wildtype GFP- tagged rGR$_{C656G}$ using this transfection technique has been described (N > C 28 % ± 9 %; C = N 18 % ± 3 %; Savory et al., 1999). This pattern differs greatly from that observed for the trout receptors. About half of the naïve GFP-rtGR1 expressed in COS-7 cells was equally distributed between the nucleus and cytoplasm (C = N 51 % ± 2 %). The other half was predominantly located in the nucleus (N > C 48 % ± 1 %; Figure 3.2 B) and when expressed in RTG-2 cells GFP-rtGR1 was mainly equally distributed between cytoplasm and nucleus (C = N 88 % ± 8 %;

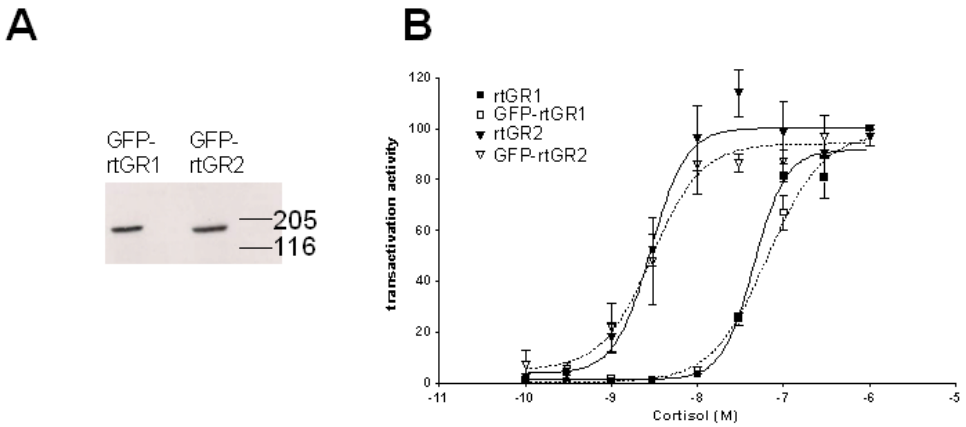


Figure 3.1: Characterisation of expression levels and function of the GFP-tagged rtGR1 and rtGR2. (A) Western Blot analysis of the levels of transiently transfected GFP-tagged trout glucocorticoid receptors. Specific bands were stained using anti-GFP antibody JL-8. Picture shown by courtesy of Dr. Armin Sturm (B) Transcriptional activation of MMTV-luciferase reporter gene in COS-7 cells co-transfected with either rtGR1 (*filled square*), GFP-rtGR1 (*open square*), rtGR2 (*filled triangle*) or GFP-rtGR2 (*open triangle*) expression vectors, as well as pSVβ plasmid. Luciferase activity was measured 24 h following treatment with varying concentrations of cortisol. Transactivation activity was normalized to the internal β-galactosidase control, and expressed as a percent of the respective construct activity measured in the presence of 10 μM cortisol. Values represent means $\pm$ S.E.M. of three independent experiments, with each experiment performed in duplicates.

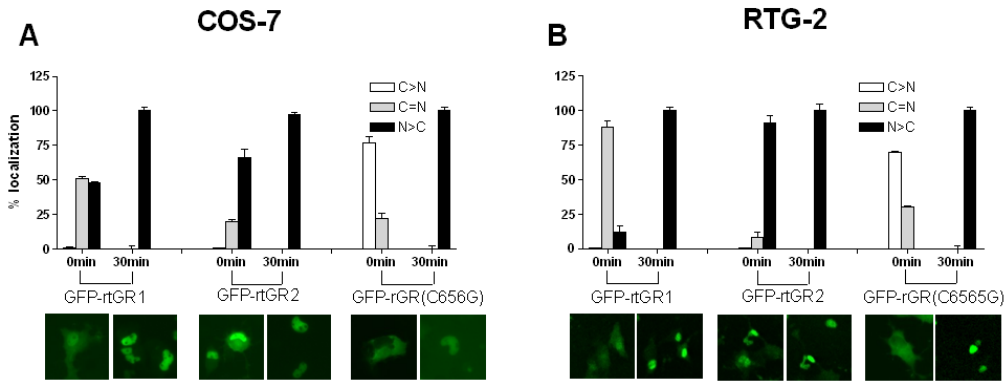


Figure 3.2: Subcellular localisation of GFP-tagged rtGR1, rtGR2, and rat GR$_{C656G}$ before and after a 30 min treatment with 1 μM cortisol. GFP-tagged receptors were transiently transfected in (A) COS-7 and (B) RTG-2 cells using Lipofectamine 2000. Localisation was determined by fluorescence microscopy. All quantifications are compilations of at least three independent experiments. Error bars indicate means $\pm$ S.E.M. Representative micrographs are shown beneath each data set. *N*, nucleus; *C*, cytoplasm.

N > C 12 % ± 8 %; Figure 3.2B). Naïve GFP-rtGR2 was predominantly located in the nucleus in COS-7 (N > C 66 % ± 10 %; C = N 20 % ± 3 %; Figure 3.2A), which was even more evident in RTG-2 cells (N > C 91 % ± 9 %; C = N 8 % ± 7 %; Figure 3.2B). Upon steroid treatment (1 μM cortisol for 30 min) the rat and both trout receptors transferred completely to the nucleus (Figure 3.2 A and 3.2 B).

Previous studies have reported that transient expression of GFP-tagged rat GR resulted in levels of expression at which receptors partially localized to the nucleus prior to exposure to ligand (Sanchez 1990, Martins et al., 1991, Savory et al., 1999). This was a result of that the GFP-tagged rat GR was over expressed in COS-7 cells by the use of Lipofectamine-mediated transfection (Savory et al., 1999). Additionally the rGR$_{C656G}$ expression plasmid replicated from SV40 replication origins which led to strong overexpression when expressed in COS-7 cells because COS-7 carries the SV40 antigen (Sanchez 1990, Martins et al., 1991, Savory et al., 1999). To verify that the unusual distribution of the trout receptors was not due to the COS-7/Lipofetamine system I also transfected the rainbow trout gonad cell line RTG-2 with the rat GR. It is noteworthy that RTG-2 does not carry the SV40 antigen and artificially enhanced expression of transiently transfected receptor is not expected. RTG-2 is reported to harbour a single population of high affinity cortisol binding sites (Lee and Bols, 1989). I was not able to distinguish between both trout GR isoforms by immunoblotting. However, results from RT-PCR indicate presence of mRNA of both isoforms, rtGR1 and rtGR2 (Table 3.1). GFP-rGR$_{C656G}$ expressed in RTG-2 cells exhibited the same pattern of distribution as that observed in COS-7 cells (Figure 3.2). GFP-rtGR1 transiently transfected in RTG-2 cells localizes predominantly in both cytoplasm and nucleus (C = N 88 %) with only a small proportion of the cells exhibiting clear nuclear distribution (N > C 15 %; Figure 3.2). In contrast GFP-rtGR2 fusion protein was localised predominantly nuclear (N > C 91 %; Figure 3.2).

3.2.2 Time and dose-dependent pattern of GFP-rtGR1 and GFP-rtGR2 nuclear transfer

The main difference in the functional characteristics between the two trout GR isoforms is their differential transactivational sensitivity to cortisol (Bury et al., 2003). The transactivational potential of the GR is related to the translocation of the receptor to the nucleus following hormonal treatment (Shank and Paschal et al., 2005; Savory et al., 1999). Therefore I evaluated the dose-dependent and temporal kinetics of the nuclear uptake of the remaining cytoplasmic GFP-rtGR1 and GFP-rtGR2 upon treatment with the natural ligand cortisol.

Previous studies showed that wild type rGR, tagged with GFP, transfers very rapidly into the nucleus upon treatment with the synthetic glucocorticoid dexamethasone (Sackey et al., 1996). The high uptake rate upon steroid treatment of rat GR has been determined to be $t_{1/2} = 4$ - 5 min (Sackey et al., 1996) and nuclear transfer is completed within 30 min (Sackey et al., 1996, Haché et al., 1998). For this reason I choose

an observation time of 30 min for studying the nuclear translocation of trout GRs. To provide a clear representation of the results, the percentage of cells with totally nuclear GFP-GR [N] or mostly nuclear GFP-GR [N > C] are displayed.

Nuclear translocation of the remaining cytoplasmatic GFP-rtGR1, transiently expressed in COS-7 cells, occurred in a dose dependent manner in the concentration range of cortisol (1 nM to 1 μM) (Figure 3.3 A). Complete nuclear transfer occurred after 5 min in the presence of 1 μM cortisol, whereas treatment with 1 nM cortisol resulted in only 60 % of the cells exhibiting GFP-rtGR1 fluorescence signal in the nucleus after 30 min of treatment (Figure 3.3 A). The remaining cytoplasmic GFP-rtGR2 fusion proteins, expressed in COS-7, transferred more efficiently from the cytoplasm to the nucleus at low cortisol concentrations compared to GFP-rtGR1 translocation (Figure 3.3 B), and 1 nM cortisol induced nuclear transfer with the same efficiency as 1 μM, with nuclear transfer being completed within 5 min upon hormone addition (Figure 3.3 B). Similar results are seen when expressed as the % of cells with a complete nuclear expression [N]. Again, rtGR2 nuclear transfer at 1 nM cortisol was very efficient compared to rtGR1 (Figure 3.3).

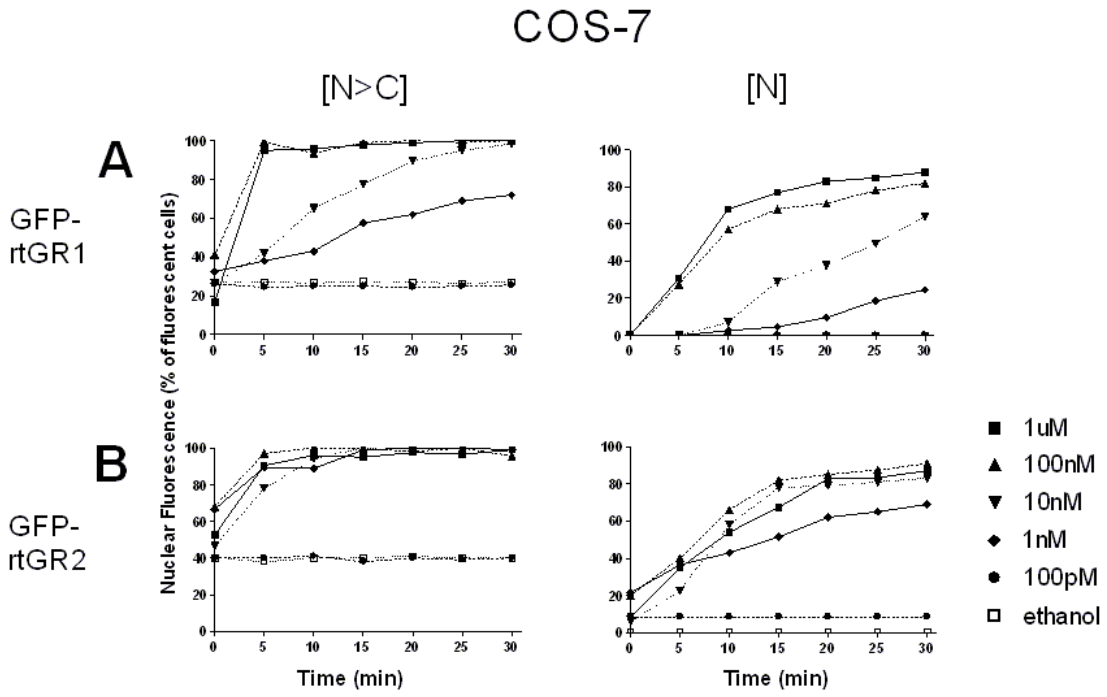


Figure 3.3: Kinetics of (A) GFP-rtR1 and (B) GFP-rtGR2 nuclear transfer transiently expressed in COS-7. Cells were transiently transfected in medium without steroid supplement using Lipofectamine 2000, and cultured for another 16 h in serum free medium prior to transfer studies. Image acquisition was performed in a temperature-controlled chamber at 37°C. Nuclear transfer of the receptors was induced upon addition of 1 μM (*filled square*), 100 nM (*triangle up*), 10 nM (*triangle down*), 1 nM (*diamond*), 100 pM (*dot*) cortisol, and the solvent ethanol (*open square*). For 30 min, changes in subcellular distribution were evaluated by confocal laser scanning microscopy. Of each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. Each data point represents a compilation of three independent experiments. *N*, nucleus; *C*, cytoplasm.

In RTG-2, transiently transfected with GFP-rtGR1, a similar dose dependent translocation of GFP-rtGR1 was observed (Figure 3.4 A). However, there appeared to be a difference between the two cell lines, with the nuclear localisation response to cortisol being less sensitive in the rainbow trout gonad cell line. No response to cortisol was seen in transfected RTG-2 cells at 1 nM cortisol which was in contrast to findings in COS-7. One explanation could be that the RTG-2 cells possess an endogenous GR population, as described by Lee and Bols (1989), which masks the answer of the GFP-tagged receptors. RTG-2 cells transfected with GFP-rtGR2 showed a mostly nuclear distribution in the absence of hormone (N > C 91 %; Figure 3.4B). On addition of hormone, a greater proportion of cells demonstrated complete nuclear distribution. This temporal pattern of complete nuclear localisation was dose dependent with concentrations above 10 nM cortisol causing 85 - 100 % nuclear distribution by 15 min of exposure (Figure 3.4 B).

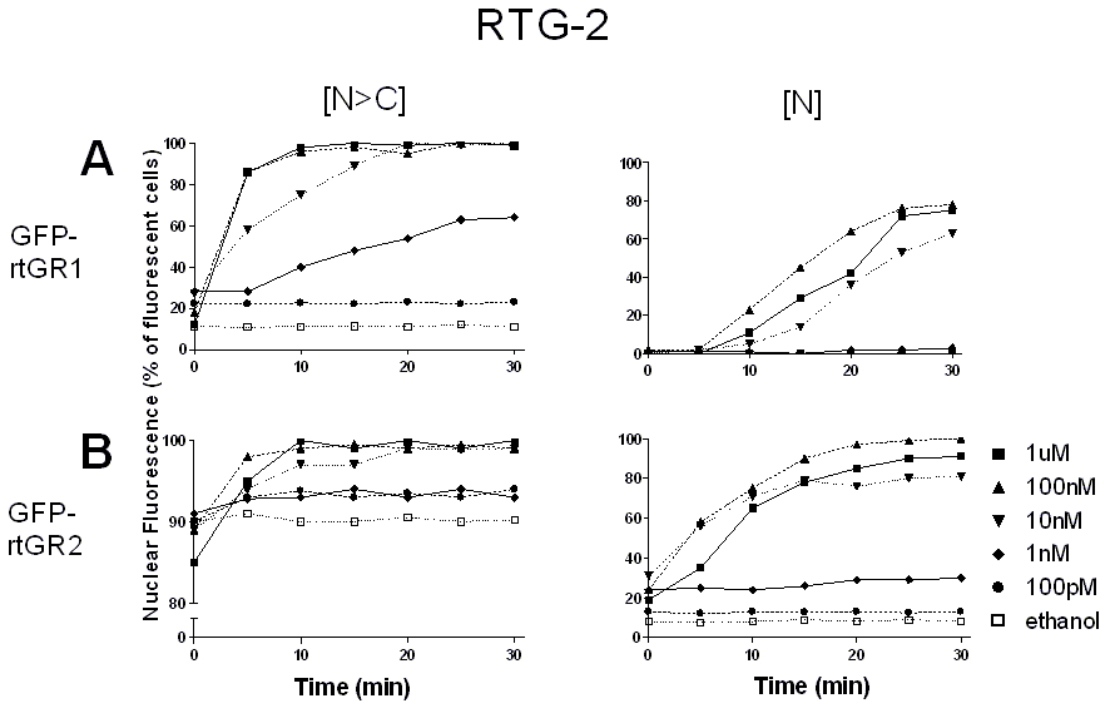


Figure 3.4: Kinetics of (A) GFP-rtGR1 and (B) GFP-rtGR2 nuclear transfer transiently expressed in RTG-2 cells. Cells were transiently transfected in medium without steroid supplement using Lipofectamine 2000, and cultured for another 16 h in serum-free medium prior to transfer studies. Image acquisition was performed at room temperature. Nuclear transfer of the receptors was induced upon addition of 1 μM (*filled square*), 100 nM (*triangle up*), 10 nM (*triangle down*), 1 nM (*diamond*), 100 pM (*dot*) cortisol, and the solvent ethanol (*open square*). For 30 min, changes in subcellular distribution were evaluated by confocal laser scanning microscopy. For each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. Each data point represents a compilation of three independent experiments. *N*, nucleus; *C*, cytoplasm

3.2.3 Ligand specificity of GFP-rtGR1 and GFP-rtGR2

In addition to cortisol, I employed treatment with dexamethasone, RU486, aldosterone, and progesterone to examine ligand specificity of GFPrtGR1 and GFP-rtGR2 transiently expressed in COS-7 cells (Figure 3.5) and RTG-2 cells (Figure 3.6). The synthetic glucocorticoid dexamethasone is a very potent inducer of nuclear transfer of GFP-rtGR1 and GFP-rtGR2 expressed in both cell lines. Progesterone, a weak agonist of the mammalian GR (Htun et al., 1995), did not induce nuclear transfer of GFP-rtGR1 in COS-7 but was able to induce nuclear import of GFP-rtGR2 with a delay of 15 min and only 50 % of the cells exhibiting the receptor in the nucleus after 30 min. Aldosterone, the natural ligand of the mineralocorticoid receptor did not induce GFP-rtGR1 nuclear transfer but induced very efficiently the transfer of GFP-rtGR2 into the nucleus. The synthetic steroid RU486 induced slower initial nuclear

transfer of GFP-rtGR1. In contrast, GFP- rtGR2 nuclear transfer was induced very efficiently, possessing dynamic rates comparable to cytoplasmic-nuclear transport of GFP-rtGR1 induced by 1 μM cortisol. These results suggest that rtGR2 nuclear transfer is stimulated more efficiently than that of rtGR1 by other natural corticosteroids and progestins as well as synthetic glucocorticoids and the mammalian GR antagonist RU486.

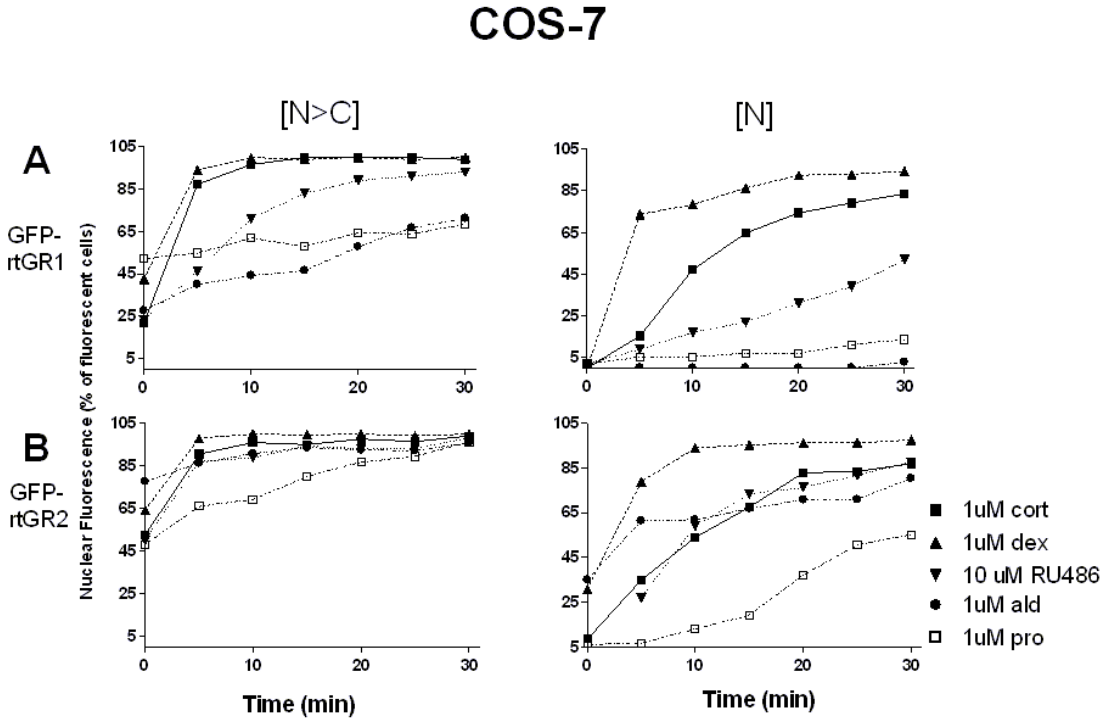


Figure 3.5: Ligand specificity of (A) GFP-rtGR1 and (B) GFP-rtGR2 transiently expressed in COS-7. Cells were transiently transfected in medium without serum and cultured for additional 16 h in steroid-free medium and then treated with 1 μM cortisol (filled square), 1 μM dexamethason (*triangle up*), 10 μM RU486 (*triangle down*), 1 μM aldosteron (*dot*), and 1 μM progesterone (*open square*). Image acquisition was performed in a temperature-controlled chamber at 37°C. For 30 min, changes in subcellular distribution were evaluated by confocal laser scanning microscopy. For each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. Each data point represents a compilation of three independent experiments. *N*, nucleus; *C*, cytoplasm.

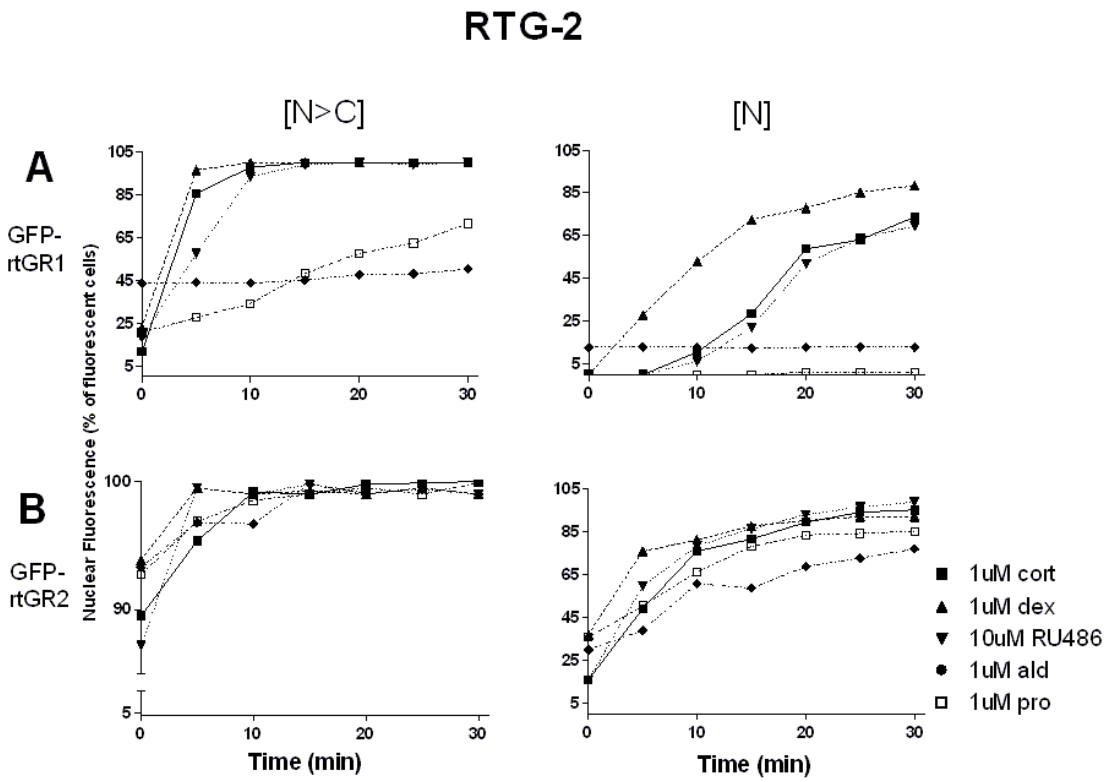


Figure 3.6: Ligand specifity of (A) GFP-rtGR1 and (B) GFP-rtGR2 transiently expressed in RTG-2. Cells were transiently transfected in medium without serum and cultured for additional 16 h in steroid-free medium and then treated with 1 μM cortisol (*filled square*), 1 μM dexamethason (*triangle up*), 10 μM RU486 (*triangle down*), 1 μM aldosteron (*dot*), and 1 μM progesterone (*open square*). Image acquisition was performed at room temperature. For 30 min changes in subcellular distribution were evaluated by confocal laser scanning microscopy. For each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. Each data point represents a compilation of three independent experiments. *N*, nucleus; *C*, cytoplasm.

Because the potential of progesterone to activate rainbow trout GR-mediated transcription had not been described, I tested progesterone in transactivation assays. Progesterone was unable to induce transactivation activity for of rtGRs (Figure 3.7).

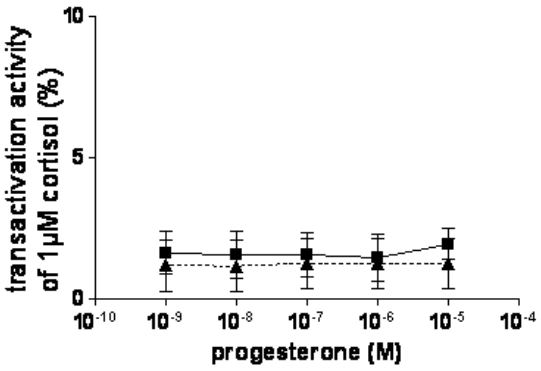


Figure 3.7: Transactivation activity of rtGR1 and rtGR2 in the presence of progesterone. COS-7 cells were co-transfected with either rtGR1 (*squares*) or rtGR2 (*triangle*) expression vectors, as well as the reporter plasmid pFC31Luc, and the pSVβ plasmid which expresses β-galactosidase. After transfection, cells were treated with varying concentrations of progesterone. Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control, and expressed as a percent of the rtGR1 or rtGR2 construct activities measured in the presence of 10 μM cortisol. Values represent means $\pm$ S.E.M. of three independent experiments, with each experiment performed in duplicate. No significant differences from 1 nM progesterone were calculated using student t-test.

3.2.4 Domain swap mutants of fish GR and rat GR

To determine which region(s) of the trout receptors caused the unexpected nuclear distribution pattern of naïve GRs, a number of chimers containing domain swaps of rtGR1 or rtGR2 and rGR$_{C656G}$ tagged to GFP were generated[1]. The aim was to introduce certain regions of the trout receptor into the well studied context of the rat GR and their distribution pattern in the absence of hormone following transient transfection of COS-7 (Figure 3.7) and RTG-2 (Figure 3.8) cells was investigated.

When the E-domain of trout rtGR1 or rtGR2 was introduced into the context of rGR$_{C656G}$, all chimeras constructed ([ABCD(rat)E(GR1/GR2)], [AB(rat)CDE(GR1/-GR2)]) demonstrated a distribution pattern reminiscent of rGR$_{C656G}$ when expressed in COS-7 and RTG-2 cells.

Chimeras containing the C- and D-domain of either rtGR1 or rtGR2 linked to the A/B and E domain of rat GR ([AB(rat)CD(GR1/GR2)E(rat)]) showed a greater nuclear distribution if compared to rGR$_{C656G}$, but the nuclear distribution was not as distinct as wildtype rtGR1 and rtGR2.

The AB domain of trout GRs had the greatest influence on the nuclear distribution of the receptor. Chimers where the A/B domain where replaced with either trout GR

[1]Domain swap mutants were constructed by Dr. Armin Sturm

A/B domain ([AB(GR1/GR2)CDE(rat)]) were found to be shifted towards the nuclear distribution in the absence of hormone in COS-7 and RTG-2 cells.

However, the chimeric constructs that mimicked the wildtype rtGR distribution pattern contained both the A/B-, as well as the C- and D-domains ([ABCD(GR1/GR2)E(rat)]) suggesting a degree of co-operation between the A/B-domain and C- and D-domain is needed to determine nuclear location of the naïve trout GR.

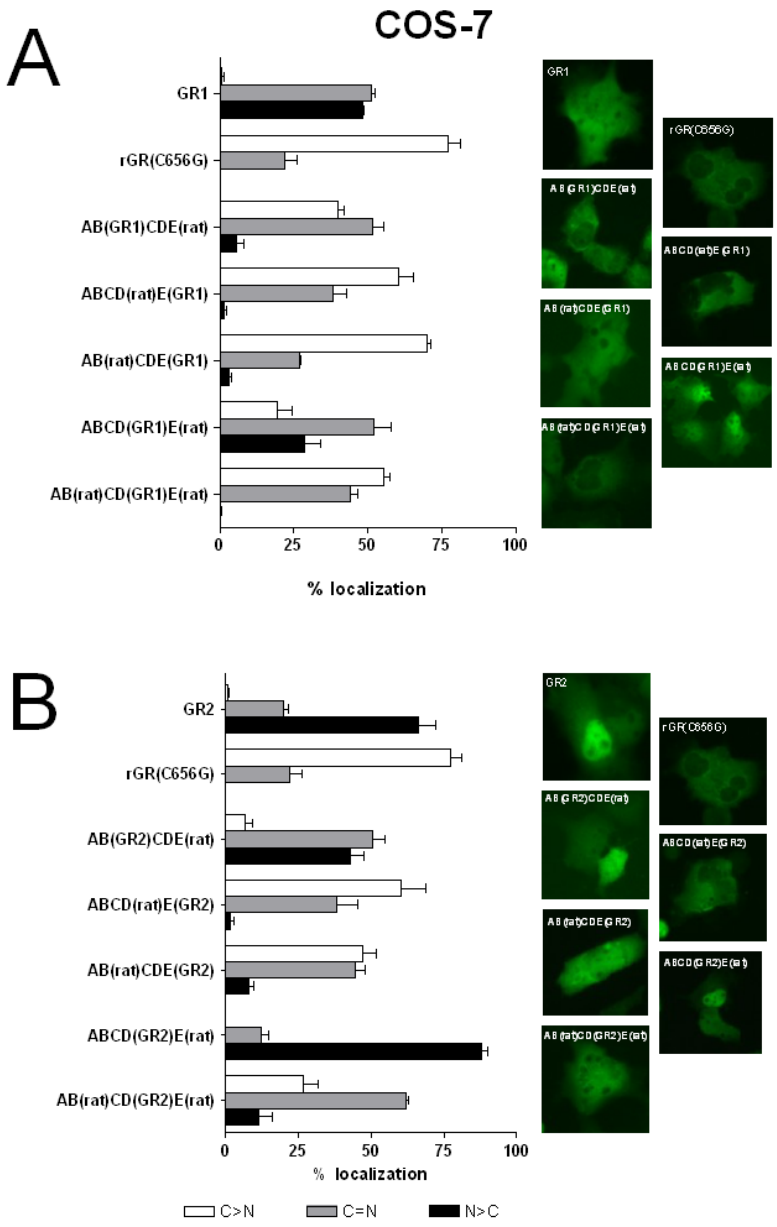


Figure 3.8: Nuclear localisation of naïve receptors can be linked to certain regions of the trout GRs. Domain-swap mutants were created introducing the A/B, CD or E domain of rtGR1 or rtGR2 into the context of the rGR$_{C656G}$ and tagged with GFP. Chimeras of rGR$_{C656G}$ and (A) rtGR1 or (B) GR2 were transiently transfected using Lipofectamine 2000 in the absence of steroids into COS-7 cells. Subcellular localisation of receptor mutants was determined using fluorescence microscopy. Of each field of view 40 - 60 cells were evaluated for subcellular distribution. All quantifications are compilations of at least three independent experiments. Error bars indicate means ± S.E.M. Representative fluorescence images are shown beside each data set. *N*, nucleus; *C*, cytoplasm.

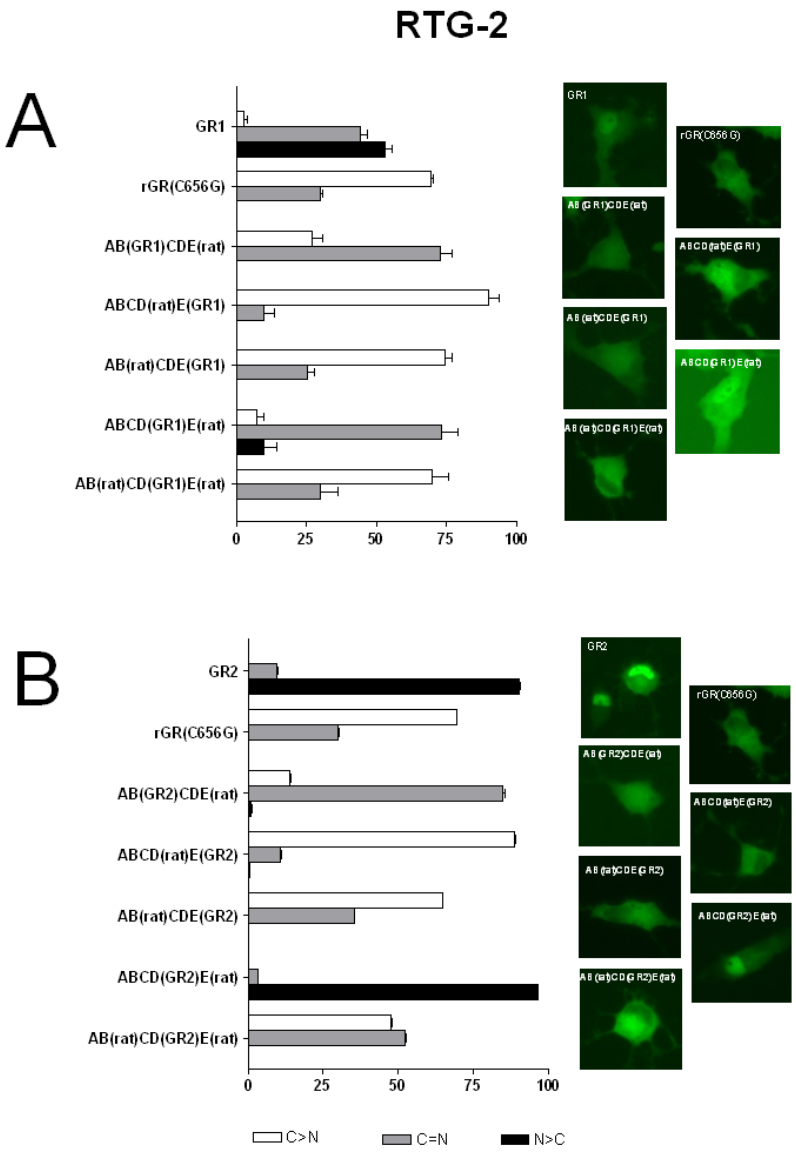


Figure 3.9: Nuclear localisation of naïve receptors can be linked to certain regions of trout GRs. Domain-swap mutants were created introducing the A/B, CD or E domain of rtGR1 or rtGR2 into the context of rGR_{C656G} and tagged with GFP. Chimeras of rGR_{C656G} and (A) rtGR1 or (B) GR2 were transiently transfected using Lipofectamine 2000 in the absence of steroids into RTG-2 cells. Subcellular localisation of receptor mutants was determined using fluorescence microscopy. Of each field of view 40 - 60 cells were evaluated for subcellular distribution. All quantifications are compilations of at least three independent experiments. Error bars indicate means ± S.E.M. Representative fluorescence images are shown beside each data set. *N*, nucleus; *C*, cytoplasm.

3.3 Toxicological analysis of fish glucocorticoid receptors

3.3.1 Screening for environmental contaminants impairing rtGR1 and rtGR2 action

After detailed description of subcellular localisation of naïve trout GRs and determining temporal and dose dependent pattern of nuclear transfer, I evaluated the possibility of environmental contaminants to impair the function of trout GRs *in vitro*.

A broad set of contaminants found in the aquatic environment, representing model substances of the different classes of environmental pollutants, were investigated. PCB126 and TCDD, the metals copper, cadmium, and nickel, as well as the benzo[a]pyrene and the pharmaceuticals, diclofenac, metoprolol, clofibric acid and carbamazepin have been included in the investigations. Hydrogen peroxide (H_2O_2) was used as a positive control for impact on fish GR because of the known impairment of human GR nuclear translocation (Okamoto et al., 1999). All substances were screened first to select pollutants with a high impact on trout GR function. Results are summarized in Table 3.2. Concentrations used of the respective substance are summarized in Table 2.5.

Table 3.2: Impact of xenobiotics on rtGR1 and rtGR2 action

	contaminant	Nuclear import		Transactivation	
		rtGR1	rtGR2	rtGR1	rtGR2
	H_2O_2	⇓	⇓	⇓	⇓
	PCB126	-	-	-	-
	TCDD	-	-	-	-
	BaP	-	-	n.d.	n.d.
metals	Copper	⇓	⇓	-	-
	Cadmium	⇓	⇓	⇓	⇓
	Nickel	n.d.	n.d.	-	-
Pharmaceuticals	Diclofenac	⇓	⇓	⇓	⇓
	Metoprolol	n.d.	n.d.	-	-
	Clofibric acid	n.d.	n.d.	-	-
	Carbamazepin	n.d.	n.d.	-	-
	Nonylphenol	⇑	⇑	⇑	⇑

(-) no change compared to control; (⇑) induction;
(⇓) repression; (n.d.) not determined

Of all substances tested, diclofenac, copper, cadmium, nonylphenol and, as expected, the positive control H_2O_2 had a clear impact on trout GR transactivation activity. These contaminants were studied in more detail for their impact on three levels of GR action. First the potency of the selected contaminants to alter the subcellular localisation was investigated. Second, I observed the impact of the selected pollutants to interfere with the nuclear import kinetics. Third, how selected contaminants impair the level of transactivation of target genes.

Using the previously described reporter gene assay for testing transactivation of target genes by rtGR1 and rtGR2, this experimental setup was used for the first time to investigate pollutants and their impact on transactivation activity of both trout GRs. Transactivation experiments were only carried out in COS-7 cells because the fish cell line RTG-2 proved not suitable to study transactivation, probably because of presence of endogenous receptors (Lee and Bols, 1989 and this study).

3.3.2 Alteration of subcellular localisation of GFP-rtGR1 and GFP-rtGR2 by selected environmental contaminants in the absence of cortisol

The potential of diclofenac, nonylphenol, copper, cadmium, as well as the positive control hydrogen peroxide (H_2O_2) to alter subcellular localisation of naïve rainbow trout GRs was investigated. COS-7 cells were transiently transfected with either GFP-rtGR1 (Figure 3.10) or GFP-rtGR2 (Figure 3.11). The subcellular localization of GFP tagged receptors was first described in 3 categories (C > N, C = N, N > C) as in the studies on naïve and cortisol activated rtGRs (see left panel in Figure 3.10 and 3.11). Additionally, the subcellular localization was described in 4 categories (C > N, C = N, N > C, N)for better representation of small changes in the nuclear localization of naïve receptors (see right panels of Figure 3.10 and 3.11). As shown in Figure 3.10, GFP-rtGR1 was partially but significantly shifted towards the nuclear component after incubation of the transfected cells for 24 h with high and low concentrations of diclofenac, the high concentration of copper and the high concentration of nonylphenol. The compounds cadmium and H_2O_2 had no influence on subcellular localisation of unliganded receptor compared to control. GFP-rtGR2 was significantly shifted towards the nucleus after incubation with both concentrations of diclofenac and nonylphenol. Cadmium and copper had no influence on GFP-rtGR2 subcellular localisation (Figure 3.11). In contrast to COS-7 expressing GFP-tagged rtGR1, cells expressing GFP-rtGR2 cells had detached after treatment with 0.5 μM H_2O_2 for 24 h, so that the effect of this compound on GFP-rtGR2 localisation could not be assessed in this cellular system (Figure 3.11).

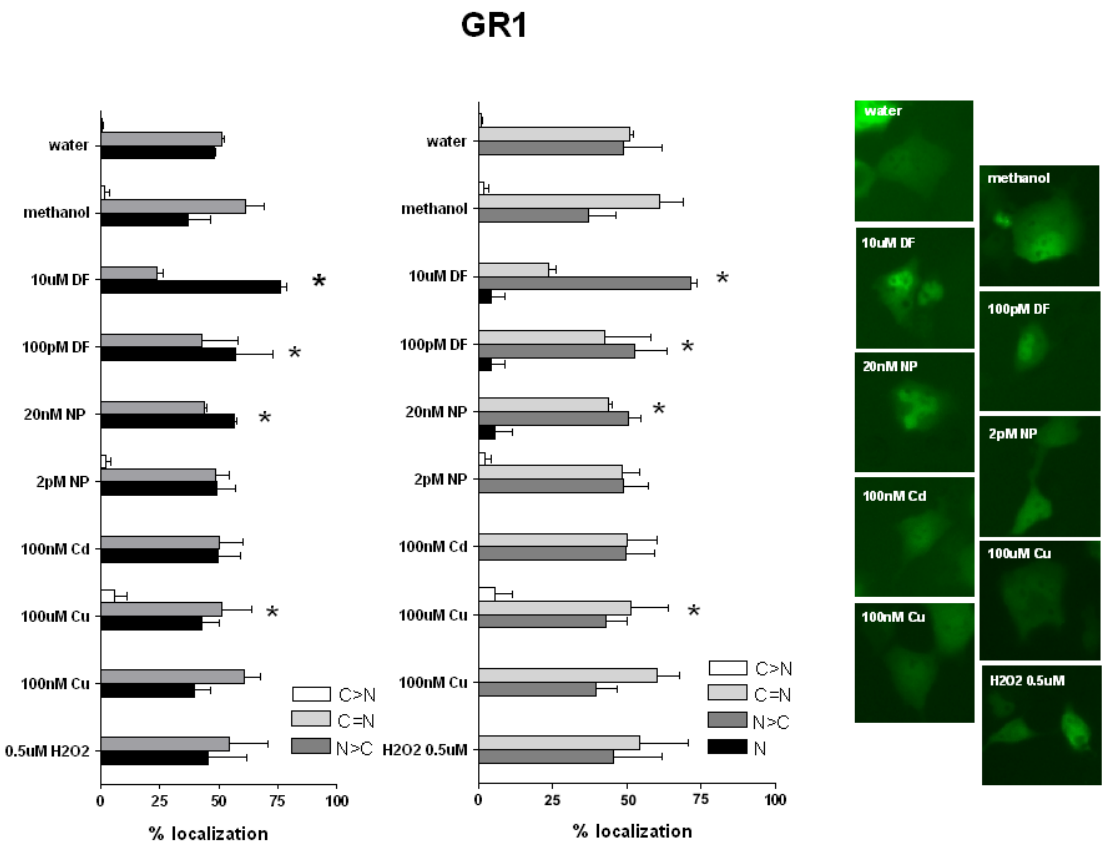


Figure 3.10: Influence of environmental contaminants on GFP-rtGR1 subcellular localisation in the absence of cortisol. Trout rtGR1 tagged with GFP was transiently transfected using Lipofectamine 2000 in the absence of steroids into COS-7. 16 h after transfection cells were treated with contaminants for 24 h. Subcellular localisation of GFP-rtGR1 was determined using fluorescence microscopy. Of each field of view 40 - 60 cells were evaluated for subcellular distribution. All quantifications are compilations of at least two independent experiments. Error bars indicate means ± S.E.M. Asterisks indicate significant differences from control calculated using Chi-square test. Representative fluorescence images are shown beside each bar graph set. *N*, nucleus; *C*, cytoplasm; DF, diclofenac; NP, nonylphenol; Cd, cadmium; Cu, copper.

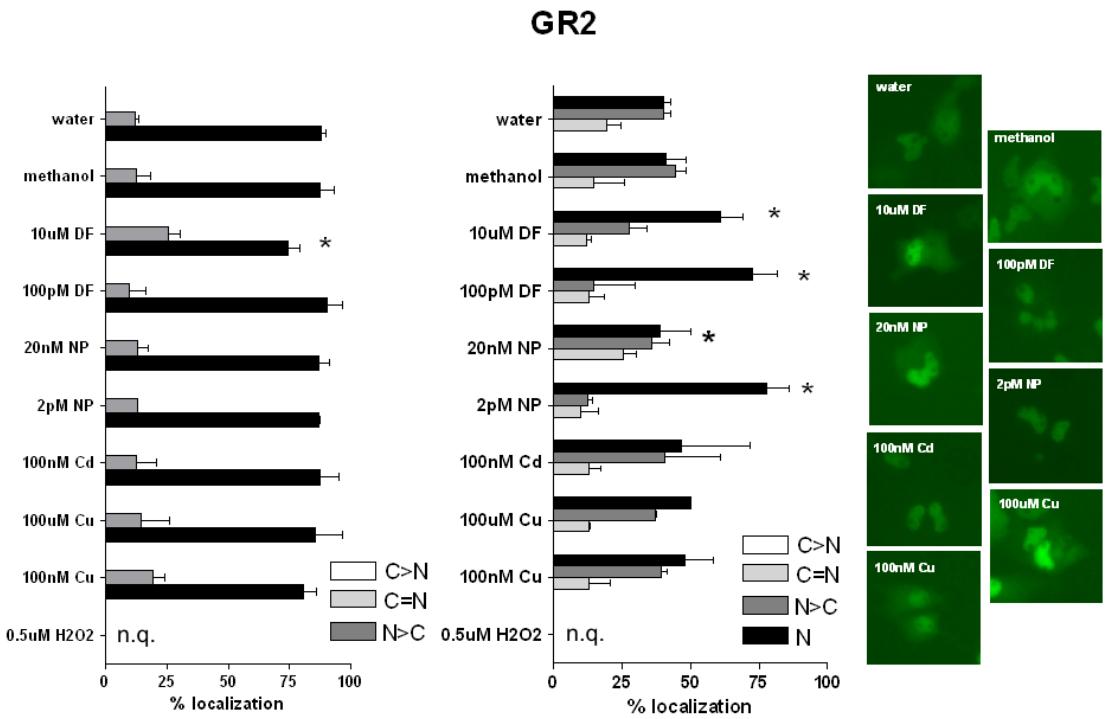


Figure 3.11: Influence of environmental contaminants on GFP-rtGR2 subcellular localisation in the absence of cortisol. Trout rtGR2 tagged with GFP was transiently transfected using Lipofectamine 2000 in the absence of steroids into COS-7. 16 h after transfection cells were treated with contaminants for 24 h. Subcellular localisation of GFP-rtGR2 was determined using fluorescence microscopy. Of each field of view 40 - 60 cells were evaluated for subcellular distribution. All quantifications are compilations of at least two independent experiments. Error bars indicate means ± S.E.M. Asterisks indicate significant differences from control calculated using Chi-square test. Representative fluorescence images are shown beside each bar graph set. *N*, nucleus; *C*, cytoplasm; DF, diclofenac; NP, nonylphenol; Cd, cadmium; Cu, copper; n.q., not quantified.

3.3.3 Kinetics of GFP-rtGR1 and GFP-rtGR2 nuclear transfer in the presence by selected environmental contaminants

I subsequently investigated the ability of diclofenac, nonylphenol, copper and cadmium to interfere with ligand-dependent nuclear translocation of both rainbow trout glucocorticoid receptors. Figure 3.12 shows that GFP-rtGR1 nuclear transfer was enhanced in the presence of the low concentration of diclofenac (Figure 3.12 A) as well as both concentrations of nonylphenol (Figure 3.12 B). Both concentrations of copper impaired nuclear translocation of GFP-rtGR1 (Figure 3.12 C). However, 30 min after cortisol addition a similar number of cells expressed the GFP-tagged receptor in the nucleus compared to control cells. Cadmium had no effect on nuclear translocation of

GFP-rtGR1 (Figure 3.12 D). Ligand-dependent nuclear translocation of GFP-rtGR1 was markedly delayed upon the addition of H_2O_2 (Figure 3.12 D).

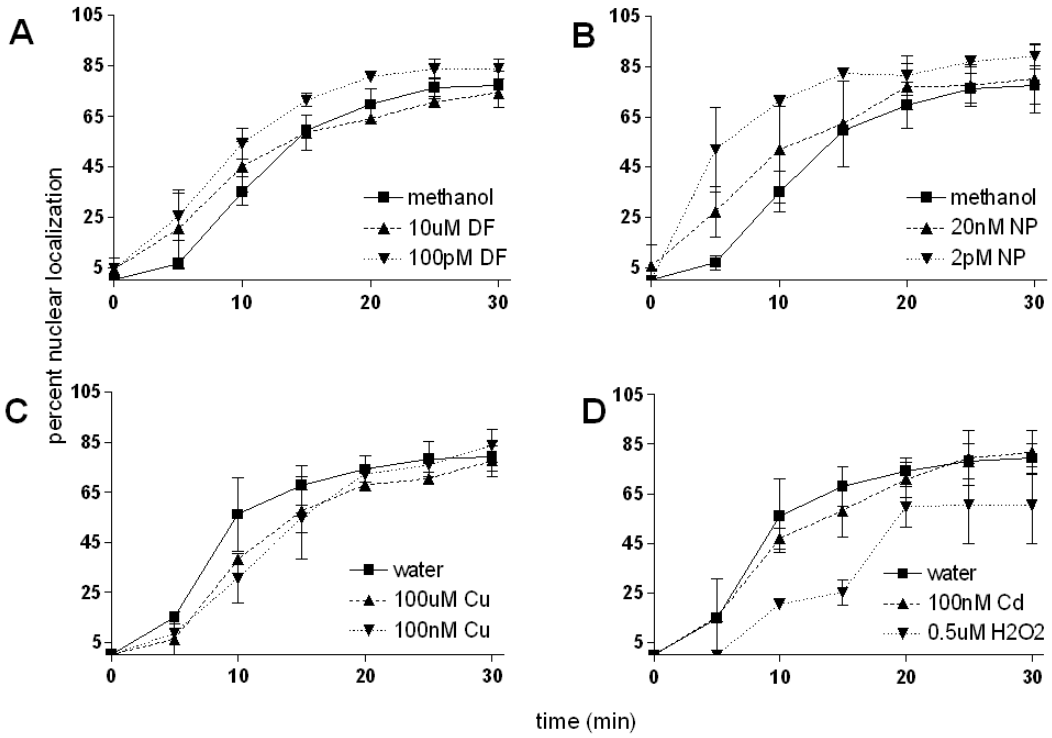


Figure 3.12: Kinetics of GFP-rtGR1 nuclear transfer is altered in the presence of contaminants. Cells were transiently transfected with GFP-rtGR1 in medium without steroid supplement using Lipofectamine 2000, and cultured for another 16 h in medium without serum supplement. Cells were then treated for 24 h with (A) diclofenac, (B) nonylphenol (C) copper, and (D) cadmium and H_2O_2. Image acquisition was performed in a temperature-controlled chamber at 37°C. Nuclear transfer of the receptors was induced upon addition of 1 μM cortisol at t = 0. For 30 min, changes in subcellular distribution were evaluated by fluorescence microscopy. At each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. The percentage of the cells with complete nuclear localisation are displayed. All quantifications are compilations of at least two independent experiments. Error bars indicate means ± S.E.M. Statistical differences were calculated using Kolmogorov-Smirnov test but did not yielded significant differences *DF*, diclofenac; *NP*, nonylphenol. DF, diclofenac; NP, nonylphenol; Cd, cadmium; Cu, copper.

GFP-rtGR2 nuclear import rate was increased in the presence of the high concentration of diclofenac (Figure 3.13 A). The low concentration of nonylphenol significantly increased the amount of cells showing a completely nuclear localisation of fluorescence already before cortisol was added (Figure 3.13 B). The low concentration of copper (Figure 3.13 C) and cadmium (Figure 3.13 D) significantly decreased the import efficiency of GFP-rtGR2. Nuclear translocation of GFP-rtGR2 in the presence of H_2O_2 could not be observed because cells were detached after treatment with H_2O_2 for 24 h.

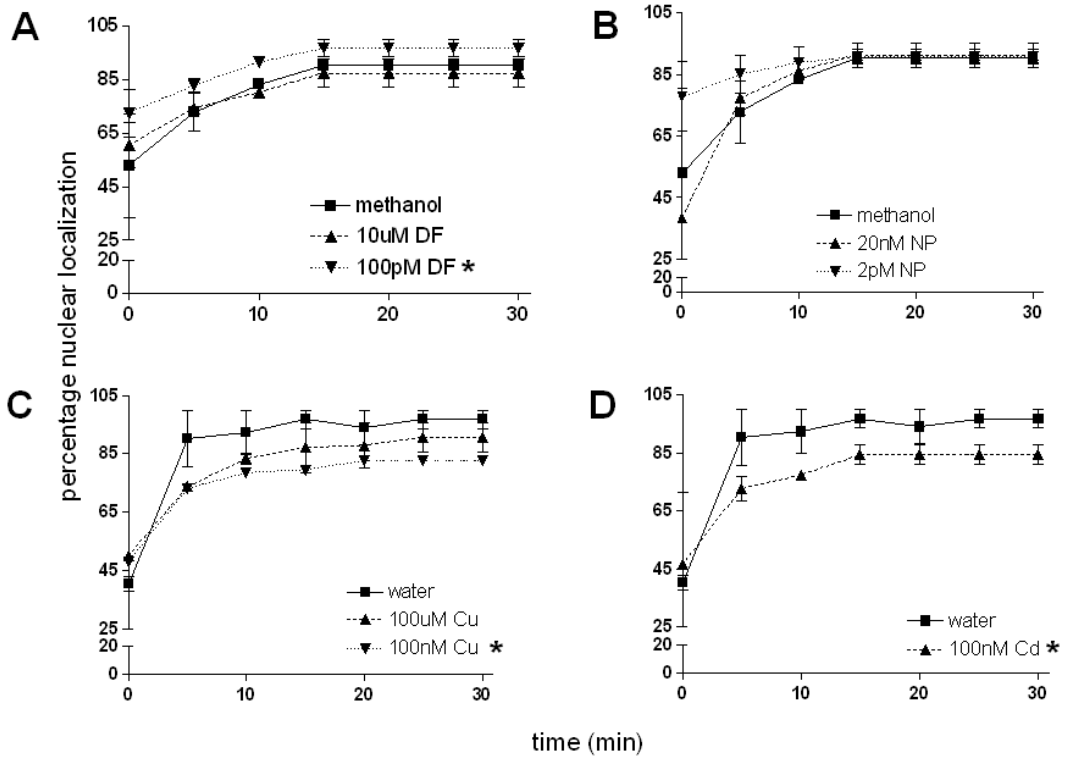


Figure 3.13: Kinetics of GFP-rtGR2 nuclear transfer is altered in the presence of contaminants. Cells were transiently transfected with GFP-rtGR2 in medium without steroid supplement using Lipofectamine 2000, and cultured for another 16 h in medium without serum supplement. Cells were then treated for 24 h with (A) diclofenac, (B) nonylphenol (C) copper, and (D) cadmium, and H_2O_2. Image acquisition was performed in a temperature-controlled chamber at 37°C. Nuclear transfer of the receptors was induced upon addition of 1 μM cortisol at t = 0. For 30 min, changes in subcellular distribution were evaluated by fluorescence microscopy. At each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. The percentage of the cells with complete nuclear localisation are displayed. All quantifications are compilations of at least two independent experiments. Error bars indicate means ± S.E.M. Asterisk in the legend indicate exposure conditions that led to significant differences from the control curve calculated using the Kolmogorov-Smirnof Test. DF, diclofenac; NP, nonylphenol; Cd, cadmium; Cu, copper.

Okamoto observed that human GR, when treated with H_2O_2 for 2 h, decreases import rates due to inhibition of certain cystein residues within the receptor. To compare the results directly to the findings of Okamoto (1999), transfected COS-7 cells, expressing the GFP-tagged receptors, were treated for only 2 h with H_2O_2. Interestingly, H_2O_2 significantly decreased nuclear transfer of GFP-rtGR1 and 30 min after addition of cortisol less than 50 % of the cells showed a predominantly nuclear signal (Figure 3.14 A). GFP-rtGR2 nuclear translocation was completely abolished in the presence of H_2O_2 (Figure 3.14 B).

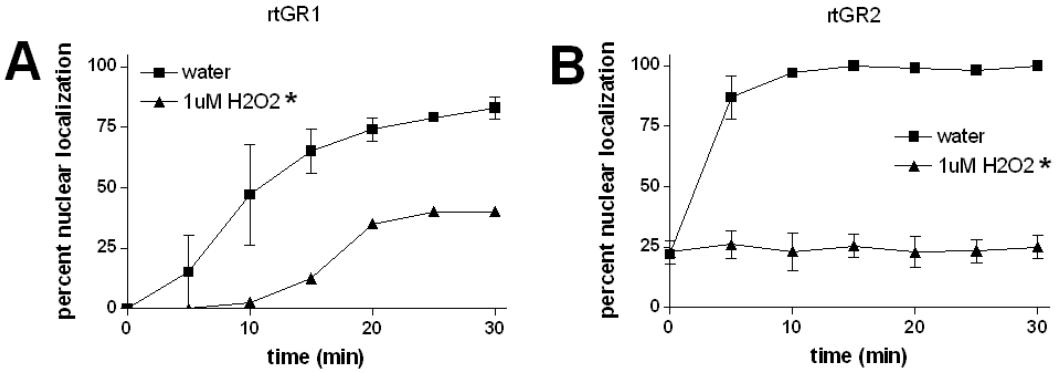


Figure 3.14: Kinetics of GFP-rtGR1 and GFP-rtGR2 nuclear transfer is altered after 2 h exposure to hydrogen peroxid. Cells were transiently transfected with (A) GFP-rtGR1 and (B) GFP-rtGR2 in medium without steroid supplement using Lipofectamine 2000, and cultured for another 16 h in medium without serum supplement. Cells were then treated for 2 h with H_2O_2 which did not lead to visible changes in cell viability. Image acquisition was performed in a temperature-controlled chamber at 37°C. Nuclear transfer of the receptors was induced upon addition of 1 μM cortisol. For 30 min, changes in subcellular distribution were evaluated by confocal laser scanning microscopy. Of each time point analysed, an average of 20 - 80 cells were quantified for the cellular localisation of the receptor. The percentage of the cells with complete nuclear localisation are displayed. All quantifications are compilations of at least two independent experiments. Error bars indicate means ± S.E.M. Asterisk in the legend indicate exposure conditions that led to significant differences from the control curve calculated using the Kolmogorov-Smirnof Test.

3.3.4 Influence of selected environmental contaminants on transactivational activity of rtGR1 and rtGR2

The impact of nonylphenol, diclofenac, cadmium and copper on transactivation activity of the two rainbow trout GRs was explored. A reporter gene assay was carried out in transiently co-transfected cells, using a reporter plasmid in which luciferase expression is under the control of the MMTV promoter that contains three classical glucocorticoid response elements. As shown in Figure 3.15, treatment of the cells with a high concentration of nonylphenol slightly induced transcriptional activity of rtGR1 in the absence of cortisol while diclofenac, copper and cadmium alone had no effect. At sub-maximal transactivation induction of rtGR1 by cortisol, treatment of the cells with the high concentration of nonylphenol significantly enhanced the transcriptional activity of the receptor, but the low nonylphenol concentration, diclofenac, and both metals did not affect receptor responses to cortisol (Figure 5.15 B). However, maximum induction by cortisol was significantly decreased in the presence of the high copper concentration. Cadmium decreased the rtGR1 response although not significantly. H_2O_2 significantly inhibited rtGR1 transactivation activity (Figure 3.15 A-C).

rtGR2 transactivational activity was induced by exposing transiently transfected cells to the high concentration of nonylphenol and diclofenac for 24 h in the absence of cortisol (Figure 3.16 A). Copper and cadmium had no effect on transactivational activity of rtGR1 (Figure 3.16 A). At sub-maximal cortisol-induced transactivation activity of rtGR2, both nonylphenol concentrations were able to enhance the receptor transactivation although not significantly (Figure 3.16 B). Further, in the presence of both concentrations of diclofenac, the transactivational activity of rtGR2 was significantly decreased (Figure 14 B). In contrast, copper and cadmium did not alter cortisol mediated activity of rtGR2 (Figure 3.16 B). The maximal receptor activity of rtGR2 was more vulnerable and significant reduction of transactivational activity was observed in the presence of high concentrations of diclofenac, nonylphenol, copper, and cadmium (Figure 3.16 C). When cells were transfected with the rtGR2 coding plasmid, all cells had detached after 24 h of exposure to H_2O_2 and transactivational activity was not quantified (Figure 3.16 A-C).

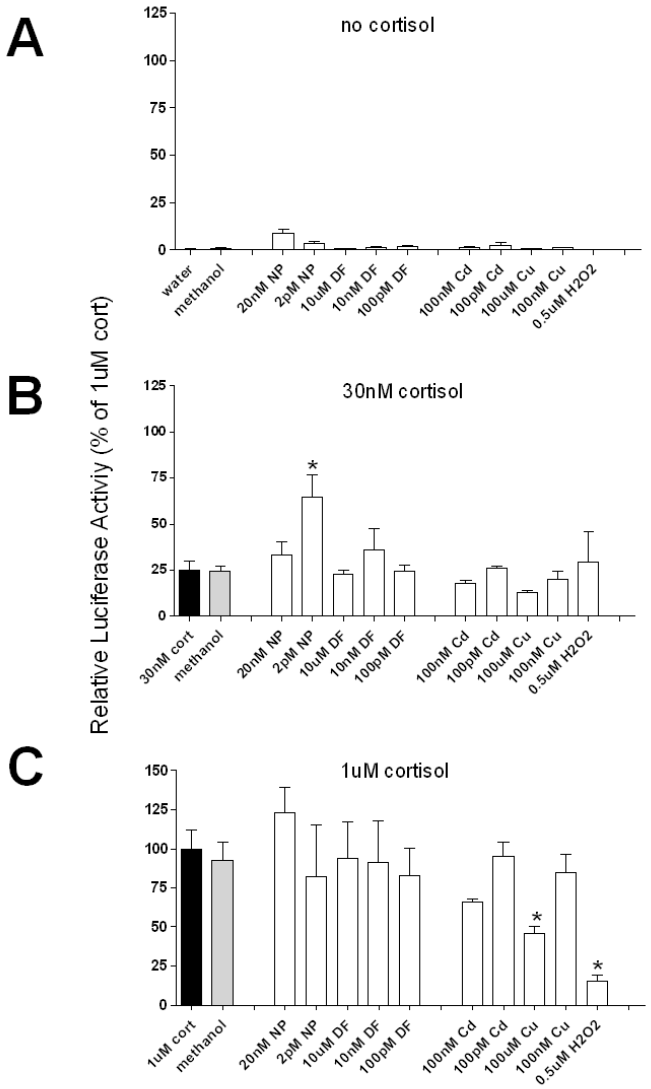


Figure 3.15: Transactivation activity of rtGR1 transiently expressed in COS-7 in the presence of contaminants. COS-7 cells were cotransfected with rtGR1 expression vector, as well as the reporter plasmid pFC31Luc, and the pSVβ plasmid which expresses β-galactosidase. After transfection, cells were treated with diclofeanc, nonylphenol, copper, cadmium and H_2O_2 in the presence of (A) ethanol as the solvent control, (B) 30 nM cortisol, and (C) 1 μM cortisol. Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control, and expressed as a percent of the GR1 activity measured in the presence of 1 μM cortisol. Values represent means $\pm$ S.E.M. of three independent experiments, with each experiment performed in duplicates. Asterisks indicate significant differences from the control when calculated using Student t-test. For comparision with NP and DF treated cell, control cells were treated with cortisol and methanol as the carrier solvent (grey bars) and control cells for Cd, Cu and H_2O_2 treatment were exposed toh cortisol and water (black bars). DF, diclofenac; NP, nonylphenol; Cd, cadmium; Cu, copper.

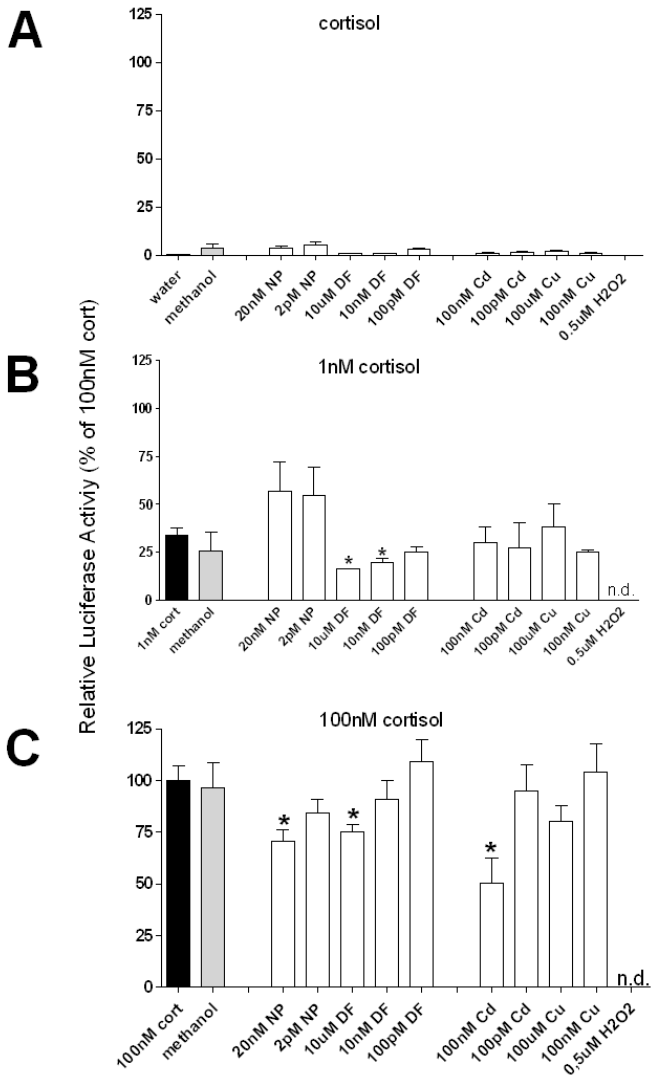


Figure 3.16: Transactivation activity of rtGR2 transiently expressed in COS-7 in the presence of contaminants. COS-7 cells were cotransfected with rtGR2 expression vector, as well as the reporter plasmid pFC31Luc, and the pSVβ plasmid which expresses β-galactosidase. After transfection, cells were treated with diclofeanc, nonylphenol, copper, cadmium and H_2O_2 in the presence of (A) ethanol as the solvent control, (B) 1 nM cortisol, and (C) 100 nM cortisol. Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control, and expressed as a percent of the GR2 activity measured in the presence of 1 μM cortisol. Values represent means $\pm$ S.E.M. of three independent experiments, with each experiment performed in duplicates. Asterisks indicate significant differences from the control when calculated using Student t-test. For comparision with NP and DF treated cell, control cells were treated with cortisol and methanol as the carrier solvent (grey bars) and control cells for Cd, Cu and H_2O_2 treatment were exposed toh cortisol and water (black bars). DF, diclofenac; NP, nonylphenol; Cd, cadmium; Cu, copper.

Chapter 4

Discussion

4.1 Mechanistic studies of fish glucocortidoid receptors

Phylogenetically diverse teleost fishes have been demonstrated to possess two GRs that probably arose following a whole genome duplication event that occurred 335 million years ago in the teleost lineage (Greenwood et al., 2003, Jaillon et al., 2004, Stolte et al., 2006, Bury and Sturm, 2007). The two rainbow trout glucocorticoid receptors, termed rtGR1 and rtGR2, display a marked difference in their hormone sensitivity in transactivation and transrepression assays, with rtGR2 exerting its activity at far lower concentration of dexamethasone or cortisol, compared to rtGR1 (Bury et al., 2003, Bury and Sturm 2007). One basic idea is that the glucocorticoid signal transduction is mediated via the subcellular distribution of the GRs (Savory et al., 1999; Shank and Paschal, 2005). Therefore, the first purpose of the present work was to explore determinants of subcellular localisation and nuclear translocation of rtGR1 and rtGR2 using GFP-tagged receptors.

4.1.1 Identification of a novel NLS in the A/B-domain of rtGR1 and rtGR2

I observed that trafficking to the nucleus showed the same differences in hormonal sensitivity between the two receptors as was observed in transactivation or transrepression studies. However, to my surprise I observed that the majority of the naïve trout GRs was constitutively localised in the nucleus. This is in strong contrast to the cytoplasmic localisation reported for all mammalian GRs.

Partial nuclear localisation of naïve mammalian GRs has been attributed to the high expression levels of GR obtained in transient expression systems using plasmid constructs with strong promoters and SV40 controlled replication (Savory et al., 1999). In the present study the rat GR (GFP-rGR$_{C656G}$) transiently expressed using the same strong promoter and SV40 controlled replication also showed partial nuclear localisation in the nucleus, but clearly less pronounced than observed for the two trout GRs, suggesting that the transfection protocol alone is not the reason for the nuclear distribution. In addition, results obtained in a second cell line derived from the same organism as the investigated receptors (rainbow trout gonad cell line, RTG2) showed a far greater shift of naïve GFP-rtGR1 and especially unliganded GFP-rtGR2 towards the nuclear compartment compared to the two receptors expressed in COS-7 cells. In RTG-2 the expression levels are probably lower than in COS-7 because RTG-2 cells are not transformed with SV40. Thus I conclude that the nuclear distribution pattern observed in cell culture is likely to resemble, at least in part, the situation *in vivo*.

The mammalian GR is reported to harbour two nuclear localisation signals. NL1 is a ligand-independent signal located within the C-terminal extremity of the C-domain and N-terminal beginning of the D-Domain that is constitutively active if uncoupled from the NL2. NL2 is a ligand-dependent nuclear localisation signal present in the E-domain (Picard and Yamamoto, 1986; Savory et al., 1999). In addition, a nuclear export signal (NES) has been mapped to 15 amino acids in the linker region between the zinc fingers of the DBD (Black et al., 2001) and recently a nuclear retention signal (NRS) that overlaps with the NL1 has been described (Carrigan et al., 2007). The naïve mammalian GR, which is predominantly located in the cytoplasm, has been shown to be in dynamic equilibrium between the nucleus and the cytoplasm (Hache et al., 1999) and the nucleo-cytoplasmic localisation of receptors is a consequence of a fine balance between operational strengths of the NLSs, NES, NRS (Black et al., 2001; DeFranco 2002; Carrigan et al., 2007), as well as a potential cytoplasmic retention signal, which has yet to be identified (Figure 4.1). To explore the unusual localisation of naïve receptors a series of domain-swap recombinant receptor mutants was used. The unusual cellular distribution was found to mainly result from an undefined components of the A/B-domain.

In detail, chimers containing the rat ABCD domains and either the rtGR1 or rtGR2 E-domain clearly demonstrated a wildtype rGR$_{C656G}$ cytoplasmic localisation, suggesting that the E-domain of the two rtGRs is not responsible for the nuclear localisation of the naïve receptor.

Replacing the receptor region containing the C- and D-domain of rGR$_{C656G}$ with that of rtGR2 caused a shift in the unliganded receptor towards the nucleus. However, introducing the same receptor regions of rtGR1 into rGR$_{C656G}$ had little effect. The observation that a chimer containing the rtGR2 C- and D-domain is partially distributed to the nucleus in the absence of hormone could mean that the rtGR2 NL1 signal is able to override the negative effect of the rGR$_{C656G}$ NL2. Because the NES is also found in this region, an alternative interpretation is that the rtGR2 NES is weaker than that of the rat or rtGR1 isoform. A third possible explanation is that NRS of

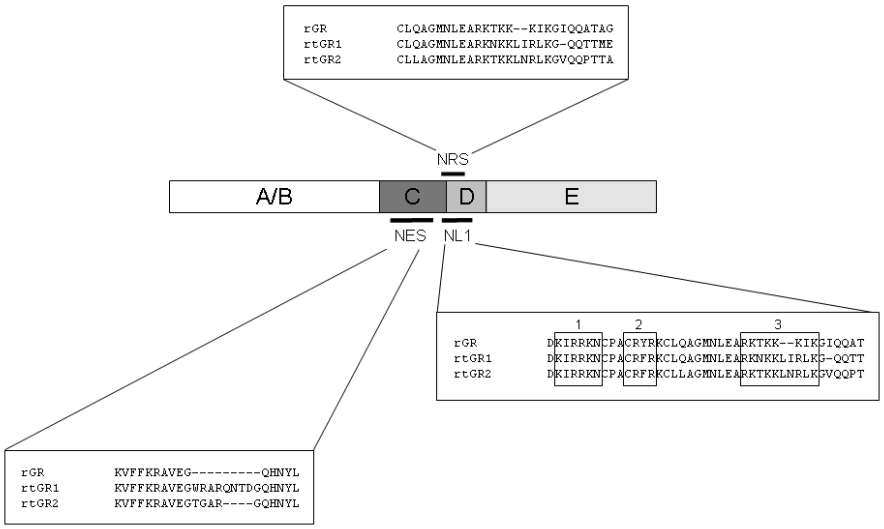


Figure 4.1: Nuclear import and nuclear export signals within the C and D domain of the glucocorticoid receptor

rtGR2 is stronger compared to the NRS in rtGR1.

Looking at the amino acid sequences of both trout receptors and comparing NL1 and NES of trout GRs with rat GR, sequence alterations become obvious (Figure 4.1).

First, adjacent to the NES both trout GRs possess an amino acid insertion. In rtGR1 the nine amino acids insertion WRARGNTG has been reported (Ducouret at al. 1995, Takeo et al., 1996, Lethimonier et al., 2002) and rtGR2 is known to harbour the five amino acid insertion TGARG (Bury et al., 2003). NES is bound by calreticulin at the highly conserved sequence KVFFKR (Holaska and Black 2001) adjacent to the amino acid insertion. For rtGR1 the nine amino acid insertion is predicted to create an additional loop which is distorted to outside the protein (Wickert and Selbig 2002). No data are available for the latest discovered rtGR2. One could hypothesize that this additional loop outside the protein could somehow disturb correct interaction of calreticulin with NES of trout GRs and therefore interfere with the transfer out of the nucleus. Interestingly, rat MR, is described to show a similar nuclear distribution of naïve receptor compared to rtGR1 and rtGR2. RatMR harbours a five amino acid insertion between the two zinc fingers and the additional residues form a loop that is distorted to outside the protein (Wickert and Selbig 2002).

Second, NL1 of rat GR is comprised of a tripartite motif (Tang et al., 1997) which is well conserved in both trout glucocorticoid receptors; except that both trout GRs NL1 possess a two amino acid insertion within the core region of the NL1, with rtGR1 having an LI insertion and rtGR2 an LN insertion. All teleost GRs identified to date posses these double amino acid insertions at this point, except for *Haplochromis burtonii* GR1 that only possess 1 amino acid insertion (Bury and Sturm personnel observation; see Stolte et al., (2006) for accession numbers for teleost GRs). In addition, the rat MR

also harbours a similar two amino acid insertion in this cluster of NL1 and as mentioned above shows a similar nuclear distribution in the absence of ligand compared to rtGR1 and rtGR2. However, mutations of the adjacent lysine residues in this region of the rat MR did not disrupt localisation and thus the NL1 was found not to be responsible for the distribution of naïve rat MR (Walther at al. 2005). From this one can infer that this region probably does not cause the rtGR distribution pattern.

Third, the NRS region, important for nuclear retention of the mammalian GR, is situated in the hinge region (D domain) of the GR that actively opposes the nuclear export of GR (Carrigan et al., 2007). The GR NRS overlaps closely with the basic NL1 but can be distinguished from NL1 by site directed mutations (Carrigan et al., 2007). Nonetheless, because NRS overlaps with NL1, the same two amino acid insertion is present within NRS. One may assume that this could alter the functioning of NRS. Nevertheless, Walther et al., 2005 showed that swapping the central domains of rat GR and MR had no influence on subcellular localisation of receptor chimers in absence of the specific ligand. The central domain of GR therefore may not be the operative point for the unusual nuclear localization of naïve trout GRs.

Although comparison of rat MR and rtGR1/rtGR2 indicates that the sequence alterations may not be important for nuclear localisation of the trout GRs in the absence of a specific ligand, still undefined components responsible for nuclear localisation are present within the C- and D domains of at least rtGR2.

Chimeric constructs in which the A/B domain of either rtGR1 or rtGR2 was combined with the remaining domains of ratGR$_{C656G}$ all showed increased nuclear localisation in the absence of hormone when compared to ratGR$_{C656G}$. These results indicate the presence of a novel NL within the A/B domain of trout GRs. If a novel NLS is present, it is unlikely to be a classical basic motif of NLS (Savory et al., 1999). While the A/B domain of rtGR1 possesses such motifs [^{274}KQENDRR^{280}], that of the more nuclear rtGR2 does not. The nucleo-cytoplasmic localisation of naïve rtGR1 and rtGR2 resembles that observed for the mammalian MR (Htun 1996, Fejes-Toth 1998, Tyagi 2000, Nichi et al., 2001). MR localisation is due to serine/threonine motifs situated within the A/B domain - most notably Ser^{601} (Walther et al., 2005). This motif is a potential phosphorylation site and implicates phophorylation for influencing corticoid receptor subcellular localisation (Walther et al., 2005). The amino acid sequence of the A/B-domain of rtGR1 and rtGR2 contains several seronine/threonine rich motif. Based on NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/), a number of them are candidate phosphorylation sites. This may indicate a conserved mechanism for the similar distribution pattern between the mammalian MRs and trout GRs.

The chimeric constructs that mimicked the wildtype rtGR distribution pattern contained both the A/B-, as well as the C- and D-domains suggesting a degree of co-operation between the A/B-domain and C- and D-domain to determine nuclear location of the naïve trout GR. This would suggest that no single nuclear localisation signal is working alone and a degree of co-operation between potential nuclear localisation sites in the A/B-domain and the C- and D-domain, presenting three important

regions, NL1, NRE and NES, determine nuclear localisation of the naïve trout GRs. To further distinguish between the single functions of NRS, NES and NL1, creation of more mutants swapping the different signals of trout GRs with rat GR would give insight into this issue.

4.1.2 Determinants of nuclear translocation of GFP-tagged rtGR1 and rtGR2

The remaining cytoplasmic GFP-tagged rtGR1 and rtGR2 transferred rapidly into the nucleus upon hormone binding in both mammalian and rainbow trout cell lines. rtGR1 and rtGR2 showed differences in their sensitivity in nuclear transfer upon exposure to cortisol which mimic closely those observed in transactivational studies (Bury et al., 2003). According to a higher sensitivity of rtGR2 in transactivation assay, nuclear translocation of rtGR2 was induced at lower cortisol concentrations compared to nuclear transfer of rtGR1. GR transcriptional properties are governed by receptor localisation (Savory et al., 1999; Shank and Paschal, 2005) and thus the difference in sensitivity for nuclear import may in part explain the difference in transactivational sensitivities observed previously. One can hypothesize that differences in hormone binding are the reasons. Bury et al., determined the binding affinities to dexamethasone for rtGR1 with K_d 5.05 ± 0.41 and for rtGR2 with K_d 3.4 ± 0.79. To further characterize hormone binding also dissociation of the hormone from the receptor needs to be identified.

The difference in sensitivity was not just restricted to natural glucocorticoids. rtGR2 shows a greater preponderance to migrate to the nucleus in the presence of the glucocorticoid antagonist RU486, aldosterone and progesterone, albeit when cells were treated with relatively high concentrations. The rtGR2 hydrophobis pocket appears to be more flexible than the rtGR1 pocket allowing binding of different ligands. However, rtGR1 in total seems to be less sensitive than rtGR2. Beyond the differences in hormone binding, I hypothesize that the different sensitivity of GFP-rtGR1 and GFP-rtGR2 in nuclear transfer studies induced by cortisol and RU486 indicate that the subcellular distribution and transactivation of rtGR2 is regulated by different pathways than rtGR1. This could be due to differences in co-chaperone/receptor complex interactions.

Phylogenetic analysis places the basal vertebrate (lamprey and hagfish) corticosteroid receptors (CR) with the MRs (Stolte et al., 2006) and transactivation assays show that the hormone sensitivity of these CRs is similar to tetrapod MRs (Bridgham et al., 2006). Duplication of the ancestral CR resulted in the appearance of an MR and GR (Thornton, 2001) with two amino acid mutations in the ancestral CR resulting in the glucocorticoid specific features observed in the GRs (Bridgham et al., 2006). The observation that teleost GRs resemble MR in their nucleocytoplasmic distribution pattern would suggest that cytoplasmic GR localisation is specific to the sarcopterygians or indeed may only be seen in the tetrapods. The molecular determinants that allowed

the retention of two GRs that emerged following the duplication of an ancestral GR gene in the teleost lineage some 350 million years ago is a puzzle, primarily because both trout GRs utilise a single hormone, cortisol. The observation that there are clear differences in the nucleocytoplasmic distribution between the two GRs suggests that receptor protein regions responsible for determining cellular distribution maybe sites for evolutionary selection. Positive mutations in these regions may have led to divergent functions.

4.2 Toxicological analysis of fish glucocorticoid receptors

Because glucocorticoids play an essential role in the regulation of key physiological processes, including immunomodulation, brain function, energy metabolism, electrolyte balance, blood pressure and stress response, I studied the potential of aquatic contaminants to disturb this fine network. Only few studies have focused so far on endocrine disruption of fish GR (see Vijayan 2005 for review).

A broad set of xenobiotics found in the aquatic environment, representing model substances of different classes of environmental contaminants, were evaluated. PCB126, TCDD, the metals copper, cadmium, and nickel, as well as benzo[a]pyrene and the pharmaceuticals, diclofenac, metoprolol, clofibric acid and carbamazepin have been included in the investigations. Of all substances tested, nonylphenol, diclofenac, copper, and cadmium caused the greatest impact on nuclear transport and/or transactivation and were selected for more in depth studies. PCB126, TCDD, nickel, metoprolol, clofibric acid and carbamazepin had no impact on rtGR1 and/or rtGR2 nuclear import and/or transactivation activity.

4.2.1 Impact of Nonylphenol on action of rtGR1 and rtGR2

Nonylphenol is among the most prevalent chemicals found in waterways of industrial countries and belongs to the most investigated xenohormones known to act at the estrogen receptor. Technical nonylphenol is a mixture of approximately 20 different isomers and is a potent inducer of ER transactivation activity. Technical nonylphenol from Acros exhibited a maximum response of 82 % of that produced by E2 at 12 - 18 μM nonylphenol in an assay using stably transfected MCF-7 cells with an ER linked luciferase reporter gene (Preuss et al., 2006). The EC20 of this dose-response curve was still 1.9 μM. Surprisingly nonylphenol concentrations of a 100 times less than 2 μM were able to increase sub-maximal transactivation activity of rtGR1 and rtGR2 elicited by cortisol. Nonylphenol did not significantly induce transcriptional activation of the trout GRs in the absence of cortisol but slightly shifted unliganded rtGR1 towards the nucleus, a response that is normally triggered by cortisol. Because nonylphenol alone was not able to significantly induce transactivational activity I suggest that nonylphenol

targets receptor associated proteins and thus alters the activity of fish GRs indirectly. Compounds have been described to bind receptor associated proteins of rat GR, for example the immunosuppressants FK506 (Ning et al., 1993) and cyclosporine A (Renoir et al., 1995). Both substances potentiated sub-maximal dexamethasone activity (Prima et al., 2000) but did not induce transactivational activity of rat GR alone. In line with the ability of nonylphenol to shift unliganded inactivated GFP-rtGR1 and GFP-rtGR2 towards the nuclear compartment was the finding that also nuclear translocation of the GRs induced by cortisol was faster in the presence of nonylphenol.

As stated above, nonylphenol is a complex mixture of isomers and the composition might differ between producers (Preuss et al., 2007) and maybe even from batch to batch. Only one batch (Sigma-Aldrich) has been tested here. To confirm that the observed effects are generally found for technical nonylphenol, future studies should repeat the experiments with different batches from the same and other producers. In this way it would also be tested if the high potential (effective concentration as low as 2 pM) found in the current study is a feature of technical nonyphenol in general. A combination of fractionation of the technical nonylphenol mixture and GR transactivation assays could then be used to identify the relevant isomer(s) of nonylphenol.

4.2.2 Impact of Diclofenac on action of rtGR1 and rtGR2

Diclofenac is one of the most frequently detected pharmaceuticals in environmental water samples (Hallare et al., 2004). The mechanism of diclofenac mediated cytotoxicity in mammals has not been fully understood but there is evidence that both uncoupling of the mitochondrial oxidative phosphorylation and cytochrome P450-mediated metabolism are involved in acute toxicity (Bort et al., 1999). In primary rainbow trout hepatocytes, diclofenac clearly inhibited P450-dependent EROD activity and cell viability after treatment of the cells with concentrations higher than 36 μM (Laville et al., 2004). After water-born diclofenac exposure of rainbow trout, renal lesions and alterations in gill integrity were observed (Schwaiger et al., 2004; Triebskorn et al., 2004) at concentration of 2 μM diclofenac that were also found in surface water.

Nuclear translocation after cortisol addition was slightly stimulated after 24 h of diclofenac exposure. Again, interactions with receptor associated proteins could be an explanation for the observed effect. It will be of pharmacological interest to study more in detail the side effect of diclofenac on the mammalian GR. It can not be excluded that the impact of diclofenac on rtGR1 and rtGR2 localisation is due to cross interaction with other cellular pathways. For example, high traffic through P450-related pathway could occupy and deplete the free pool of accessory proteins for the GR pathway. Although the observed effect is quite small, chronic exposures to diclofenac in the aquatic environment may, also combined with previously observed effects on accumulation of diclofenac (Schwaiger et al., 2004) and inflammation (Hoeger et al., 2005), still have a profound impact on the fish.

4.2.3 Impact of Cadmium on action of rtGR1 and rtGR2

Cadmium is a highly toxic nonessential metal which can be found wide spread in the environment. Cd has been reported to effectively prevent formation of intramolecular and intermolecular disulfide bonds of the mammalian GR which in turn impacts ligand binding, DNA-binding and transactivation activity (Dundjerski et al., 2000; Elez et al., 2001). In the present study 100 nM cadmium decreased nuclear transfer of GFP-rtGR1 and GFP-rtGR2 and impaired both trout receptors to activate gene expression in the reporter gene assay. This was in contrast to findings in mouse 2305 cells containing a stably incorporated MMTV-promoter regulating the expression of the reporter gene chloramphenicol-acetyltranferase (CAT) in which 500 nM of $CdCl_2$ did not alter transactivation activity in response to dexamethasone (DeMoor et al., 2001). The conflicting observations may be due to differences between the human and fish GR and the fact that different cellular expression systems were used.

4.2.4 Impact of Copper on action of rtGR1 and rtGR2

Copper significantly reduced maximal transactivation activity of rtGR1 while rtGR2 transactivation activity was only slightly decreased. In contrast, studies of DeMoor (2001) using mouse 2305 cells found no change of dexamethasone induced activation of CAT-reporter enzyme activity after $CuCl_2$ exposure with concentrations of 0.25 μM - 20 μM prior to dexamethasone treatment. Possible reasons accounting for the differences could be that the investigated receptors are from different species and that different *in vitro* systems have been used. Very high doses of copper (100 μM) have been described to induce formation of reactive oxygen species (Manzl et al., 2003), disrupt calcium homeostasis by displacing calcium from biological ligands, and disrupting membrane permeability in epithelial cells of the fish gill (Mazon et al., 2004) and trout hepatocytes (Manzl et al., 2003) thereby inducing apoptosis or necrosis (Bury et al., 1998; Mazon et al., 2004; Manzl et al., 2003). Negative effects of copper exposure could therefore be also a reason of ROS formation in the cells.

4.2.5 Impact of H_2O_2 on action of rtGR1 and rtGR2

Treatment of cells with H_2O_2 is known to inhibit ligand-dependent nuclear translocation and Hsp90 release from GFP-hGR (Okamoto et al., 1998). In agreement with findings of Okamoto et al., (1998), I observed an inhibition of nuclear transfer of GFP-rtGR1. Intriguingly, for GFP-rtGR2 expressing cells, treatment with 0.5 μM H_2O_2 was cytotoxic to the cells. Treatment of the transfected cells for only 2 h with 1 μM H_2O_2 completely abolished nuclear translocation of GFP-rtGR2 while cytotoxic effects have not been observed. One of the key regulatory mechanisms of protein localisation within the cells are thought to be redox-dependent modifications of cysteine residues (Okamoto et al., 1998). It therefore may be feasable that different redox-regulation of

rtGR2 function is present compared to rtGR1. Further, rtGR2 could be involved in cell death processes, for example apoptosis.

4.2.6 Additional pollutants tested for their impact on trout GRs

Polychlorinated biphenyls (PCBs) have been known as environmental endocrine disrupting chemicals that cause various abnormalities in many organs (Vijayan et al., 2005). PCB-methyl-sulphones (mPCBs) are known to suppress transactivational activity of a recombinant human GR (Johansson et al., 1998). In the presented study PCB126, which does not belong to the mPCBs, did not impair transactivational activity of rtGR1 and rtGR2. PCP126 has been reported to increase GR1 mRNA abundance after 6 h exposure of primary cultures of rainbow trout anterior kidney cells (Quabius et al., 2005). On the other hand, Aroclor 1254, a synthetic PCB mixture, did not affected transactivational activity of the mammalian GR in an *in vitro* transcription-based reporter assay (Portigal et al., 2002). Moreover, PCB was not able to suppress GR-mediated transcription in various cell lines (Iwasaki et al., 2002).

TCDD is reported to significantly reduce the maximal binding capacity of GR in rat liver (Sunahara et al., 1989). Further, TCDD treatment altered the GR protein content in rat ovary (Mizuyachi et al., 2002). Despite these two studies indicating an effect of TCDD on rat GR, no impact of TCDD on fish GR transactivation activity was observed in the presented study.

Till to date no reports exist about the impact of benzo[a]pyrene (BaP) on the function of glucorticoid receptors either of mammals or fish and no impact of BaP was found in the present study.

In contrast to cadmium and copper, nickel had no impact on the action of fish GRs even at very high concentrations. No previous data are available on the impact of nickel on the GR(s) of mammals or fish.

Aside from diclofenac three other pharmaceuticals have been tested on the ability to interfere with the cortisol mediated response of the two trout GRs and none of them were able to do so. Carbamazepine, an antiepileptic therapeutic, has been shown to reduce cell viability of primary rainbow trout hepatocytes and as a side effect reduce EROD activity (Fent et al., 2006). Clofibric acid, a blood lipid regulator, is one of the human drugs already detected in ground and drinking water (Fent et al., 2006). Clofibric acid is reported to induce ROS and decrease cell viability of primary rainbow trout hepatocytes (Fent et al., 2006) but a reduction of cell viability was not found in the present study. Metoprolol, is a cardio-selective beta blocker, and effects on GR has never been tested.

Chapter 5

Concluding Remarks and Future Directions

Previous studies have revealed that rainbow trout and likely a majority of teleosts possess two glucocorticoid receptors, encoded by distinct genes. In rainbow trout, the two disctinct GRs display a marked difference in their sensitivities in transactivation assays as well as transrepression assays (Bury et al., 2003; Bury and Sturm, 2007).

The current study, using GFP tagged receptors, revealed an unexpected partial nuclear localisation of both naïve trout glucocorticoid receptors rtGR1 and rtGR2 compared to a predominately cytosolic localisation of the mammalian GR (Htun et al., 1999, Sackey et al., 1996). This was directed by the receptors A/B domain, but it is not clear whether this is due to a novel NLS. Investigation of the function of the A/B domain in a recombint protein outside the GR context could answer such question.

rtGR2 nuclear transfer occurred at lower concentrations of cortisol than rtGR1. This difference in sensitivity was not just restricted to natural glucocorticoids. One can speculate that differences in hormone binding are the reasons. Bury et al. determined the binding affinities to dexamethasone with rtGR1 K_d being similar but little lesser for rtGR2. To characterize hormone binding also dissociation of the hormone from the receptor needs to be identified to fully understand the importance of ligand binding.

Furthermore, rtGR2 exhibited effective nuclear translocation in the presence of aldosterone and RU486 and rtGR2 acted more sensitive in terms of the impact of environmental contaminants compared to rtGR1. Maybe subcellular distribution and transactivation of rtGR2 is regulated by different pathways than rtGR1. This is could be due to differences in co-chaperone/receptor complex interactions. An interesting approach to study the differences in the composition of the heteromeric protein complexes would be the tandem affinity purification assay.

Further, rtGR2 may also be involved in the regulation of different cellular pathways and may also be important in processes such as including programmed cell death. Microarray analysis would give an interesting insight into the different regulatory functions

of rtGR1 and rtGR2 within the cell. For this purpose a cell model would be needed that expresses only one of both trout GRs. Because all rainbow trout cell lines tested expressed both mRNAs, an interesting technique to by-pass this difficulty would be mRNA silencing of one of each receptors.

The presented study showed the potential of known endocrine disruptors of the ER to also impair GR action. Although effects of the tested environmental contaminants on the trout GRs are small compared to the cortisol-mediated action, the results are disconcering because these endocrine disruptors are ubiquitous in the environment and chronic exposure of fish can be assumed to occur. These impacts of pollutants on trout GR further emphasize the necessity to develop and include additional environmental parameters, like the GR action, in the safety evaluation risk assessment of chemicals. The introduced transactivational assay would be an inexpensive and direct method for defining the impact of environmental contaminants on the action of trout GRs. For a scaling up for the screening of high numbers of samples, the development of a stably transfected cell line, carrying the reporter plasmids and one of the two receptor plasmids, would be an advantage. Developing a trout cell line expressing functional endogenous GRs stably transfected with the reporter plasmid would be a further asset.

Chapter 6

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Appendix A

Appendix

A.1 Desteroidized FBS

- 50 ml FBS (Invitrogen, Karlsruhe, Germany)

  0.5 g active coal (Invitrogen, Karlsruhe, Germany)
  0.05 g dextran (Invitrogen, Karlsruhe, Germany)

  56°C 30 min
  centrifuge for 20 min at 3000 rpm or 15 min at 5000 rpm
  repeat 2 - 3 times
  filter sterilize with 20 μm filters
  store at -20°C

A.2 Reverse transcription of RNA to first strand cDNA

- reaction mixture

10 µl DNA digested RNA
2 µl Oligo dT primer (10 µM, Invitrogen, Karlsruhe, Germany)
4 µl 5X RevertAid RT reaction buffer (MBI Fermentas, St. Leon-Rot, Germany)
0.5 µl ribonuclease inhibitor (recombinant, 40 U/µl, MBI Fermentas)
2 µl 10 mM dNTP mix (5 mM each, MBI Fermentas, St. Leon-Rot, Germany)
1 µl RevertAid reverse transcriptase (200 U/µl,
MBI Fermentas, St. Leon-Rot, Germany)
1.5 µl deionised water, nuclease free
20 µl per RNA sample

A.3 Polymerase chain reaction

- 10X RT-PCR buffer

500 mM Tris/HCl (pH 9) (Serva Chemicals, Heidelberg, Germany)
15 mM $MgCl_2$ (Sigma-Aldrich, Munich, Germany)
150 mM $NH_4)_2SO_4$ (Sigma-Aldrich, Munich, Germany)
1 % (v/v) Triton X-100 (Merck, Darmstadt, Germany)
(store in aliquots at -20°C)

- reaction mixture

5 µl 10X RT-PCR buffer
1 µl 10 mM dNTP mix (5 mM each, MBI Fermentas,
St. Leon-Rot, Germany)
0.2 µl taq polymerase (5 U/µl, Promega, Mannheim, Germany)
40.8 µl deionised water, nuclease free
1 µl primer forward (20 µM, Invitrogen, Karlsruhe, Germany)
1 µl primer reverse (20 µM, Invitrogen, Karlsruhe, Germany)
1 µl cDNA sample
50 µl per cDNA sample

- TAE buffer (50 X, 100 ml)

  2 M Tris base (Carl Roth, Karlsruhe, Germany) 24.2 g
  0.5 M sodium EDTA (Serva Chemicals, Heidelberg, Germany) 1.86 g
  acetic acid (Merck, Darmstadt, Germany) 5.71 ml

  add just to pH 8.0
  store at room temperature and dilute 50 X for
  electrophoresis and preparation of agarose gels

- agarose gel

  1 - 2 % (v/v) agarose (Carl Roth, Karlsruhe, Germany) in 1 X TAE buffer
  approx. 2 μl of ethidium bromide (1 %, Serva Chemicals,
  Heidelberg, Germany)
  (alternative: staining of the gel in a bath with 0.001 % (v/v)
  ethidium bromide)

- DNA loading buffer (5 X)

  50 % (v/v) glycerol (Sigma-Aldrich, Munich, Germany)
  1 mM EDTA (MP Biomedicals, Eschwege, Germany)
  0.1 % (w/v) orange G (Sigma-Aldrich, Munich, Germany)

A.4 Cloning

- Luria Bertani (LB) medium and plates

  Liquid medium (1 L)
  1 % (w/v) tryptone (Becton, Dickinson and Company, New Jersey, USA)
  0.5 % (w/v) yeast extract (Merck, Darmstadt, Germany)
  1 % (w/v) sodium chloride (Merck, Darmstadt, Germany)

  pH 7.0, autoclave, cool to 55°C, add ampicillin (50 μg/ml),
  pour into plates
  for LB plates, add 1.5 % (w/v) bacto agar
  (Becton, Dickinson and Company, New Jersey, USA)

- SOC medium

  20 g tryptone (Becton, Dickinson and Company, New Jersey, USA)
  5 g yeast extract (Merck, Darmstadt, Germany)
  0.5 g sodium chloride (Merck, Darmstadt, Germany)
  10 ml 250 mM KCl (Merck, Darmstadt, Germany)
  per liter distilled water

  pH 7.0, autoclave, cool to 55°C, add 5 ml 1 M $MgCl_2$ and 25 ml
  1 M glucose (Merck, Darmstadt, Germany)

A.5 Maps of expression vectors

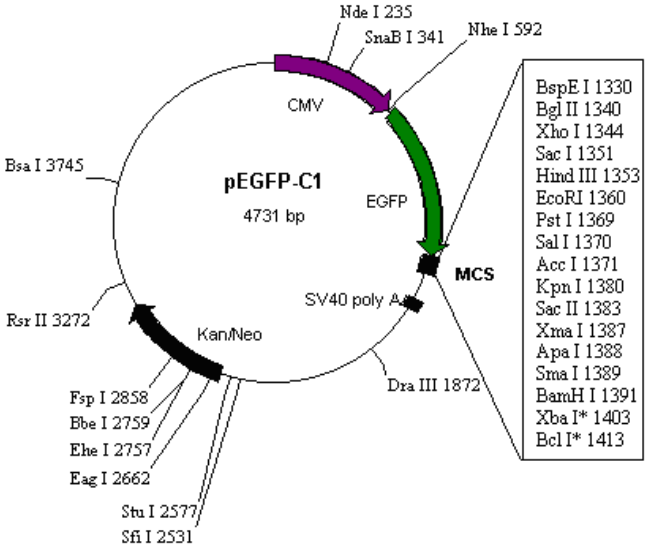

Figure A.1: Map of expression vector pEGFP-C1 (Clontech, Montain View, USA)

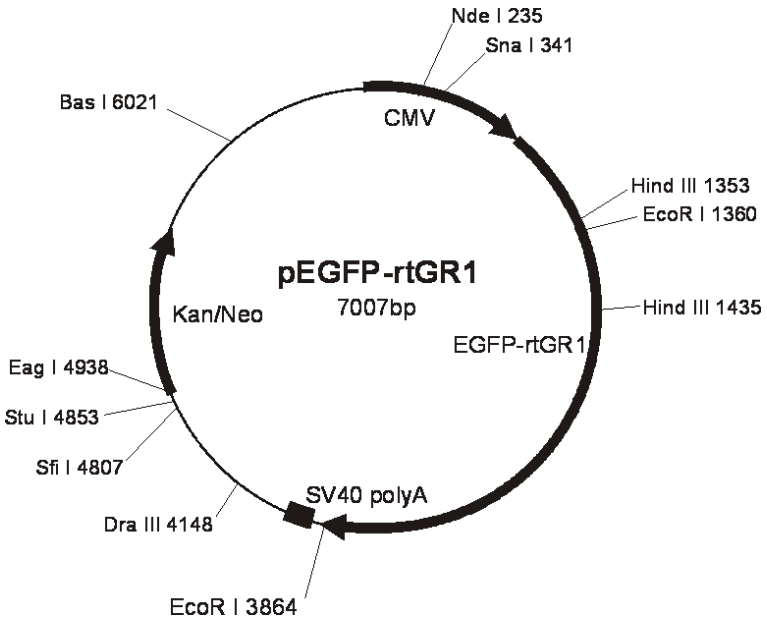

Figure A.2: Map of expression vector pEGFP-rtGR1

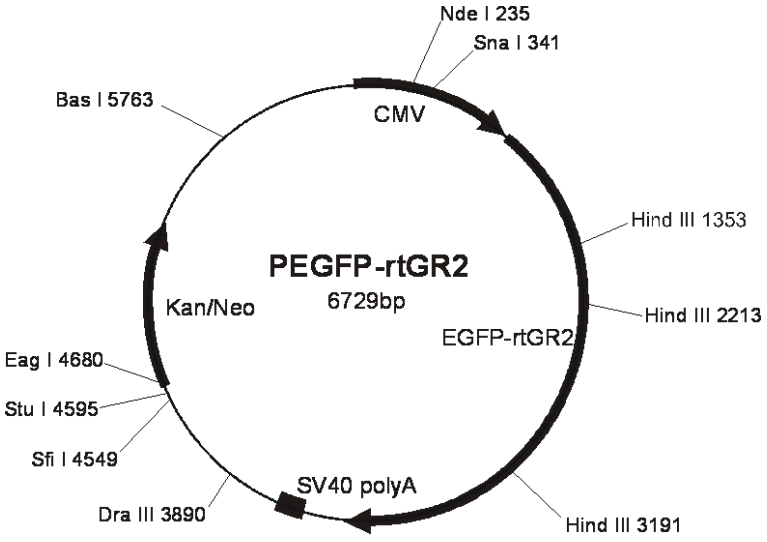

Figure A.3: Map of expression vector pEGFP-rtGR2

A.6 Competent cells

E.Coli DH5α
LB steril
1 M $CaCl_2$ (BDH Chemical Ldt. Dorset, UK)
sterilized and chilled
15 % glycerol (BDH Chemical Ldt. Dorset, UK) sterilized

inoculate 5 ml LB with DH5α
incubate at 37°C overnight
transfer into 750 ml LB
incubate at 37°C 220 rpm
measure OD_{600} after 1 hour, then at 30 min
intervals until OD600 has reached 0.4
put the tube on ice for 10 min
spin down at 2000 - 3000 rpm
resuspend each pellet with 4 ml ice cold 0.1 M $CaCl_2$ (do not vortex)
combine resuspended cells in two 50 ml tubes
pellet the cells (3000 rpm, 5 min)
resuspend pellets in 30 ml ice cold 0.1 M $CaCl_2$ (do not vortex)
pellet the cells (3000 rpm, 5 min)
resuspend pellets in 30 ml ice cold 0.1 M $CaCl_2$ with 15 % glycerol (do not vortex)
pellet the cells
resuspend both pellets in 7.5 ml ice cold 0.1 M $CaCl_2$ with 15 % glycerol
dispense 110 μl aliquots into sterile eppendorfs
store at -80°C

A.7 Transactivation assay

- 2 x HBS

280 mM $NaCl_2$ (BDH Chemical Ldt. Dorset, UK)
1.5 mM Na_2HPO_4 (BDH Chemical Ldt. Dorset, UK)
50 mM Hepes (BDH Chemical Ldt. Dorset, UK)

pH 7.1
filter sterilize

- 2 M $CaCl_2$

  5.88 g $CaCl_2$ (BDH Chemical Ldt. Dorset, UK)
  20 ml steril water

- KP-buffer

  a) 1 M K_HPO_4 (BDH Chemical Ldt. Dorset, UK)
  b) 1 M K_HPO_4 (BDH Chemical Ldt. Dorset, UK)

  mix 6.15 ml a) with 3.85 ml b)
  add 90 ml water
  pH 7.0

- ONGP

  400 mg O-nitrophenyl β-D-galactopyranoside (Sigma-Aldrich, Munich, Germany)
  100 ml KP-buffer

  filter sterilize
  aliquote in 15 ml
  store at -20 °C

- 100 x $MgCl_2$ / β-ME

  1.02 g $MgCl_2$ x $6H_2O$ (BDH Chemical Ldt. Dorset, UK)
  15.7 ml β-mercaptoethanol (Sigma-Aldrich, Munich, Germany)

  add 50 ml steril water
  store at 4 °C

Appendix B

Acknowledgement

I would like to thank all the people, who came along with me during my period as doctoral candidate, who stood by me and made me feel comfortable at the UFZ, King's and at home. I would especially like to acknowledge the following people:

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Finally, I would like to thank my parents and my grandma who always help me going my way and whom I can always rely on.

Appendix C

Versicherung an Eides statt

Versicherung gemäß § 5b

der Promotionsordnung der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg.

Hiermit versichere ich eidestattlich, dass die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Innland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Erklärung gemäß § 4b

Die vorliegende Arbeit wurde am Helmholtzzentrum für Umweltforschung UFZ unter Betreuung von Dr. Kristin Schirmer (Department für Zelltoxikologie), am King's College London, UK unter Betreuung von Dr. Nic Bury und unter Betreuung von Prof. Dr. H.-J. Ferenz (Institute für Zoologie, Universität Halle-Wittenberg) angefertigt.

Leipzig, 07. November 2007

.....
(Unterschrift Heidi Becker)

Appendix D

Curriculum Vitae

Name: Heidi Becker
Geburtstag: 16. März 1979
Geburtsort: Schkeuditz

Ausbildung

Januar 2004 - November 2007

> Promotion am Helmholtzzentrum für Umweltforschung UFZ, im Department Zelltoxikologie
>
> Thema der Dissertation: *Die Glucocorticoid-Rezeptoren der Regenbogenforelle - Nuklearer Transfer, Wirkungsweise und Einfluss von Umweltschadtstoffen.*
>
> unter Betreuung von Dr. Kristin Schirmer, Department Zelltoxikologie, Helmholtzzentrum für Umweltforschung - UFZ, Dr. Nic Bury, King's College London, sowie Prof. H.-J. Ferenz, Institut für Zoologie, Universität Halle-Wittenberg

Oktobter 2004 - September 2005

> Gastwissenschaftler im Rahmen der Promotion am King's College London, UK, School of Biomedical and Health Sciences, Nutritional Sciences Research Division

Oktober 2002 - September 2003

Diplomarbeit am Helmholtzzentrum für Umweltforschung UFZ, im Department Zelltoxikologie

Thema der Diplomarbeit: *Einfluss von Benzo[a]pyren auf die Gluconeogenese der Regenbogenforelle - Eine In-vitro Studie mit der Leberzelllinie RTL-W1.*

unter Betreuung von Dr. Kristin Schirmer, Department Zelltoxikologie, Helmholtzzentrum für Umweltforschung - UFZ

Oktober 1997 - September 2003

Studium der Biologie an der Universität Leipzig, Fakultät für Biowissenschaften und Psychologie

Vertiefungsrichtung: Genetik, Immunbiologie, Mikrobiologie und Biochemie
Diplom erlangt mit der Note 1,6
Vordiplom erlangt mit der Note 2,1

April 2003 - Juli 2003

Auslandspraktikum an der Universität Waterloo in Canada, Department Biologie

1997

Abitur am Gymnasium Engelsdorf, Abschlussnote 2,1

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Okt. 2003 - Dez. 2003 und 2001 - 2002

Anstellung als wissenschaftliche Mitarbeiterin am Helmholtzzentrum für Umweltforschung UFZ, im Department Zelltoxikologie