Protection Against Oxidative Glutamate Toxicity Mediated by the Mouse (*Mus musculus*) Orphan Receptor GPR39

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1 Aim of this Study

The aim of this study was to demonstrate protection offered against oxidative glutamate toxicity by G-protein coupled receptors, and to characterize the mechanism of protection mediated in particular by the mouse orphan receptor, GPCR mGPR39. To begin with, the influence of cell culture conditions on glutamate-induced death was investigated. The results implied that HT22 cells, depending on cell density, modify the growth medium protecting other cells in the vicinity from glutamate-induced cell death.

Furthermore, the inherent differences between a glutamate-sensitive and resistant cell line were used, by virtue of contrast, to analyze the role of G-protein coupled signaling pathways in this paradigm. The results of these initial experiments led to the investigation of orphan receptors, identifying new receptors possibly involved in glutamate-induced oxidative toxicity. These receptors were further tested using toxicity assays and the receptor offering the greatest apparent protection, namely, mGPR39, was chosen for further analysis.

In order to understand its role during the development, in situ hybridizations were carried out. To understand its role in glutamate toxicity, stable cells overexpressing the receptor were generated and analyzed. Moreover, a possible role of mGPR39 in another disease was investigated.
2 Introduction

2.1 Oxidative Stress

2.1.1 Oxygen and sources of reactive oxygen species

The survival of aerobic organisms critically depends on the availability of oxygen (O₂). In this process, dioxygen, O₂, acts as the final acceptor of electrons in the course of being reduced to two H₂O molecules. Concomittant to this reaction occur other processes within the electron transport chain of the inner mitochondrial membrane. In particular, a proton gradient is formed that serves as an energy source for the generation of adenosine triphosphate (ATP) (Slater 1977). The chemical energy stored in the electrostatically stressed terminal phosphoanhydride bond is used for many purposes, among which the synthesis of biomolecules, transport of molecules against concentration gradients, cellular movement, heat generation and establishment of electric potentials across membranes describe only a few notable examples.

Ironically, life-giving oxygen can also induce tissue injury (Capellier et al. 1999). The toxicity of O₂ arises from its electronic structure and related reactivity (Fridovich 1998). In particular, the paramagnetism in its ground state indicates that it contains two unpaired electrons bearing parallel spins. Since the electron pairs of any other stable organic molecule have antiparallel spins, they are preferentially transferred to O₂, apparently one at a time. Thus, in the course of completely reducing O₂ to two H₂O molecules, the four electrons and four protons involved necessarily generate transient intermediates like superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and the hydroxyl radical (HO⁻).

\[
\begin{align*}
O₂ & \rightarrow O₂⁻ \rightarrow H₂O₂ \rightarrow OH⁻ + HO⁻ \rightarrow 2H₂O₂ \\
& \quad 2H⁺ & \quad 2H⁺
\end{align*}
\]

These reactive oxygen species (ROS) are believed to be responsible for oxygen toxicity (Fridovich 1978; Davies 1995; Mates and Sanchez-Jimenez 2000).

The discovery of superoxide dismutases (SODs), which are O₂⁻ scavenging enzymes, led to the proposal that O₂⁻ is the major component of oxygen toxicity (Fridovich 1989;
Fridovich 1998). Superoxide is constantly produced by the electron transport chain of the mitochondria (Turrens and Boveris 1980). Here, direct leakage of $O_2^-$ occurs during the stepwise reduction of $O_2$ to $H_2O$, possibly amounting to no more than 5% but increasing significantly at higher $O_2$ concentrations (Fridovich 1989; Fridovich 1998). The mechanisms by which $O_2^-$ exerts its toxic effects were proposed to be mediated by direct oxidation and inactivation of enzymes that contain [4Fe-4S] clusters and by the reaction with nitric oxide (NO), from which the powerful oxidant peroxynitrite (ONOO$^-$) is formed (Fridovich 1999). SOD’s remove $O_2^-$ by converting it to $H_2O_2$.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Though overexpression of several SOD isoforms has been observed to protect from certain oxidative stressors (Brockhaus and Brune 1999; Ilizarov et al. 2001; Wheeler et al. 2001), deleterious effects can develop nonetheless under normal conditions. The major pathway appears to be governed by a subsequent increased production of $H_2O_2$ (Fullerton et al. 1998; Midorikawa and Kawanishi 2001).

$H_2O_2$ is known to be toxic in many systems, but it does not qualify as a radical because it has no unpaired electrons and it itself is a poorly reactive oxidizing agent (Halliwell 1992). Instead, it mainly acts as the precursor of the highly reactive hydroxyl radical (HO$^-$), which is typically formed by the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + OH^-$$

Copper ions also react with $H_2O_2$ to form HO$^-$ (Halliwell and Gutteridge 1990). The $Fe^{3+}$ or $Cu^{2+}$ generated by this reaction can be reduced by $O_2^-$:

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

Combining the above two equations to the metal ion catalysed Haber-Weiss reaction:

metal catalyst

$$H_2O_2 + O_2^- \rightarrow HO^- + OH^- + O_2$$

This reaction implies that much of the combined toxicity of $O_2^-$ and $H_2O_2$ is mediated by the production of HO$^-$. This assumption is substantiated by the efficient biological mechanisms that have evolved to limit this reaction. One mechanism uses enzymes such as
the catalases and selenium-dependent glutathione peroxidases (GPx) to metabolize away the H$_2$O$_2$ (Hayes and McLellan 1999). In addition, the labile ion pool is suppressed by special iron binding proteins. By virtue of this mode of action, excess iron is bound to either the storage protein ferritin or to transferrin (Crichton and Charloteaux-Wauters 1987).

Hydroxyl radical generated by the Fenton or Haber-Weiss reaction reacts with great speed with almost every molecule found in living cells. In the case of DNA, reaction leads to chemical alterations and potentially irreversible strand breakage (Halliwell and Aruoma 1991).

### 2.1.2 Oxidative Stress and Neurological Diseases

The brain is particularly vulnerable to damage via oxidative processes for several reasons (Behl, 1999). Firstly, the brain is an organ characterized by high-energy consumption. It almost completely depends on oxidative phosphorylation to generate ATP, what leads to a high production of ROS by the mitochondria. Secondly, the brain contains a high concentration of polyunsaturated fatty acids, which are potential substrates for peroxidation. Lastly, it contains relatively high amounts of iron and copper. Thus, it is not surprising that oxidative stress is assumed to play a role in neuronal diseases like Alzheimer’s dementia, Parkinson’s disease, and ischemic stroke.

Alzheimer’s dementia is a chronic neurodegenerative disease which is neuropathologically characterized by extracellular senile plaques mainly loaded with amyloid β protein (Aβ), intracellular neurofibrillary tangles, and degeneration of neurones (Glenner and Wong 1984; Braak and Braak 1991). The discovery that defects of the gene coding for the Aβ precursor protein (APP) can cause a hereditary form of this disease substantiated the opinion that Aβ plays a causative role in the neurodegenerative process (St George-Hyslop 1995; Tanzi et al. 1996). Furthermore, Aβ peptides, which are generated by protease cleavage of APP, are toxic to nerve cells in vitro and in vivo (Yankner et al. 1989; Kowall et al. 1992).

The observation that antioxidants can inhibit Aβ-induced cell death in vitro (Behl et al. 1992; Behl et al. 1994) and the generation of nerve cell lines resistant to Aβ expression leading to increased amounts of antioxidant enzymes (Sagara et al. 1996), gave way to the
opinion that Aβ somehow induces oxidative stress. How this is accomplished remains unknown, but the mechanism appears to involve induction of a particular superoxide generating enzyme system, which is sensitive to inhibitors of flavin-containing oxidases (Behl et al. 1994; Schubert et al. 1995).

In Parkinson’s disease the loss of the dopaminergic neurones in the substantia nigra pars compacta leads to a movement disorder characterized by a difficulty to initiate movements and related tremors (Hornykiewicz and Kish 1987). The pathological hallmark of this disease is the intraneuronal accumulation of α-synuclein in Lewy bodies (Spillantini et al. 1997; Olanow and Tatton 1999).

The assumption that oxidative stress is involved in the pathophysiology of Parkinson’s disease is based on several observations. Firstly, it was recognized early that the metabolism of dopamine involves the generation of ROS, either by monoamino oxidase-promoted production of H₂O₂ (Maker et al. 1981) or by oxidation of the catechol ring to form dopamine quinines, semiquinones and ROS such as H₂O₂ and O₂⁻ (Graham 1978; Hastings 1995). The quinones and semiquinones can react subsequently, causing depletion of glutathione (GSH) and formation of glutathionyl conjugates (Spencer et al. 1998; Spencer et al. 2002). In vitro dopamine was found to be neurotoxic, inducing oxidative stress (Basma et al. 1995; Si et al. 1998; Jones et al. 2000). Recently, the overexpression of either wild type or mutant α-synuclein was observed to induce cell death via oxidative stress in dopaminergic neurons. This observation, along with its dependency on dopamine synthesis, described the missing link between α-synuclein dysregulation, oxidative stress, and selective degeneration of dopaminergic neurones (Xu et al. 2002).

2.1.3 Oxidative glutamate toxicity as a model for neuronal death due to oxidative stress

Several in vitro models have been employed to investigate the steps leading to oxidative stress. The basic strategy is to introduce specific chemical insults, which appear to imitate events leading to in vivo neurological diseases, and to assess the biological consequences. Target diseases such as Alzheimer’s dementia and ischemic stroke can be explored in this way. Cell death due to oxidative stress, protective interventions using either primary neuronal cultures or neuronal cell lines, and stressors like Aβ, hydrogen peroxide, glutamate and other parameters have been examined. In all available models, oxidative
glutamate toxicity in particular describes a potentially useful in vitro-model to study neuronal death due to oxidative stress (Tan et al. 2001a).

After the initial description in a neuroblastoma-primary retina hybridoma cell line (N18-RE-105) (Murphy et al., 1989), oxidative glutamate toxicity was observed in other neuronal cell lines (Schubert et al. 1992; Davis and Maher 1994; Maher and Davis 1996; Froissard et al. 1997). Oxidative glutamate toxicity was particularly observed in the cell lines of immature primary neurons (Murphy et al. 1990; Davis and Maher 1994; Ratan et al. 1994), oligodendroglia (Oka et al. 1993), and astrocytes (Chen et al. 2000). Furthermore, some cell death observed after initial excitotoxic insult in mature neurones can be attributed to a secondary death of cells resistant against excitotoxicity but susceptible to oxidative glutamate toxicity. This susceptibility has been found to be induced by the high levels of glutamate released from the neurones dying from excitotoxicity (Schubert and Piasecki, 2001).

Most studies, which contributed to characterizing the programming of cell death following oxidative glutamate toxicity, were performed using the murine hippocampal cell line, namely, HT22. This cell line was specifically selected for its high sensitivity to oxidative glutamate toxicity (Davis and Maher 1994; Maher and Davis 1996; Li et al. 1997b; Tan et al. 1998a; Tan et al. 1998b; Ishige et al. 2001; Maher 2001). In sharp contrast to glutamate excitotoxicity, oxidative glutamate toxicity is independent of ionotrophic glutamate receptor activation (Murphy et al. 1989; Schubert et al. 1992).

In oxidative glutamate toxicity, elevated extracellular glutamate blocks cystine, that is normally imported via the System Xc− glutamate/cystine antiporter (Murphy et al. 1989; Tan et al. 2001a). Subsequent cystine depletion within the cells induces reduction in GSH levels. Due to this impairment of the cells’ antioxidant defence, ROS accumulate (Tan et al. 1998b). The metabolism of catecholamines present in the cell culture medium further increases oxidative stress (Maher and Davis 1996). GSH depletion, in turn, activates 12-lipoxygenase, which contributes to ROS production (Li et al. 1997b). The metabolites of 12-lipoxygenase activate soluble guanylate cyclase, inducing a calcium influx via cGMP-operated channels, which further increases the production of ROS (Li et al. 1997a; Tan et al. 2001a). This leads to a form of programmed cell death, which morphologically shares characteristics with both apoptosis and necrosis (Tan et al. 1998a; Tan et al. 1998b). However, hallmarks of classical apoptosis like nuclear fragmentation and DNA-laddering
do not occur in this type of cell death and caspase-3 activation plays no significant role either. Rather, this form of cell death is critically dependent on gene transcription and translation and is blocked by inhibitors of caspases other than caspase-3 and serine proteases.

2.1.4 Oxidative glutamate toxicity and G-protein coupled signaling

Activation of some G protein-coupled receptors (GPCRs) is shown to protect in diverse models of neurodegeneration (Yasui and Kawasaki 1995; Bond et al. 1998; O’Neill et al. 1998; Takei et al. 1998; Jolkkonen et al. 1999; Pizzi et al. 1999). In contrast, activation of other GPCRs exacerbates neuronal cell death (Campbell 2001; Kimura et al. 2001). This fundamental difference might be explained by the capability of GPCRs to couple to different heterotrimeric G proteins. Receptor coupling to Gi/o seems to be detrimental as a result of oxidative stress, while Gs-coupling, at least in part, results in protection of neuronal cells (Lewerenz et al. 2003).

Pharmacologically distinct pathways have been described by which some GPCRs or GPCR-regulated kinases offer protection against oxidative glutamate toxicity in HT22 cells and immature primary neurones (Ikeda et al. 1994; Papadopoulos et al. 1997; Sagara and Schubert 1998; Dargusch and Schubert 2002). G protein-coupled receptors that confer protection include the metabotropic glutamate receptors 1 and 5, which couple to Gq and block GSH depletion (Sagara and Schubert 1998), and the dopamine D4 receptor which blocks the cGMP-operated calcium channel (Ishige et al. 2001). Metabotropic glutamate receptors (mGluR) play roles in synaptic plasticity (Bashir et al. 1993; Manzoni et al. 1994; Riedel and Reymann 1996), seizure activity (Thomsen et al. 1994), and excitotoxicity (Bruno et al. 1995a; Bruno et al. 1995b). Furthermore, a role for mGluRs in oxidative glutamate toxicity has been described (Sagara and Schubert 1998).

GPCR-mediated regulation proceeds mainly via activation of protein kinase C (PKC) by phorbol esters, which in turn offer protection by activating the extracellular signal-regulated kinase (ERK) without modifying glutathione depletion or ROS accumulation. ERK, in turn, activates c-Jun NH2-terminal kinase (JNK) and blocks p38 mitogen-activated protein kinase (p38 MAPK) (Davis and Maher 1994; Maher 2001). Analysis of HT22 cells, specifically selected for their high resistance to glutamate, revealed the upregulation of catalases, enzymes of the GSH-metabolic system, and enzymes involved in the synthesis of the antioxidant bilirubin (Sagara and Schubert 1998). A detailed investigation of
glutamate-resistant HT22 cells or Aβ–resistant PC 12 (rat pheochromocytoma) cells revealed a high cross-resistance between Aβ and glutamate or related cellular insults such as cystine depletion and H₂O₂ (Sagara and Schubert 1998). Thus, it would follow to reason that these cells employ mechanisms of broad scope in the general defence against oxidative stress.

2.2 G-Protein Coupled Receptors

G-protein-coupled receptors (GPCRs) are the largest family of cell-surface receptors, and transduce signals elicited by a diverse range of signaling molecules, including ions, biogenic amines, peptides and lipids, as well as photons, leading to alterations of cellular function (Bockaert et al. 2002). These receptors share a common general structure, which consists of an extracellular amino terminus, an intracellular carboxyl tail, and seven transmembrane helices connected by three extracellular and three intracellular loops. GPCRs can exist in an active or inactive form. The inactive conformation is favored in most cases, although some GPCRs exhibit constitutive activity under normal circumstances, that is, in the absence of respective activating ligands (Parnot et al. 2002; Holst and Schwartz 2003).

Upon activation by a ligand, GPCRs associate with distinct classes of heterotrimeric G proteins composed of three subunits, namely, the α-subunit that has the guanine-nucleotide binding site and GTPase activity, and the β- and γ- subunits that form a tightly bound dimer. The subunits of G proteins show a wide range of heterogeneity. There are more than 20 different mammalian Ga subunits and 5 β and 14 γ subtypes currently known, which are found in nature in many possible combinations (Radhika and Dhanasekaran 2001).

The specific combination of α, β, and γ subunits, in combination with other regulatory and scaffolding proteins, connects a particular receptor to a specific effector pathway. The variety of Gβγ dimers that can be formed adds to the large diversity among G proteins and thus presents another mechanism regulating receptor-G protein specificity (Neves et al. 2002). G-protein classes are defined according to the primary sequences of the α subunits, resulting in four main families: Gαi/o, Gαs, Gαq/11, and Gα12/13.

Although the classification is rather arbitrary, there is a general mechanism among the members of the subfamily. Stimulation of the Gs subfamily activates adenylyl cyclase
whereas stimulation of the Gi subfamily leads to its inhibition. Stimulation of the Gq subfamily activates phospholipase C (PLC), and the G12 family is implicated in the regulation of small GTP binding proteins.

In many cases, these G proteins can couple to more than one receptor subtype, with differing affinities. GPCRs act as GEFs (guanine nucleotide exchange factors) for their cognate Ga subunits. Ga subunits bound to activated receptors undergo a conformational change, resulting in the release of guanosine diphosphate (GDP), which is the rate-limiting step in G protein activation. The transient high-affinity empty state is followed by binding of GTP to Ga and its dissociation from Gβγ and receptors. The GTP-bound Ga subunit and dissociated Gβγ dimer can then interact with multiple downstream effectors, such as adenyl cyclase, PLCβ, inward rectifier G protein-gated potassium channels, voltage-sensitive calcium channels, PI3 kinase, and molecules in the MAPK pathway (Garcia et al. 1998; Maudsley et al. 2005). In the absence of other regulatory factors, the intrinsic guanosine triphosphatase (GTPase) activity of the Ga subunit dephosphorylates GTP to GDP, thereby returning to the inactive state, which allows reassociation of Ga and Gβγ (Figure 2.1).
Figure 2.1: G-protein cycling. Rate-limiting receptor-promoted GDP dissociation (a) is followed by ternary complex formation (b). The GPCR then catalyzes the binding of GTP to Ga (c), which disrupts the ternary complex, causing dissociation of the G-protein heterotrimer into Ga and βγ. Both entities regulate the activity of effector systems (d). G-protein activation is terminated by hydrolysis of the Ga-bound GTP to GDP and phosphate, P (e). (adapted from Nature Rev. (Participants. 2004))

GPCRs can be divided into 5 different families based on their structural and genetic characteristics. Family 1 consists of receptors related to Rhodopsin and the adrenergic receptor, family 2 of the receptors are related to the calcitonin and PTH receptors. Family 3 consists of receptors related to the metabotropic receptors. Family 4 is made up mainly of pheromone-related receptors, while family 5 is formed by cAMP receptors. Sequences within each family generally share over 25% sequence homology in the transmembrane core region, and a distinctive set of highly conserved regions and motifs. Among these families, little similarity is evident beyond the predicted seven-transmembrane architecture (7TM) (Joost and Methner 2002).
2.2.1 Orphan G-Protein Coupled Receptors

At present, many open reading frames encoding putative members of the GPCR family have been identified, for which the ligands are not known. These receptors, commonly known as orphan receptors, have caught the interest of the pharmaceutical industry as drug targets, because it has been estimated that over 50% of all modern drugs modulate GPCR activity (Wilson and Bergsma 2000; Howard et al. 2001). In fact, the human genome sequencing project has identified around 800 genes that belong to the GPCR superfamily (Lander et al. 2001; Venter et al. 2001) wherein half of these genes are thought to encode sensory receptors. Of the remaining 360 receptors, the natural ligands for approximately 210 have been identified. This leaves around 150 orphan GPCRs that have no known ligand or function (Wise et al. 2004).

The first stage of any orphan GPCR screening experiment is to express the cloned receptor in a recombinant expression system that can provide the necessary transmission and G protein signaling machinery to enable the successful identification of an activating ligand. Irrespective of the expression system, the success of a ligand screening experiment depends entirely upon expressing the receptor at the cell surface and coupling the ligand successfully to the signal transduction machinery of that cell to generate a detectable signal in the desired assay (Lewerenz et al. 2003; Civelli 2005).

2.2.1.1 Mouse GPR15

2.2.1.1.1 Chromosomal Localization and Sequence Analysis

The gene for mouse GPR15 (mGPR15) codes for 1080 basepairs and is localized on Chromosome 16 (ENSMUST00000023425). The deduced protein is 360 amino acids long with a calculated MW of 40541.22 Daltons. GPR15 is an orphan receptor that belongs structurally to the Family 1 GPCRs, i.e. the rhodopsin and angiotensin receptors (Joost and Methner 2002). Following its identification (Heiber et al. 1996), it was isolated under the name of BOB (also known as GPRF), a co-receptor for SIV, HIV-1, and HIV-2 (Deng et al. 1997). Both Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV) infect the cells through their interaction with the CD4 Receptors and several other co-receptors, mainly chemokine receptors. CCR5 and CXR4 are the first chemokine receptors identified, with a function as co-receptors for HIV- and SIV-infection (Davis et al. 1997).
Although showing structural differences (Farzan et al. 1997), GPR15 has a similar function to the chemokine receptors as a co-receptor for HIV-infection (Deng et al. 1997). Recently, it was described to be one of the major co-receptors in HIV-1 and HIV-2, together with CCR5 and CXCR6 (Blaak et al. 2005). The mouse sequence shows 81% identity to the human sequence (Figure 2.2).

![Alignment of mouse and human GPR15 sequences](image)

**Figure 2.2: Alignment of mouse and human GPR15 sequences.** Identical residues are presented in purple, similar residues in red, and non-conserved residues are not shaded. Amino acid sequences were aligned with ClustalW within Workbench (http://workbench.sdsc.edu)

### 2.2.1.1.2 Phylogenetic Analysis

Despite its known function, GPR15 still lacks a specific ligand. In principle, a ligand can be envisaged fairly efficiently by turning to established receptors within this protein family, particularly those which could bear design similarities, for instance the Apelin, Angiotensin, and Bradykinin Receptors (Figure 2.3). As all the receptors within the family are peptide receptors, one can deduce from this that mGPR15 might bind a peptide ligand (Joost and Methner 2002).
The expression of human GPR15 has been described in the liver, prostate, intestine and immune system, examples of the latter being related to CD4-T-lymphocyte, macrophage and leukocyte production (Deng et al. 1997; Farzan et al. 1997). Anna Pantlen, from the group of Dr. Axel Methner, found in quantitative PCR analysis that in contrast with its human homologue, mouse GPR15 was predominantly expressed in the brain.

**Figure 2.3: Phylogenetic tree of GPR15 (GPRF).** AG2R: Type-1A Angiotenin II Receptor, AG2S: Type-1B Angiotensin II Receptor, BRB1: B1 Bradykinin Receptor, BRB2: B2 Bradykinin Reseptor, APJ: Apelin Receptor, AG22: Type-2 Angiotensin II Receptor, GPR25: G Protein-Coupled Receptor 25, GPRF: G Protein-Coupled Receptor 15. The scale bar indicates a maximum likelihood branch length of 0.1 inferred substitutions per site and the numbers represent the pairwise distances between two sequences on the branch. Adapted from (Joost and Methner 2002).

### 2.2.1.2 Mouse GPR39

#### 2.2.1.2.1 Chromosomal Localization and Sequence Analysis

The gene for mGPR39 (ENSMUSG00000026343) codes for 1660 basepairs and is localized on Chromosome 1. The resulting protein is 456 amino acids long with a calculated MW of 51584.32 Daltons. GPR39 is an orphan receptor that belongs to the Family 1 GPCRs, a family consisting of rhodopsin and angiotensin receptors (Joost and Methner 2002).
Analysis of the gene shows several sites for N-myristoylation, N-glycosylation, and phosphorlyation sites for Protein kinase C (PKC), Casein kinase II (CK2), and cAMP- and cGMP-dependent protein kinase (CAMP).

Phosphorylation occurs through the serine-, threonine- or tyrosine-residues, and is mainly responsible for the internalization of GPCRs (Hanyaloglu et al. 2001; Dale et al. 2002). Arrestin binds to the phosphorylated receptors (Krupnick and Benovic 1998) and prevents them from binding to G-proteins and other effectors, thereby attenuating signaling through these receptors (Pippig et al. 1993; Metaye et al. 2005). Through the act of binding, GPCRs are targeted into clathrin-coated vesicles, for subsequent internalization into endosomes (Goodman et al. 1996).

Myristoylation describes the condensation of myristate (a C14-saturated fatty acid) and the N-terminal moiety of glycine via an amide linkage (Towler et al. 1988). Myristoylation alters the interaction between protein and cell membrane (Resh 2004). N-glycosylation is one of the major post-translational modifications that occur in GPCRs and is carried out by the glycosylation of asparagine residues in the consensus sequence Asn-X-Ser/Thr, where X is any amino acid other than proline (Gavel and von Heijne 1990). Glycosylation plays not only an important role in the cell surface expression of the receptors (Duverney et al. 2005), but it may also be crucial for the proper folding as in the case of the AT1 receptors (Deslauriers et al. 1999). In particular, glycosylation at the second extracellular loop seems to be required for the proper folding of all GPCRs (Lanctot et al. 2005). The receptor mGPR39 has three of its five glycosylation sites located at its second extracellular loop.

Thus, it can be concluded that mGPR39 bears all modification sites that are characteristically present in GPCRs. The mouse sequence shows 83% identity to the human sequence (Figure 2.4).
2.2.1.2.2 Phylogenetic Analysis

GPR39 was first identified through the growth hormone secretagogue receptor (GHSR), and placed into the group of peptide receptors using nucleotide sequence comparisons (McKee et al. 1997). After the identification of Ghrelin, as another GHSR-binding peptide (Kojima et al. 1999), and the identification of its receptors (Kojima et al. 2001a; Kojima et al. 2001b) a phylogenetic analysis showed that the Ghrelin Receptor, Neurotensin Receptors 1 and 2, and Neuromedin U Receptors 1 and 2 were additional members of the human GPR39 receptor family (Holst et al. 2004). The same findings had been noted previously in a phylogenetic analysis carried out (Joost and Methner 2002) (Figure 2.5).
**Figure 2.5: Phylogenetic Analysis of GPR39.** NMU2R: Neuromedin U Receptor 2, NMU1R: Neuromedin U Receptor 1, NTR1: Neurotensin Receptor Type 1, NTR2: Neurotensin Receptor Type 2, TRFR: Thyrotropin-Releasing Hormone Receptor, GHSR: Growth Hormone Secretagogue Receptor Type 1, BRS: Bombesin Receptor Subtype-3, NMBR: Neuromedin-B Receptor, GRPR: Gastrin-Releasing Peptide Receptor, ETBR: Endothelin B Receptor, ET1R: Endothelin-1 Receptor, ETBR-LT2: Endothelin B Receptor-Like Protein-2, GPR37: G Protein-Coupled Receptor 37, GPR39: G Protein-Coupled Receptor Gpr39. The scale bar indicates a maximum likelihood branch length of 0.1 inferred substitutions per site and the numbers represent the pairwise distances between two sequences on the branch. Adapted from (Joost and Methner 2002)).
3 Materials and Methods

3.1 Buffers

10xMOPS running buffer
0.4 M MOPS, pH 7.0
0.1 M sodium acetate
0.01 M EDTA

PBS
137 mM NaCl
2.7 mM KCl
7.4 mM Na₂HPO₄
1.5 mM KH₂PO₄

50xTAE
1 M Tris base
5.71% (v/v) acetic acid
50 mM EDTA
Cell biology

3.2 Molecular Biology Methods

3.2.1 Cloning of GPR15, GPR39, VPAC2

Full-length cDNAs of mGPR15, mGPR39 and hVPAC2 cloned in frame with a signal peptide and a hemaglutinin tag at the amino terminus were previously cloned into pDNR loxP SP HA (modified from original Clontech vector) by Anna Pantlen from the group of Dr. Axel Methner.

To obtain the vectors that were used for transient transfections, full-length cDNAs were cloned from pDNR loxP SP HA vectors into pcDNA5 FRT/TO loxP expression vectors (Invitrogen) using a loxP site present in both vectors. This system is based on Cre Recombinase, a Type I topoisomerase from bacteriophage P1, which catalyzes the site-
specific recombination of DNA between loxP sites (Abremski et al. 1983). The enzyme requires no energy cofactors and Cre-mediated recombination quickly approaches equilibrium between substrate and reaction products. The loxP recognition element is a 34 base pair (bp) sequence comprised of two 13 bp inverted repeats flanking an 8 bp spacer region. The spacer in particular confers directionality (Metzger and Feil 1999).

To obtain the vector that was used for stable transfections, pDNR SP HA mGPR39 was used as a template for PCR. The product was cloned into a pGEM-T Easy vector (Promega) and then digested using Agel / BamHI sites (Figure 3.1). The digested fragment was then ligated with the pQCMX vector (BD Biosciences), affording pQCMX SP HA mGPR39. All constructs were verified by DNA sequencing.

![Figure 3.1: Cloning of pQCMX SP HA mGPR39. Agarose gel showing evidence of pQCMX (left), and pGEM-T Easy SP HA & mGPR39 (right), after incubation with restriction enzymes Agel / BamHI. All bands described the expected sizes (molecular weight markers not shown).](image)

### 3.2.2 Polymerase Chain Reaction

The full-length mGPR39 sequence obtained from pDNR loxP SP HA mGPR39 was amplified, together with Signal Peptide (SP) and Hemaglutinin tag (HA), by using the following primers designed with Agel (underlined) / BamHI (underlined) digestion sites:

- Forward 5’- ACCGGTCCACCATGAAGACGATCATCG - 3’
- Reverse 5’- GGATCCTCAAGATTCTCGCTCTGTAA - 3’

In every case, template DNA (1 μg), 10xPCR Buffer (5 μl, 100 mM Tris-HCl, pH 8.4; 500 mM KCl; 0.8% NP-40), MgCl₂ (1.5 μl, 50 mM), primer (1 μl of each, 10 μM), dNTP
mixture (1 μl, 10mM), and Platinum Taq DNA Polymerase (2.5 U, Invitrogen) were combined in a reaction tube and the total volume was brought to 50 μl with dH2O. Using the Biometra UNO II Thermocycler, PCR amplification was achieved using the following protocol: Unfolding/melting (94°C, 30 sec); annealing (67°C, 30 sec); extension (72°C, 30 sec); 25 cycles; and a final extension period (72°C, 10 min).

3.2.3 Protein Preparation and Western Blotting

HT22 sensitive and resistant cells were harvested after treatment with PBS buffer (5 min, 2 mM in EDTA). They were collected subsequently by centrifugation, and solvated in Lysis Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% SDS, 1% NP40, and 0.5% Sodium deoxycholate) containing a protease-inhibitor cocktail (complete-mini, Roche Molecular Biochemicals). Following incubation (20 min, 4°C), the lysates were ultracentrifuged (10.000 g, 10 min). The lysates were boiled in SDS Sample Buffer (100 μl; 5 mM Na2HPO₄, 2% SDS, 10 mM DTT, 5% β-mercaptoethanol, 10% glycerol, and bromphenolblue; 5 min). The resulting samples were migrated in a denaturing SDS polyacrylamide gel (12% crosslinking, 125 V). The electrophoretic transfer of the proteins onto nitrocellulose membrane was accomplished via semi-dry blotting (125 mA, 50 min).

After the transfer of the proteins, the nitrocellulose membrane was blocked using blocking solution (overnight; 4°C, 5% nonfat skim milk powder in TBS containing 0.1% TritonX100). The following day, primary antibodies (rabbit polyclonal anti-GPR39 (Acris) or mouse monoclonal anti-Actin (Covance)) were added to the blocking solution (final dilution of 1:2000) and incubated (RT, 1 hour). After incubation the membrane was thrice washed (10 min) in TBS containing 0.1% TritonX100, followed by incubation (RT, 1 h) with the second antibodies (final dilution 1:125000; goat anti-mouse or anti-rabbit IgG horse reddish peroxidase coupled antibody (Alexa)). After another washing cycle (thrice with TBS containing 0.1% TritonX100), the protein-antibody complex was detected with the chemiluminescent reagent Lumi-Phos (Perbio Science). The Lumi-Phos reagent was evenly distributed onto the membrane and allowed to incubate (5 min), before exposing the blot to X-ray film for the indicated times (please refer to the results section).

3.2.4 in situ Hybridization

For in situ hybridization, pDNR SP HA GPR39 was digested using EcoRI / PstI. The resulting 926 bp fragment was purified and inserted into pBluescript SK II + vector. The
construct was verified by sequencing. After linearization with XhoI (sense) or NotI (antisense), the DNA was purified by phenol/chloroform extraction. The radioactively labelled RNA was synthesized by PD Dr. Irm Hermans-Borgmeyer using the MAXScript in vitro transcription kit (Ambion, Frankfurt, Germany) with $^{35}$S-UTP, according to the manufacturer’s instructions. The labelled RNA was purified with Sephadex-G50 quick spin columns (Boehringer, Mannheim, Germany) in order to separate the unincorporated radioactive nucleotides from the transcripts. The antisense direction comprised a large part of the open reading frame (nucleotides 1-926). Activity of the labelled RNA probe was determined by taking a sample and measuring the counts per minute (cpm) using a scintillation counter (Beckmann). The probe was diluted to a concentration of 5000 cpm/µl in hybridization solution containing 50% formamide, 1x Denhardt’s solution, 4x SSC, 5% dextran sulphate, 500 µg/ml herring sperm DNA, 250 µg/µl yeast tRNA, and 10mM DTT. A minimum of 2×10⁶ cpm/ml hybridization solution were used. Cryosections of 10 µm were fixed in 4% paraformaldehyde in phosphate buffered saline, acetylated, dehydrated, and subjected to in situ hybridization at 55°C for 18 hours. The slides were washed with 4x SSC and subsequently treated with RNase buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 20 µg/ml RNase) for 30 min at 37°C, to digest single stranded non-hybridized RNA. The slides were then washed at decreasing salt concentrations. A 30 min high-stringency wash was performed in 0.1x SSC at 55°C- Sections were dehydrated and exposed to high-resolution X-ray film (Kodak Biomay MR). Subsequently, the slides were dipped in Kodak NTB-2 nuclear track emulsion, developed after 3 weeks in Kodak Dektol, and stained with Giemsa (Sigma). Specificity of the signals was verified by comparing antisense with the sense controls.

3.3 Cell Biology Methods

3.3.1 Cell culture and viability assays

HT22 cells were obtained from Prof. Paschen, Max-Planck-Institute for Neurological Research, Cologne, Germany. These cells comprise a subclone of the murine hippocampal cell line HT4 that has been immortalized by a temperature-sensitive SV-40 T antigen (Morimoto and Koshland, 1990) selected for high sensitivity to glutamate (Maher and Davis, 1996). Cells were cultivated at 37°C in an 5% CO₂-atmosphere and high glucose-Dulbecco’s modified minimal essential medium (high glucose-DMEM, PAA Laboratories)
containing 5% fetal calf serum (FCS, Gibco), 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, and 100 μg/ml streptomycin (both from Life Technologies).

Cells were removed by dissociation with 0.5% trypsin and 0.2 % EDTA (Life Technologies) every other day. For viability assays 5 x 10^3 cells in 100 μl were seeded into 96-well microtiter plates (Falcon). L-Glutamatic acid (Sigma) was solubilised in H2O at a concentration of 250 mM, adjusted to pH 7.4 with sodium hydroxide and stored at –20° C. It was added 24 hours later for 8 hours as indicated. Other experimental agents were also added after 24 hours, unless indicated. Cell survival was judged by phase contrast microscopy and assayed using the methylthiazoltetrazolium (MTT) method (Hansen et al., 1989). Briefly, MTT (Sigma) solubilised in phosphate buffered saline (PBS, 5 mg/ml) was added to the cells 24 hours after addition of glutamate to a final concentration of 1 mg/ml. Two hours later, cells were lysed by addition of 125 μl of lysis buffer containing 50 % dimethyl formamide, 20% sodium dodecyl sulfate, 2% acetic acid, adjusted to pH 4.7. Absorbance was measured after 24 hours at 550 nm using a microplate reader (SLT labinstruments, Crailsheim, Germany).

Each experiment was done in quadruplicates if not otherwise mentioned and repeated at least twice. Using GraphPadPrism™ software, the mean optic density obtained in the control sample, namely, that neither treated with glutamate nor experimental agents, was normalised to 100% and data of three experiments were pooled and subjected to statistical analysis as per the unpaired student’s t test.

3.3.2 Transfection of DNA into mammalian cells

Adherent HT22 cells were trypsinized in 1 ml Trypsin/EDTA for 2 min at RT, followed by resuspension in 4 ml serum-containing DMEM, and centrifuged for 4 min at 1000 rpm. The cell pellet was resuspended in 5 ml PBS and re-centrifuged for 4 min at 1000 rpm. The cell pellet was resuspended in 500 μl ice-cold PBS, and mixed with 10 μg of the DNA of interest. The tube containing the cells and DNA was incubated on ice for 15 min. After incubation, the mixture was transferred into an electroporation cuvette (Invitrogen, 4 μm) and placed in the holder of the electroporation apparatus at room temperature. A single pulse shock with 250 mV and 960 μF was applied. The time constant was kept between 16-22 msec for all the transfections. After electroporation, cells were taken up in 10 ml of
serum-containing DMEM and incubated for 24 hours at 37°C, before continuing with the experiments.

In order to transfect pQGXIP SP HA mGPR39 and pQGXIP EGFP into the Phoenix-Eco cells (Orbigen), calcium phosphate method was used. Briefly, the cells were grown to 60-70% confluency and 5 minutes prior to transfection, cell media was replaced with 3 ml fresh media supplemented with 25 μM chloroquine for each plate. The transfection reagent (10 μg DNA, 438 μl dH2O, 61 μl 2M CaCl2) was prepared in a 15 ml Falcon tube. Before transfection, 0.5 ml 2xHBS solution (50 mM HEPES, ph 7.05; 10 mM KCl, 12 mM Dextrose; 280 mM NaCl; 1.5 mM Na2HPO4) was added into the transfection reagent and combined by vigorous pipetting. The finished transfection reagent was quickly added dropwise onto the dishes containing Phoenix-eco cells, and the dishes were rocked gently to distribute DNA/CaPO4 particles evenly. The dishes were then incubated at 37°C for 24 hours and then with fresh medium for another 24 hours.

3.3.3 Stable Cell Line Production

To obtain a stable HT22 cell line overexpressing mGPR39, the packaging cell line Phoenix-Eco (Orbigen Inc) was co-transfected with pQGXIP SP HA mGPR39 and pQGXIP EGFP, using the Calcium Phosphate Method. Following transfection, cells were incubated for 48 hours before adding Puromycin (final concentration: 2.5 μg/ml) to the medium. The cells were kept in this medium for 2 weeks, with changes of the medium every 48 hours. After this time, all the cells in culture were expressing EGFP and featured Puromycin resistance. These cells were passaged and incubated at 37°C until they reached 60-70% confluence. At this stage, the medium was removed and centrifuged at 2000xg for 5 min, to remove cell debris. In order to achieve viral transfection, the resulting medium was put onto 24-hour-old HT22 cells in culture. After waiting for 48 hours, Puromycin was added into the medium at a final concentration of 2.5 μg/ml. The cells were kept in this medium for 3 weeks, with fresh medium being added every other day. Cells building colonies were isolated using cloning cylinders (Sigma) and transferred into 96-well plates, where they were further propagated.
3.3.4 Flow Cytometry

3.3.4.1 Measurement of ROS levels

For analysis by flow cytometry, 1 x 10⁵ cells were seeded in six-well plates (Falcon) and grown for 24 hours. The next day, the cells were collected using PBS/EDTA and centrifuged at 1000 rpm for 4 minutes. Then the cells were incubated with 20 μM DCFDA for 30 minutes at 37°C during which time they were protected from light. After incubation, the cells were washed once with PBS and centrifuged at 1000 rpm for 4 minutes and the pellets were resuspended in PBS containing 1 μg/ml 7-amino-actinomycin D (7-AAD, Calbiochem-Novabiochem). Cells were stored on ice until measurement at 488 nm with an FACS Calibur flow cytometer (Becton Dickinson). Median fluorescence of viable cells was calculated by the Cell Star™ software for each substance and time point.

3.3.4.2 Measurement of mGPR39 and HA expression

For analysis by flow cytometry, 1 x 10⁵ cells were seeded in six-well plates (Falcon) and grown for 24 hours. The next day, the cells were collected using PBS/EDTA and centrifuged at 1000 rpm for 4 minutes. Then the cells were incubated with the specific antibodies (mouse monoclonal anti-HA or rabbit polyclonal anti-GPR39) in PBS containing 2% FCS for 20 minutes at 4°C. After 20 minutes, cells were washed twice with PBS + 2% FCS, and incubated with the secondary antibodies (Alexa Fluor anti-mouse or anti-rabbit) for further 20 minutes. Cells were washed once more by centrifugation at 1000 rpm for 4 minutes and the pellets were resuspended in PBS + 2% FCS containing 1 μg/ml 7-amino-actinomycin D (7-AAD, Calbiochem-Novabiochem). Cells were stored on ice until measurement at 488 nm with an FACS Calibur flow cytometer (Becton Dickinson). For CRE-mediated EGFP measurements, the cells were directly stained with 7-AAD and measured. Dead cells were identified by 7-AAD staining and excluded from the analysis. Median fluorescence of viable cells was calculated by the Cell Star™ software for each substance and time point.

3.3.5 Measurement of total GSH

Five thousand HT22 and HT22R cells were plated in white, non-transparent 96 well-microtiter plates. After 24 hours of cultivation, cells were incubated with glutamate or standard medium for six hours. Cells were then carefully washed twice with phenol red-free media and incubated with 20 μM MCB for five minutes at 37°C during which time they
were protected from light. MCB fluorescence was then measured by a SpectraMax Gemini (Molecular Devices, Ismaning, Germany) using the SoftmaxPro 3.1.1 software (Softmax, San Diego, USA). Excitation wavelength was 393 nm and emission wavelength was 485 nm. After measurement, one volume normal medium and MTT were added immediately and cell viability assessed two hours later as described above. Results indicate MCB fluorescence per well normalized to the corresponding viability as measured by the MTT-test.

3.4 Preparation of RNA

3.4.1 Cell cultivation for RNA preparation

HT22 and HT22CR cells (5x10^5) were seeded in 92 mm-cell culture dishes. HT22CR were cultivated in the presence of 10 mM glutamate. Cells were harvested after 24 hours of cultivation. Additionally, HT22 cells were treated with 10 mM glutamate after 24 hours and harvested six hours later. For harvesting, cells were washed once with PBS and detached by PBS with 2 mM EDTA. After centrifugation with 200 x g for four minutes cells were washed with PBS and again pelleted by centrifugation. Supernatant was removed, cells were shock-frozen in liquid nitrogen and stored at –70°C until RNA-preparation.

3.4.2 Isolation of total RNA

Total RNA was prepared by single step isolation using TRIzol™ Reagent (Life Technologies). The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate and an improvement of the method originally described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Each cell pellet weighting ~200 mg was thawed and homogenized in 2 ml TRIzol under constant aspiration through a 20 gauge-needle. To permit the dissociation of the nucleoprotein complexes samples were incubated five minutes at room temperature. 0.4 ml of chloroform was added and tubes shaken vigorously for 15 seconds to mix the phases and then again incubated at room temperature for three minutes. Aqueous and organic phase were separated by centrifugation at 12.000 x g for 15 minutes at 4°C. The upper aqueous phase that contains the RNA was carefully aspired and transferred into a fresh tube. The total RNA in the aqueous phase was precipitated by mixing with 1 ml isopropyl alcohol. After mixing and incubation at room temperature for ten minutes, the tubes were centrifuged at 12.000 x g at 4 °C for ten
minutes. The supernatant was removed and the RNA pellet was washed once with 75% 
ethanol by short vortexing, followed by centrifugation at 7,500 x g for five minutes at 4°C. 
The pellet was air dried at 37°C and redissolved in five to ten μl RNase-free H2O.

3.4.3 Qualitative analysis of total RNA by agarose electrophoresis

To detect contamination with RNA-degrading enzymes each RNA-sample was mixed with 
ethidium bromide to a final concentration of 0.25 μg/ml and size fractionated using 1% 
Seakem LE™ agarose (BMA) in 1xTAE and 20 Volt/cm for 10 minutes in an ice-water 
bath. RNA samples with clearly visible non-degraded 18S and 28 S RNA and tRNA were 
pooled.

3.4.4 Quantification of RNA by absorption spectrometry

To determine RNA-concentration, the absorption after appropriate dilution of samples in 
H2O was measured by spectrometry at 260 nm wavelength and contamination with 
proteins was estimated by the 260/280 nm-ratio of absorption using a UV-160A 
spectrophotometer (Shimadzu). RNA-concentration (μg/ml) was calculated by division of 
the absorption at 260 nm by 0.025. This equation uses 0.025 (g/ml)^{-1}cm^{-1} as a specific 
absorption coefficient of single-stranded RNA.

3.5 Quantitative PCR

Templates for PCR were single stranded cDNA from glutamate sensitive and resistant 
HT22 cells. To determine the exponential phase of amplification, aliquots of the PCR- 
reaction were removed after 15, 20, 25, 30, 35 and 40 cycles and visualized on ethidium 
bromide stained agarose gels. Then, aliquots were removed every other cycle starting five 
cycles before the first appearance in ethidium bromide stained gels and dot blotted onto 
nylon membranes with a 96 well pin tool. Oligonucleotides specific for differentially 
expressed GPCRs, as judged by ethidium bromide stained gels, were end-labeled with 5 
μCi (γ32P) ATP using the Megaprime DNA labeling system (Amersham Pharmacia 
Biotech, UK) and hybridized to the membranes overnight at 42°C. Specific activity was 
>10⁷ cpm/pmol oligonucleotide. Membranes were washed under high-stringency 
conditions, exposed to phosphoimaging plates, and analyzed with Tina Version 2.10h 
(Raytest). The density of each spot on the blot was plotted on a semi logarithmic scale 
against the cycle number for each reaction to estimate the PCR efficiency. Values were
presumed to be valid if compared curves were parallel at a given cycle number, indicating both reactions being in the exponential phase of amplification. The difference in PCR cycles between glutamate sensitive and resistant HT22 cells was multiplied with the PCR efficiency to calculate the magnitude of regulation. This was then normalized to β-actin and gapdh expression.

All experiments showing no difference between glutamate sensitive and resistant HT22 cells were replicated once; differences were reproduced three times with two different mRNA preparations to calculate the mean of regulation ± SEM.

Specific primers corresponding to the given part of the receptors were synthesized by MWG Biotech: VPAC₁ (accession number NM_011703, forward primer (fp) 325-345 bp, reverse primer (rp) 438-457 bp, probe (p) 356-378 bp, Tm 59°C), VPAC₂ (NM_009511, fp 897-918, rp 1058-1076, p 985-1007, Tm 59°C), PAC₁ (NM_007407, fp 1004-1022, rp 1124-1144, p 1065-1086, Tm 63°C) AT₂ (NM_007429, fp 645-667, rp 781-803, p 697-717, Tm 59°C), A1 (XM_129465, fp 728-746, rp 865-886 Tm 55°C), A2A (XM_125720, fp 921-940, rp 1247-1267, p 961-980, Tm 63°C), A2B (NM_007413, fp 605-627, rp 743-762 Tm 55°C), A3 (AF069778, fp 5036-5055, rp 5289-5311, Tm 55°C), P2Y₁ (NM_008772, fp 481-500, rp 692-712, Tm 55°C), P2Y₂ (NM_008773, fp 943-963, rp 1215-1233, p 1002-1019, Tm 55°C), P2Y₄ (NM_020621, fp 771-791, rp 910-928, Tm 55°C), P2Y₆ (XM_133678, fp 744-764, rp 927-945, p 858-879, Tm 63°C), S1P₁ (NM_007901, fp 1117-1139, rp 1216-1233, p 1189-1208, Tm 55°C), LPA₁ (BC025425, fp 989-1009, rp 1121-1142, p1007-1023, Tm 55°C), S1P₃ (NM_010101, fp 683-702, rp 810-831, p 736-755, Tm 55°C), PAR₁ (NM_010169, fp 659-679, rp 1041-1057, p 989-1007, Tm 55°C), PAR₂ (NM_007974, fp 463-482, rp 586-605, Tm 55°C), PAR₃ (BC037126, fp 1137-1154, rp 1241-1262, p 1181-1203, Tm 55°C), mGlu₁ (AF320126, fp 1743-1764, rp 1826-1846, p 1784-1805, Tm 55°C), mGlu₃ (U31444, fp 5083-5101, rp 5176-5199, Tm 55°C), mGlu₅ (AF170699, fp 395-415, rp 522-541, p 415-437, Tm 59°C), mGlu₇ (XM_194317, fp 2910-2931, rp 2973-2993, p 2939-2957, Tm 55°C), CB₁ (NM_007726, fp 1073-1091, rp 1276-1296, Tm 55°C)CASR (AF128842, fp 2967-2988, rp 3118-3116, Tm 55°C), GAPDH (XM_122186, fp 1007-1026, rp 1204-1225, p 1110-1130, Tm 55°C), β-ACTIN (NM_007393, fp 817-838, rp 946-967, p 863-884, Tm 59°C)
4 Results

4.1 Oxidative Glutamate Toxicity

4.1.1 HT22 cell death from oxidative glutamate toxicity upon exposure to glutamate

In order to determine the dose- and time response of glutamate-induced cell death, 5x10^3 HT22 cells per well were seeded in 96-well microtiter plates and treated with 2.5 mM, 5 mM, and 10 mM glutamate after 24 hours. Glutamate exposure was stopped by changing the medium. Cell survival was quantified by the MTT test, 24 hours after addition of glutamate (Figure 4.1).

![Graph showing cell viability over glutamate exposure time](image)

**Figure 4.1: Time and concentration dependence of glutamate-induced cell death.** HT22 cells were treated with the indicated concentrations of glutamate for the indicated times. Glutamate exposure was stopped by changing the medium, and cell survival was measured by MTT after the completion of 24 hours. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

Two or four hours of glutamate treatment resulted only in limited cell death at all concentrations tested, whereas the next four hours led to a rapid decline in survival to ~14% and ~9% at 5 and 10 mM concentrations, respectively. Extending treatment to 24
hours led to complete cell death in the presence of 10 mM glutamate, whereas only ~50% died in the presence of 2.5 mM glutamate.

In summary, HT22 are sensitive to glutamate under the chosen conditions and the induction of cell death by either 5 mM or 10 mM glutamate was nearly completed within the first eight hours of treatment.

4.1.2 Influence of cell culture conditions on glutamate susceptibility

Next, it was investigated whether the susceptibility to glutamate was modulated by cell culture conditions. In order to answer this question, first the influence of cell density was investigated. HT22 cells were plated at different densities and treated with different concentrations of glutamate for 8 hours (Figure 4.2).

![Graph showing the effect of glutamate concentration on cell viability for different cell densities.](image)

**Figure 4.2: Glutamate susceptibility depends on culture density.** HT22 cells were seeded with different densities and treated with the indicated concentrations of glutamate for 8 hours. Glutamate exposure was stopped by changing the medium, and cell survival was measured by MTT after the completion of 24 hours. Viability of untreated cells was normalized to 100%. The graph represents the data of relative cell viability obtained from four independent experiments, each done in quadruplicate and expressed as mean ± SEM.
The LD$_{50}$ drastically increased from 2.41 mM glutamate (SEM 0.3559, n=16) for cells seeded at a density of 5x10$^3$ per well to 9.786 mM (SEM 0.2015 n=16) when 1x10$^4$ cells were seeded, and dropped to 1.912 mM (SEM 0.0109 n=16) when 2.5x10$^3$ cells were seeded.

It followed to conclude that the observed modulation of glutamate toxicity by cell density might result from increased cell-cell interactions, either in dense cultures or from pronounced modification of the growth medium by the increased numbers of cells.

To characterize the influence of growth medium, normal growth medium was replaced with defined medium either with or without different concentrations of serum, simultaneous with glutamate treatment. In defined medium, limited cell death occurred during 8 hours of glutamate application. Cell death was prominently increased by the presence of 2.5% serum (Figure 4.3). The effect was further enhanced by increasing serum concentration to 5%. Under the latter conditions, a ~10-fold reduction of cell survival induced by 5 mM glutamate was observed compared to serum-free conditions. Therefore, it also appeared that serum contained essential cofactors that modulate the susceptibility of HT22 cells to glutamate.
Figure 4.3: The effect of serum concentration on glutamate susceptibility. HT22 cells were seeded at 5x10^3 cells per well with different serum and glutamate concentrations for 8 hours. Glutamate exposure was arrested by changing the medium, and cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

To answer this question, a test was conducted to ascertain if media enriched with factors released by HT22 cells imparted protective properties. Normal growth medium was conditioned for 24 hours by confluent HT22 cultures. Twenty-four hours after plating 5x10^3 HT22 cells per well, the conditioned medium was used for glutamate treatment and cell death was compared against cultures with fresh medium or glutamate in fresh medium. Cells treated 8 hours with glutamate in the presence of HT22-conditioned medium did not die at 2.5 mM glutamate concentrations. In addition, survival at 10 mM glutamate concentrations increased significantly from ~5% to 25% (Figure 4.4).
Figure 4.4: Conditioned medium alters glutamate sensitivity. HT22 cells were seeded at 5x10^3 cells per well with different glutamate concentrations in either conditioned or fresh medium for 8 hours. Glutamate exposure was stopped by changing the medium with either fresh or conditioned medium from sub-confluent HT22 cultures, and cell survival was measured by MTT after the completion of 24 hours. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

Thus, the results appear to indicate that HT22 cells secrete factors into the growth medium that protect other cells in the vicinity from glutamate-induced cell death.

4.1.3 The depleting action of oxidative glutamate toxicity on cell glutathione levels

Oxidative glutamate toxicity, unlike glutamate excitotoxicity, is known to proceed through a mechanism independent of ionotropic glutamate-receptor activation (Murphy et al. 1989). In oxidative glutamate toxicity, elevated extracellular glutamate blocks cystine import via the System X_c glutamate/cystine antiporter (Tan et al. 2001a). This decrease of cystine levels results in GSH depletion. Due to the subsequent impairment of cellular antioxidant defence mechanisms, ROS accumulate (Tan et al. 1998a).
To verify that depletion of GSH is followed by ROS accumulation, cells were treated with or without different concentrations of glutamate for 6 hours, and then incubated with the fluorescent probe Monochlorobimane (MCB). Following incubation, glutathione levels in the cells were measured in a GEMINI Fluorescence Plate Reader, using indicated wavelengths.

Unlike older methods utilizing 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman’s reagent) in a classic oxidation or oxidation-reduction scheme, monochlorobimane permits facile detection of intracellular glutathione S-transferase (Rice et al. 1986; Cook et al. 1989). This membrane-permeable reagent has been used to measure GSH in tissue homogenates (Kamencic et al. 2000), in cultured neural cells (Devesa et al. 1993; Reichelt et al. 1997) and also in HT22 cells (Tan et al. 1998a), using flow cytometry and other fluorometric systems. It has been found that monochlorobimane readily enters cells to form a fluorescent GSH–monochlorobimane adduct that can be measured fluorometrically and that this reaction is catalyzed by glutathione S-transferase.

To measure ROS levels, cells were incubated with another fluorescent probe, DCFDA. After incubation, the cells were incubated in 7-amino-actinomycin D (7-AAD). Reactive oxygen species levels were measured at 488 nm with a flow cytometer. DCFDA is both cell-permeable and non-fluorescent until oxidized in the cytoplasm of live cells. After entering live cells, the diacetate group is cleaved by intracellular esterases. Oxidation of the reduced dye can then occur in the presence of ROS, causing the dye to fluoresce (Zhu et al. 1994).

Incubating the cells with 5 mM glutamate reduced the glutathione levels from 100% (SEM 12.244 n=12) to 10.8% (SEM 2.141 n=12), while increasing ROS levels by 108% (SEM 15.179 n=12) (Figure 4.5). In conclusion, glutamate does in fact suppress the glutathione levels within the HT22 cells, potentiate the ROS levels, and lead to cell death.
Figure 4.5: Glutathione and ROS levels change under glutamate toxicity. HT22 cells were seeded in 96-well or 6-well plates with different glutamate concentrations. (A) For GSH-measurements, the cells in 96-well plates were incubated with Monochlorobimane and then analyzed using a GEMINI Fluorescence Plate Reader. Cell survival was determined by MTT directly after the measurements. (B) For ROS measurements, cells in 6-well plates were collected and incubated with DCFDA and 7-AAD, followed by FACS analysis at 488 nm. Starting levels of untreated cells were normalized to 100%. The graphs represent the data of the relative fluorescences obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

4.1.4 Tolerance of glutamate resistant HT22 cells towards other cell death inducers

Cell selection is widely used to identify cells bearing mechanisms of resistance towards toxic substances, e.g. to characterize resistance to anti-cancer drugs in neoplastic cell lines and the cellular defence against Aβ toxicity and other forms of oxidative stress (Mulcahy et al., 1994; Sagara et al., 1998; Sagara et al., 1996).
Glutamate resistant cell lines were previously generated by Dr. Jan Lewerenz from the group of Dr. Axel Methner. Briefly, HT22 cells that were to be selected for resistance against glutamate toxicity were repetitively exposed to glutamate for 24 hours. In the time between two sequential treatments, surviving cells were allowed to multiply until the pool reached a reasonable number for the next selection. For the first round, the cells were treated with 10 mM glutamate, while the next three treatments were done with 20 mM glutamate. The last selection was expanded to 48 hours and those, which survived, were cultivated further in 10 mM glutamate. These cells were referred to as chronically resistant HT22 cells (HT22CR).

In contrast to wild type HT22 cells, HT22CR cells tolerate up to 20 mM glutamate without substantial reduction in viability (HT22 2.45% survival (SEM 0.243 n=52) vs. HT22CR 96.56% survival (SEM 4.282 n=52)) (Figure 4.6).

![Graph showing cell viability vs. glutamate concentration](image)

**Figure 4.6: Glutamate resistant and sensitive cells differ in viability under glutamate.** HT22 cells were seeded at 5x10^3 cells per well with increasing glutamate concentrations for 8 hours. Glutamate exposure was stopped by changing the medium, and cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from thirteen independent experiments, each done in quadruplicate and expressed as mean ± SEM.
To test further resistance in HT22CR, the wild type and resistant cell lines were subjected to H₂O₂ stress. Most of the HT22CR cells (62.5% (SEM 3.367 n=18)) survived a treatment with 750 µM H₂O₂, whereas only 23.4% of HT22 cells (SEM 6.091 n=18) survived at the same concentration (Figure 4.7). Consequently, LD₅₀ increased from 564 µM (SEM 3.58 n=18) in HT22, to 874 µM (SEM 3.467 n=18) in HT22CR cells.

In order to test the capacity of resistance, HT22CR cells were subjected to other kinds of stress inducers that directly induce oxidative stress. The first stress-inducing agent was a pro-apoptotic signaling sphingolipid, ceramide, that is capable of modifying the activity of a number of proteins, including receptors, ion channels, and enzymes as well as intracellular calcium levels (Colombaioni and Garcia-Gil 2004).

![Graph showing cell viability at various H₂O₂ concentrations](image)

**Figure 4.7: Glutamate-resistant cells show also resistance against hydrogen peroxide.** HT22 cells were seeded at 5×10³ cells per well with different hydrogen peroxide (H₂O₂) concentrations for 24 hours. Cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.
Ceramide has been known to act in different cellular processes leading to apoptosis, differentiation, or survival, depending on the amount that is used (Goodman and Mattson 1996; Irie and Hirabayashi 1998; Lu and Wong 2004). The apoptotic effect is mediated mainly by activation of components of the MAPK family (ERK, JNK and p38) and inhibition of the phosphatidylinositol-3-kinase/Akt pathway (Xia et al. 1995; Verheij et al. 1996; Blazquez et al. 2000; Salinas et al. 2000).

Various concentrations of ceramide for 24 hours clearly showed protection in the HT22CR cell line (Figure 4.8). At 5 μM concentration, only 27% (SEM 3.455 n=12) of the HT22 cells survived, while at the same concentration 74.2% (SEM 1.870 n=12) viability was observed in HT22CR cells. Moreover, LD_{50} increased from 2.375 μM (SEM 5.064 n=12) in HT22, to 10.23 μM (SEM 2.143 n=12) in HT22CR cells.

![Graph showing cell viability vs ceramide concentration](image)

**Figure 4.8: Glutamate-resistant cells are also resistant against ceramide.** HT22 cells were seeded at 5x10<sup>3</sup> cells per well with different ceramide concentrations for 24 hours. Cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.
Another stress-inducing agent used in this study was tunicamycin, an inhibitor of glycosylation of newly synthesized proteins in the endoplasmic reticulum (ER), which causes the accumulation of unfolded proteins in the ER leading to ER stress (Tordai et al. 1995). ER stress triggers three compensatory responses, namely, the unfolded protein response, which is mediated by increased expression of molecular chaperones such as GRP94 and GRP78/Bip (Mori 2000; Urano et al. 2000), the generalized suppression of translation (Harding et al. 1999), and mechanisms related to ER-associated degradation (Ng et al. 2000). These three protective responses act transiently to control the accumulation of misfolded proteins within the ER. Not surprisingly, sustained ER stress leads to apoptosis, which in turn results in cell death (Annis et al. 2001; Wei et al. 2001).

Application of different concentrations of tunicamycin for 24 hours was tested. High concentrations of tunicamycin, like 25 μM, led to almost complete cell death in HT22 cells (2.14% (SEM 0.691 n=12)), while reducing the viability of HT22CR cells by only 29% (SEM 3.311 n=12) (Figure 4.9).

Thus, the results show that HT22CR cells are not only resistant to oxidative glutamate toxicity, but they are also resistant to other forms of oxidative stress and apoptosis.
Figure 4.9: Glutamate resistant cells show also tunicamycin resistance. HT22 cells were seeded at 5x10^3 cells per well with increasing tunicamycin concentrations for 24 hours. Cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

4.1.5 Induction of glutamate resistance via HT22CR cell-conditioned media

After testing the resistance capacity of HT22CR cells, a test was performed to ascertain if media enriched with factors released by these cells imparted protective properties, as in the case of HT22 cells. To achieve this end, normal growth medium was conditioned for 24 hours by confluent HT22 and HT22CR cultures. Twenty-four hours after plating the HT22 cells, conditioned media were used for glutamate testing and compared to fresh media with glutamate. Interestingly, cells treated with conditioned medium from HT22CR had much higher tolerance against glutamate toxicity when compared to cells treated with conditioned medium from HT22 (Figure 4.10). Cells not only survived 2.5 mM glutamate without any loss in viability (96.08% survival (SEM 6.993 n=12)), but also the amount of glutamate tolerated increased to 40 mM (34.88% survival (SEM 12.165 n=12)). When
exposed to 10 mM glutamate, a concentration fatal under normal conditions, the conditioned medium of resistant cells showed 81.95% survival (SEM 7.964 n=12).

![Graph showing cell viability against glutamate concentration]

**Figure 4.10: Glutamate resistance can be induced by conditioning the medium.** HT22 cells were seeded at 5x10^3 cells per well with increasing glutamate concentrations in either fresh or conditioned medium from sub-confluent HT22 or HT22CR cultures for 8 hours. Glutamate exposure was stopped by changing the medium and cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

In view of these findings, a hypothesis was formed in which HT22CR cells were envisaged to release protective substances into the medium, imparting greater tolerance to cells.

### 4.1.6 Activation of CRE-dependent pathways in glutamate-resistant cells

Recent studies (Davis and Maher 1994; Maher 2001; Lewerenz et al. 2003) suggested that the protective substances, released into the medium, might protect by activating G-protein coupled membrane receptors. To better understand this possibility, a comparison was performed between the activation of signal transduction pathways involved in GPCR signaling in glutamate-sensitive versus resistant cells. With the aid of phosphorylation-specific antibodies, more phosphorylated cAMP-response element binding protein (CREB)
and decreased phosphorylation of extracellular-signal regulated protein kinases 1 and 2 (ERK1/2) were noted in the glutamate-resistant cells. In contrast, the amount of phosphorylated c-Jun N-terminal kinases/stress-activated protein kinases, total protein, and total ERK remained unchanged (Figure 4.11). CREB is mainly phosphorylated in response to the activation of Gi-coupled receptors. ERK1/2 phosphorylation can occur downstream of receptor tyrosine kinase activation, through Gi-coupled mechanisms or via activation of protein kinase C (PKC) by Gα coupled receptors (Gutkind 1998b).

**Figure 4.11: Distinct signaling pathways are activated in resistant cells compared to sensitive cells.** HT22 and HT22CR cells were seeded at a density of 5x10⁵ cells in 92 mm cell culture dishes and grown for 24 hours. Whole cell extracts were made using boiling lysate buffer and analyzed by Western blotting. The primary antibodies used were monoclonal phosho-MAPK44/42 (1:2000), a polyclonal total MAPK44/42 (ERK1/2) (1:1000), and a monoclonal phosho-CREB (1:2000). Total ERK (α-ERK) is shown as a loading control.

Next, the presumed increase of CRE activation in HT22CR cells was quantified using an independent method by transfecting a reporter vector containing a CRE element controlled EGFP (Roeder *et al.* 2004) into HT22 and HT22CR cells. HT22CR cells exhibited a 55% increase in CRE activation as judged by mean CRE-EGFP dependent fluorescence (Figure 4.12). CMV-driven EGFP fluorescence was not different in HT22 and HT22CR cells (data
not shown). Forskolin treatment served as positive control and induced CRE-EGFP fluorescence to a similar extent in both cell lines.

![Graph showing CRE induction](image)

**Figure 4.12: Glutamate resistance functions over the CRE pathway.** HT22 and HT22CR cells were transfected with a CRE-EGFP construct and incubated. The next day they were seeded at a density of 1x10^4 cells in 6-well plates and grown for another 24 hours. Cells were collected and incubated for 20 minutes with either 5μM forskolin or PBS, followed by FACS analysis at 488 nm. The graph represents the data of the relative CRE induction obtained from three independent experiments each done in triplicates as mean ± SEM.

In summary, HT22CR cells demonstrate an activation of Gₛ-coupled signal transduction pathways, which could at least in part underlie the mechanism of resistance towards oxidative stress.

### 4.1.7 Expression of GPCRs in resistant HT22 cells versus parental cell lines

The activation of Gₛ-coupled signal transduction pathways could theoretically occur via paracrine or autocrine activation of protective GPCRs by their cognate ligands or by an increase in the amount of protective GPCRs as described for group I metabotropic glutamate receptors (Sagara and Schubert 1998). To distinguish between the possibilities, a strategy was adopted in which the relative mRNA expression of 24 candidate receptors was quantified in wild type and HT22R cells by real-time PCR.
Those receptors chosen were previously reported to be involved in cell differentiation, survival, and apoptosis, or as in the case of metabotropic glutamate receptors, because that their ligand plays a role in glutamate-mediated cell death. The vasoactive intestinal peptide (VIP) receptors VPAC₁ and VPAC₂ were chosen since the peptide itself was previously reported to be involved in fetal growth (Waschek 1995) and in the suppression of inflammation and immunomodulation (Delgado and Ganea 2001). Angiotensin receptor 2 (AG₂₂) was taken, because it was reported to confer excellent neuroprotection against β-Amyloid (1-42) induced stress (Shaw et al. 2002; Shaw et al. 2003). This choice was made despite other findings that implicated angiotensin receptors in ROS production (Strawn 2002) and blocked versions of the receptor in cardiovascular protection (Ruilope et al. 2005). The nucleotide receptor P2Y6 has been described to offer neuroprotection against tumor necrosis factor α-induced apoptosis and shown to activate PKC (Kim et al. 2003a; Kim et al. 2003b). The cannabinoid receptor, CB1, was chosen since it was described to decrease glutamatergic and GABAergic synaptic transmissions resulting in protection against excitotoxicity in hippocampal neurons (Marsicano et al. 2002; Azad et al. 2003; Marsicano et al. 2003).

In the course of comparing expression levels, four receptors were upregulated less than five-, but more than two-fold. Two of these, namely, the VPAC₁ (3.6-fold, SEM 0.59) and PAC₁ receptor (2.7-fold, SEM 0.85), are activated by VIP too (Laburthe and Couvineau 2002; Laburthe et al. 2002; Gutierrez-Canas et al. 2003). The nucleotide receptor P2Y6 (3.9-fold, SEM 0.86) was also found to be upregulated while the adenosine receptor A₂A (2.2-fold, SEM 0.66) was downregulated in resistant cells. The nucleotide receptor P2Y4, the metabotropic glutamate receptor mGlu₅ and the cannabinoid receptor CB1 were found to be expressed scarcely in resistant cells. No receptor was found to be expressed only in sensitive or resistant cells (Figure 4.13).

Out of the 24 receptors chosen, three were noted to be reproducibly regulated but nonetheless differently in HT22 and HT22CR cells (Fig 4.13). The receptors for the vasoactive intestinal peptide (VIP), VPAC₂ (12.6-fold, SEM 1.8), and the metabotropic glutamate receptor mGlu₁ (5.3-fold, SEM 0.11) were found to be more prominently expressed in resistant cells, whereas the angiotensin receptor AT2 (6-fold, SEM 1.23) in sensitive cells.
Figure 4.13: GPCR regulation in resistant cells. Regulation was calculated by ΔCT R and S normalized to gapdh and β-actin expression. Expression levels between the dashed lines were defined as equal. Relative expression level is depicted to the left as: 0 for no expression; + for first detection at 40 cycles PCR; ++ for detection at 35 cycles; +++ for detection at 30 cycles. The graph represents the data of three independent quantitative PCR experiments of two different mRNA preparations and the results are expressed as mean ± SEM. Adapted from (Sahin et al. 2006).

The findings implied a clear difference in GPCR expression between the glutamate sensitive and resistant cells.

4.1.8 Activation of VIP receptors in HT22 cells

As a next step, the effect of the natural ligands of the most prominently regulated receptors on CREB and ERK phosphorylation were examined, to assess their role in the constitutive activation or inhibition observed in glutamate-resistant cells. The effect of VIP on sensitive
HT22 cells was studied under serum-free conditions to exclude the influence of VIP contained in the serum. Western blot analyzes of HT22 cells treated with VIP showed increased CREB and decreased ERK1/2 phosphorylation (Figure 4.14). Treating the cells with glutamate for 20 minutes had the same effect, probably mediated by metabotropic glutamate receptors, as these cells do not express ionotropic glutamate receptors.

![Western Blot Image](image)

**Figure 4.14: VIP and glutamate act synergistically and induce Bcl-2.** HT22 cells were seeded at a density of 5x10^5 cells in 92 mm cell culture dishes and treated with VIP (10-8 M), glutamate (2.5 mM) or both for 20 minutes. Whole cell extracts were made using boiling lysate buffer and analyzed by Western blotting. The primary antibodies used were monoclonal phospho-MAPK44/42 (1:2000), a polyclonal total MAPK44/42 (ERK1/2) (1:1000), a monoclonal phospho-CREB (1:2000), a monoclonal Bcl-2 clone 100 (1:2000), and a monoclonal β-actin (1:5000). Total ERK (α-ERK1/2) and β-actin are shown as loading controls.

Taken together, these receptors and their ligands yielded the same phosphorylation pattern that was constitutively active in glutamate-resistant HT22 cells.

### 4.1.9 Converging signaling pathway for VIP and glutamate

The antiapoptotic protein Bcl-2 is upregulated in a cAMP-dependent manner (Hui et al. 2003) and is capable of suppressing the formation of ROS (Kane et al. 1993; Ellerby et al. 1996). It could thus represent a suitable downstream mediator of GPCR signaling. Therefore, the induction of Bcl-2 by VIP and glutamate was investigated (Figure 4.14). VIP
increased the amount of Bcl-2 just like glutamate, although to a lesser extent. Again, Bcl-2 had clearly increased in resistant cells, whereas the amount of the housekeeping protein β-actin remained unchanged (Figure 4.14). The findings led to the hypothesis that VIP and glutamate signaling converge, at least in part, on the induction of Bcl-2.

To test whether this induction could confer a resistance against glutamate, Bcl-2 was transiently overexpressed in glutamate-sensitive HT22 cells (Figure 4.15). An explicit increase (up to fivefold) in viability of HT22 cells was observed under 40 mM glutamate, when transiently transfected with Bcl-2 (survival 19.15%; SEM 1.99, n=12) or an empty vector (survival 3.79%; SEM 0.89, n=12).

**Figure 4.15: Bcl-2 transfected cells show resistance against glutamate.** HT22 cells were transfected with bcl-2 or empty vector (mock) and seeded at a density of 5x10³ cells. The next day they were exposed to the indicated amounts of glutamate for 8 hours. Glutamate exposure was stopped by changing the medium, and cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.
This protection was accompanied and probably caused by a significant increase in intracellular glutathione content as monitored by monochlorobimane fluorescence in increasing amounts of glutamate (Figure 4.16).

![Figure 4.16: Glutathione levels are higher in Bcl-2 and hVPAC2 transfected cells.](image)

**Figure 4.16: Glutathione levels are higher in Bcl-2 and hVPAC2 transfected cells.** HT22 cells were transfected with bcl-2, hVPAC2 or empty vector (mock) and seeded at a density of 5x10⁵ cells. The next day they were exposed to the indicated amounts of glutamate and incubated with Monochlorobimane (MCB) before analysis using a GEMINI Fluorescence Plate Reader. Cell survival for 96-well plates was measured by MTT directly after the measurements. Starting levels of untreated cells were normalized to 100%. The graph represents the data of MCB Fluorescence obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

These findings confirmed the previous results on converging signaling pathways for Bcl-2 induction through VIP and glutamate.

### 4.2 Orphan Receptors

After identifying differing GPCR concentrations in the sensitive and resistant HT22 cell lines, the relative mRNA expression of 82 mouse orphan receptors were quantified by real-time PCR (Table 4.1). Orphan receptors are proteins that share GPCR structural motifs, but lack a defined ligand and function.
| Not Detected | BAIR3, Emr1, ETBR-LP, GPR102, GPR17, GPR20, GPR25, GPR26, GPR27, GPR30, GPR40, GPR41, GPR42, GPR43, GPR44, GPR49, GPR50, GPR55, GPR57, GPR58, GPR6, GPR62, GPR7, GPR72, GPR80, GPR81, GPR86, GPR88, GPR90, GPR91, GPR92, GPRC5C, GPRC5D, H963, HM74, LGR7, P2Y10, PNR, PSP24, SALPR, SREB3 |
| Not regulated | A-2, BAI1, BAI2, CML1, EBI2, GPR#, GPR1, GPR103, GPR18, GPR19, GPR21, GPR22, GPR3, GPR34, GPR35, GPR48, GPR52, GPR56, GPR61, GPR63, GPR73, GPR75, GPR82, GPR84, GPR85, GPR87, GPRC5B, LGR6, P2Y5, RAI3, RDC1, RE2, RRH, TM7SF3, TPRA40 |
| Regulated | Not Reproduced | GPR37, GPR101 |
| Reproduced | P2Y9, GPR15, GPR39 |

Table 4.1: Orphan GPCR regulation in resistant cells. Orphan GPCRs that were not detected were marked as not detected, while receptors that were detected were grouped according to their regulation. Regulation was calculated by the difference in cycle thresholds (ΔCT) of HT22CR and HT22, normalized to GAPDH and β-actin expression. The table represents the data of three independent quantitative PCR experiments with two different mRNA preparations.

Most had been identified by methods like degenerate PCR, exploiting conserved amino acid residues (Methner et al. 1997; Hermey et al. 1999). Two receptors, namely GPR39 and GPR15, were found to be upregulated 4-fold, while another receptor, P2Y9, was upregulated 2.5-fold (Figure 4.17).
Figure 4.17: Quantification of regulation observed for GPCRs in resistant cells over sensitive cells. Regulation of orphan receptors was calculated by the difference in cycle thresholds (ΔCT) of HT22CR and HT22, normalized to gapdh and β-actin expression. The graph represents the data of three independent quantitative PCR experiments with two different mRNA preparations.

The P2Y9 receptor was recently characterized by another group as the lysophosphatidic acid receptor 4 (LPA4) (Noguchi et al. 2003). Therefore, the two-upregulated orphan receptors were used for further studies.

4.2.1 Orphan GPCR induction of glutamate resistance towards hydrogen peroxide

In order to test whether the cloned GPCRs had an effect on cell survival under oxidative stress, HT22 cells were transiently transfected by electroporation. To realize transfection, pcDNA5 FRT/TO loxP SPHA mGPR15, pcDNA5 FRT/TO loxP SPHA mGPR39, pcDNA5 FRT/TO loxP SPHA hVPAC2 and pcDNA5 FRT/TO loxP SPHA (mock) were used.
Cells transfected with mGPR39 showed the highest amount of cell survival, similar to resistant HT22 cells, while mGPR15 transfected cells had almost no effect at all (Figure 4.18). At 20 mM glutamate concentration, a 40% survival rate (SEM 2.264 n=12) was observed in mGPR39 transfected cells, whereas only 2% (SEM 0.683 n=12) of mGPR15 transfected cells could survive. The human vasointestinal peptide receptor, hVPAC₂, was used as a control and showed similar protection (27% survival at 20 mM (SEM 3.190 n=12)) against glutamate-induced oxidative toxicity as mGPR39.

![Graph showing cell viability vs glutamate concentration](image)

**Figure 4.18: Transient GPCR transfection confers resistance against glutamate.** HT22 cells were transfected with hVPAC₂, mGPR15, mGPR39, or empty vector (mock) and seeded at a density of 5x10³ cells. The next day they were exposed to the indicated amounts of glutamate for 8 hours. Glutamate exposure was stopped by changing the medium, and cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

Moreover, mGPR39 and hVPAC₂ protected the cells against hydrogen peroxide (H₂O₂), even under high concentrations (Figure 4.19). At 934 μM, mGPR39 conferred a 2-fold protection compared to mock transfected cells (mGPR39 66.6% survival (SEM 5.445 n=12) vs. mock 34.8% survival (SEM 3.288 n=12)). As mGPR15 did not confer any resistance, further experiments were focused on mGPR39.
Figure 4.19: Resistance against hydrogen peroxide induced by transient transfections. HT22 cells were transfected with mGPR39, hVPAC2, or empty vector (mock) and seeded at a density of 5x10⁵ cells in 96-well plates. The next day they were exposed to the indicated amounts of hydrogen peroxide for 24 hours. Cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

In conclusion, these findings implied that resistance against oxidative glutamate toxicity, and hydrogen peroxide, could be induced by activating or overexpressing GPCRs.

4.2.2 Mechanism of protection in HT22 cells

To elucidate the mechanisms involved at the cellular level for the protection observed by mGPR39, ROS and glutathione levels were measured in HT22 cells using transient transfections of hVPAC2, Bcl-2, mGPR39, and an empty vector.

Glutathione levels in mGPR39 and hVPAC₂ transfected HT22 cells were higher than in mock transfected HT22 cells, and Bcl-2 transfected cells had higher glutathione levels than the rest (Figure 4.20). At 2.5 mM glutamate, Bcl-2 transfected cells had 40% more glutathione than mGPR39 or hVPAC₂ transfected cells (Bcl-2 71.70% (SEM 8.909 n=24) vs. mGPR39 49.56% (SEM 4.720 n=24) and hVPAC₂ 33.67% (SEM 2.767 n=24)).
**Figure 4.20: Glutathione levels are elevated in mGPR39 transfected cells.** HT22 cells were transfected with bcl-2, mGPR39, hVPAC2 or empty vector (mock) and seeded at a density of 5x10^3 cells. The next day they were exposed to the indicated amounts of glutamate and incubated with Monochlorobimane before analysis using a GEMINI Fluorescence Plate Reader. Cell survival was determined by MTT directly after the measurements and used for normalizations. The graph represents the data of the relative MCB fluorescence obtained from six independent experiments, each done in quadruplicate and expressed as mean ± SEM.

Moreover, mGPR39 transfected cells had less intracellular ROS than mock-transfected cells (mock, 242.7% survival; SEM 13.332 n=12) vs. mGPR39, (183.8% survival; SEM 8.319 n=12)), and the rest of the transfected cells (Bcl-2 (213.6% survival; SEM 22.916 n=10) and hVPAC2 (225% survival; SEM 9.703 n=10)) (Figure 4.21).
**Figure 4.21: mGPR39 protects cells against glutamate by increasing the glutathione content.**

HT22 cells were seeded in 6-well plates with indicated glutamate concentrations, collected, and incubated with DCFDA and 7-AAD, followed by FACS analysis at 488 nm. Starting levels of untreated cells were normalized to 100%. The graph represents the data of the relative DCFDA fluorescence obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

As a result, mGPR39 protected the cells as well as Bcl-2 by increasing the glutathione content and reducing ROS levels within the cells.

### 4.2.3 Expression of mGPR39 throughout the development

The GPR39 receptor was detected in and cloned from the mouse hippocampal cell lines HT22 and HT22CR. To get insight into a possible role in development, *in situ* hybridizations on sections of mouse embryos were performed. Starting from E12.5, mGPR39 mRNA was detected in both central and peripheral nervous systems, and epithelial tissues (Figure 4.22).
Figure 4.22: Expression of mGPR39 during mouse embryogenesis. Autoradiograms of parasagittal sections through mouse embryos are shown at the stages indicated (E12.5, E14, E16.5, P0). Abbreviations: cc, cerebral cortex; i, intestine; k, kidney; l, lung; li, liver; mle, myelencephalon; msc, mesencephalon; ret, retina; sc, spinal cord; thy, thymus. Scale bar is 2 mm.

The cerebral cortex and midbrain showed more intense labeling than other brain areas. Starting at E12.5 the hybridization intensity of mGPR39 in the cerebral cortex and midbrain regions increased at each stage. The most intense labeling was observed in epithelial tissues (liver, kidney, stomach, intestine) and declined at further stages. Further signals were detected in trigeminal ganglion, spinal cord, olfactory bulb, tongue epithel.

Figure 4.23: Photoemulsion-dipped sagittal sections of mouse embryos. Expression of mGPR39 in distinct epithelial tissues throughout the development is shown in sagittal sections. Abbreviations: bl, bladder; drg, dorsal root ganglion; fi, future intestine; i, intestine; k, kidney; l, lung; li, liver; sc, spinal cord; te, tongue epithelial cell layer.

Analysis of emulsion-dipped sections revealed strong signals in dorsal root ganglia, spinal cord, kidneys, lung, liver, intestine, and bladder (Figure 4.23A-E). Analysis of the brain and
central nervous system revealed signals in the retina and retinal pigment epithelium (RPE) (Figure 4.24D), and cerebral cortex, hippocampus, amygdala, piriform cortex, Purkinje and Granular cell layers, as well as dentate gyrus (Figure 4.24A-C).

![Figure 4.24: Photoemulsion-dipped sections through mouse brain.](image)

**Figure 4.24: Photoemulsion-dipped sections through mouse brain.** Dark-field photomicrographs of photoemulsion-dipped sections through adult and developing (C) mouse brains are shown. Abbreviations: a, amygdala; cc, cerebral cortex; dg, dentate gyrus; gc, Granular cell layer; hi, hippocampus; hn, medial habenular thalamic nuclei; pc, Purkinje cell layer; pir, piriform cortex; r, retina; rpe, retinal pigment epithelium.

### 4.2.4 Stable HT22 cell lines

After observing the induced protection and the expression pattern through different developmental stages, an HT22 cell line overexpressing mGPR39 was created. For this purpose, the Retroviral Gene Transfer and Expression System (BD Biosciences) vector pQCXIP and the Phoenix Retroviral Expression System (Orbigen Inc) were used together. Both systems consist of a HEK-293-based packaging cell line that stably expresses the viral gag, pol, and envelope genes. To produce infectious virus, a retroviral expression vector has to be transfected into this cell line, using the viral envelopes’ ability to infect other cells. The retroviral expression vector, pQCXIP, contains the extended retroviral packaging signal, which promotes high-titer virus production. The vector itself is a self-inactivating bicistronic expression vector that is driven by a CMV promoter and has an internal ribosome entry site (IRES) followed by the Puromycin gene for conferring antibiotic resistance. The viruses produced possess an ecotropic envelope (gap70), and thus can infect only mouse and rat cells.
In total, 55 colonies were removed from the transfected HT22 cells that survived puromycin stress. These colonies were further propagated until they reached confluency and then transferred into 24-well plates and from there to 6-well plates. In the end, there were 37 surviving colonies, and these were amplified until they could be tested for mGPR39 expression.

4.2.4.1 Verification of the Stable Cell Lines

To verify the stable integration of SP HA mGPR39 into the HT22 cells, FACS analysis was performed. HT22 clones that were kept constantly under Puromycin stress were analyzed by FACS for surface expression, using a monoclonal mouse antibody against the hemagglutinin (HA) tag (Covance) and Alexa Fluor anti-mouse secondary antibody (data not shown).

Among the 37 clones, six clones were utilized in glutamate toxicity experiments: Clones 1 and 34 for low-level expression, clones 7 and 26 for mid-level expression, and clones 17 and 18 for high-level expression. Of the six clones, the least and most HA expressing clones turned out to confer the lowest and highest level of protection against glutamate toxicity, respectively (data not shown).

The two clones that conferred the most and least protection were then analyzed by FACS, using a polyclonal rabbit anti-GPR39 antibody (Acris Antibodies). Clone 1 showed an upregulation of 243-fold in mGPR39 surface expression, while clone 17 showed an upregulation of 345-fold (Figure 4.25). In contrast, HT22CR cells had a 1.8-fold upregulation in mGPR39 expression over HT22 cells.
**Figure 4.25: mGPR39 upregulation in stable HT22 cells.** The most and the least HA-overexpressing clones were selected and seeded in 6-well plates at a density of 1x10⁶ cells. The next day they were incubated with a polyclonal anti-GPR39 antibody (1:1000) and analyzed by FACS at 488 nm. The graph represents the data of the relative GPR39 expression obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

### 4.2.4.2 HT22 cells stably overexpressing mGPR39 show resistance to stress inducers

In order to test the capacity of resistance, newly created stable cell lines were subjected to different kinds of stress inducers. The first one to be tested was oxidative glutamate toxicity, the classical paradigm where mGPR39 was identified. Both clones conferred resistance even under 40 mM glutamate after 8 hours, while HT22 cells could not survive 20 mM glutamate for the same duration (Figure 4.26). Clone 1 had a viability of 51.83% (SEM 2.453 n=12), while clone 17 yielded 66.71% survival (SEM 4.317 n=12) under 40 mM glutamate.
Figure 4.26: Glutamate resistance in selected clones. HT22 clones overexpressing mGPR39 stably were seeded at a density of 5x10^3 cells in 96-well plates. The next day they were exposed to the indicated amounts of glutamate for 8 hours. Glutamate exposure was stopped by changing the medium and cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

After testing for glutamate, the protection potential of the clones against hydrogen peroxide was investigated. Although both clones protected the cells from hydrogen peroxide, the protection was less than expected (Figure 4.27). After 24 hours of 1mM hydrogen peroxide treatment, 9.85% (SEM 1.542 n=12) of the cells in clone 1 and 16.54% (SEM 2.646 n=12) of the cells in clone 17 could survive.
Figure 4.27: Overexpression of mGPR39 resulted in hydrogen peroxide resistance. HT22 clones 1 and 17 were seeded at a density of 5x10^3 cells in 96-well plates. The next day they were exposed to the indicated amounts of hydrogen peroxide for 24 hours. Cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graph represents the data of the relative cell viability obtained from three independent experiments, each done in quadruplicate and expressed as mean ± SEM.

Ceramide and tunicamycin toxicities did not change the protection observed in the clones. Both clones were protected against high doses of ceramide and tunicamycin (Figure 4.28). Interestingly, ceramide stress had no effect on all the cells until a concentration of 2.5 μM, and even at this concentration clones 1 and 17 showed very little loss in viability (Clone 1 82.92% (SEM 8.115 n=12) vs. clone 17 88.40% (SEM 9.624 n=12)). Increasing the concentration to 5 μM decreased the viability of the clones 1 and 17 to 32.64% (SEM 1.859 n=12) and 37.15% (SEM 6.893 n=12), respectively.

Tunicamycin decreased the viability of HT22 cells abruptly to 12.85% (SEM 4.603 n=12), starting at a concentration of 5 μM, while clone 1 had a viability of 73.53% (SEM 1.963 n=12) and clone 17 a viability of 76.01% (SEM 2.641 n=12). Both of the clones could protect the cells over 50% until a concentration of 50 μM, where the viabilities decreased to 6.54% (SEM 1.577 n=12) and 11.47% (SEM 1.022 n=12).
In conclusion, stable transfections of mGPR39 conferred protection against different kinds of cell death inducers, as observed by the resistant cell line.

Figure 4.28: Tunicamycin and ceramide toxicities in stable cell lines. HT22 clones 1 and 17 were seeded at a density of 5x10^3 cells in 96-well plates. The next day they were exposed to the indicated amounts of (A) tunicamycin or (B) ceramide for 24 hours. Cell survival was measured by MTT. Viability of untreated cells was normalized to 100%. The graphs represent the data of the relative cell viabilities obtained from five independent experiments, each done in quadruplicate and expressed as mean ± SEM.

4.2.5 Implications related to mGPR39 and its potential participation in other diseases

Since mGPR39 was expressed abundantly on epithelial tissues and the retina during development (Figure 4.24D), tests were carried out to determine where mGPR39 was expressed in the adult retinal pigment epithelium. The retinal pigment epithelium (RPE) describes a monolayer of pigmented cells forming a part of the blood/retina barrier (Strauss 2005). The apical membrane of the RPE faces the photoreceptor outer segments. Long
apical microvilli surround the light-sensitive outer segments, establishing a complex of close structural interaction.

As a layer of pigmented cells, the RPE absorbs the light energy focused by the lens on the retina (Boulton and Dayhaw-Barker 2001). It also transports ions, water, and metabolic products from the subretinal space to the blood (Marmor 1999; Hamann 2002). Most importantly, retinal is constantly exchanged between photoreceptors and the RPE (Baehr et al. 2003). As oxygen and light energy are readily available, the RPE also defines another main site to potentially produce oxidative stress agents. An increase in oxidative stress due to a reduction of protective mechanisms or an increase in number and concentration of active photo-oxidative reaction species can contribute to a disease called age-related macular degeneration (AMD), the most common cause for blindness in industrialized countries (Beatty et al. 2000; Ambati et al. 2003). The disease starts mostly with the accumulation of lipofuscin in the RPE, and develops by a chain of events that alter the RPE in an age-dependent manner (Delori et al. 2001; Hageman et al. 2001). These events include, but are not limited to, reduction in cell density due to apoptosis (Delori et al. 2001) and change in pigmentation (Feeney-Burns et al. 1984), resulting in RPE and photoreceptor loss over large areas.

To show the presence of mGPR39 in the RPE, cDNA from RPE cells with specific GPR39 primers was used in PCR amplification. The data obtained by this approach showed that the RPE contains mGPR39 (Figure 4.29A). In order to verify the results, FACS-Analysis using a GPR39-specific antibody was carried out (Figure 4.29B). RPE cells, containing vast amounts of pigments, are dark in colour and able to fluorescence. An increase in fluorescence could be observed when the specific antibody was added, indicating the presence of mGPR39 in the RPE.
**Figure 4.29: mGPR39 is present in the RPE.** (A) Agarose gel showing evidence of mGPR39 PCR-product. cDNA prepared from HT22 (control) and RPE cells were used as a template for a PCR experiment with GPR39 specific primers. The bands described the expected sizes (molecular weight markers not shown). (B) RPE cells were freshly prepared and incubated with a polyclonal GPR39 antibody followed by FACS analysis at 488 nm. The graph represents the data of the relative GPR39 fluorescence obtained from three independent experiments each done in triplets as mean ± SEM.
5 Discussion

5.1 HT22 cells die from oxidative glutamate toxicity upon exposure to glutamate

Glutamate can induce cell death in neuronal cells either via excitotoxicity or oxidative glutamate toxicity (Choi 1988; Murphy et al. 1989; Murphy et al. 1990; Coyle and Puttfarcken 1993). Both ways differ in the concentration of glutamate and the duration of exposure needed to induce cell death.

Mature cortical neurones that are susceptible to excitotoxic glutamate toxicity face a rapid cell death, prominent within the first hour after glutamate exposure, at micromolar concentrations of glutamate with an exposure of only a few minutes, via activation of ionotropic glutamate receptors (Schubert and Piasecki 2001).

HT22 cells express neither an ionotropic glutamate receptor nor die in response to agonists of ionotropic glutamate receptors (Maher and Davis 1996). The glutamate-induced cell death observed in HT22 cells has been shown to occur via oxidative glutamate toxicity (Tan et al. 2001b). The glutamate concentration and duration of treatment sufficient to induce a cell death program in HT22 cells by oxidative glutamate toxicity were reported to be 5 mM glutamate for seven hours (Davis and Maher 1994; Tan et al. 2001a). In our hands, 10 mM and eight hours of glutamate treatment were sufficient to exert almost maximal cell death (Figure 4.1). This increase in tolerance can be attributed to different kinds and concentrations of serum used (Figure 4.3).

Analyzes using fluorescent dyes (DCFDA and MCB) in flow cytometry and fluorescence plate readers confirmed the decrease of glutathione and ROS accumulation after glutamate challenge in HT22 cells (Figure 4.5). Thus, findings related to glutamate-induced cell death under the conditions used in this work are consistent with the work done by other groups in the sense that cell death is mediated by the oxidative pathway.
5.2 Glutamate susceptibility of HT22 cells is modulated by cell culture conditions

Many neuronal cells are dependent on secreted survival signals that counteract otherwise detrimental effects resulting in programmed cell death (Raff et al. 1993). Furthermore, factors promoting survival were described to act in autocrine and paracrine manners (Desire et al. 2000; Kriegstein et al. 2002). On the other hand, decreased cell density can trigger apoptosis by soluble factors or may induce an increased susceptibility to apoptosis-inducing agents in non-neuronal cells (Saeki et al. 1997; Washo-Stultz et al. 2000). In retinal epithelial cells, programmed cell death due to oxidative stress was reduced in denser cultures and accompanied by an increased synthesis of FGF2, which acts as a survival-promoting factor for these cells (Bryckaert et al. 1999; Wada et al. 2001).

Thus, the attenuation of glutamate-induced cell death by a higher cell density (Figure 4.2), might be explained through the release of survival promoting factors by the HT22 cells in an auto- and paracrine fashion. This hypothesis could be further substantiated by the protective effect of medium conditioned by confluent cultures (Figure 4.4 and 4.10). Further experiments comparing the conditioned media taken from sensitive and resistant HT22 cells could identify the factors important for cell survival in this context. Alternatively, HT22 cells might remove substances from the growth medium that otherwise promote programmed cell death.

Studying the impact of serum concentration on glutamate-induced cell death, it was clearly shown that major cofactors for the initiation of glutamate-induced cell death originating from fetal calf serum are present in the growth medium (Figure 4.3). Although the identity of these substances is not known, catecholamines may represent, at least partially, these death-promoting cofactors: They are present in serum and, their metabolism with monoamine oxidases produces H2O2 (Weyler et al. 1990; Singer and Ramsay 1995). In addition, the inhibition of catecholamine transporters in HT22 cells was reported to protect these from oxidative glutamate toxicity (Maher and Davis, 1996).

Moreover, serum may contain growth factors that were shown to intensify glutamate-toxicity (Schubert et al. 1992). Serum and growth factors are potent inducers of the transcription factor AP-1. AP-1 was recognized as one of the key regulators of life and death in a cell. It was reported to induce the transcription of both pro- and antiapoptotic
gene products whose final balance was context-dependent (Shaulian and Karin 2002). This is supported by the observation that growth factors mediating survival in serum deprivation can also induce cell death in models of ischemic stroke and excitotoxicity (Koh et al. 1995). The context-dependence of the biological action of some factors can be further exemplified by nitric oxide. Nitric oxide was reported to switch from a protective agent to a death promoter in GSH-depleted mid-brain cultures (Canals et al. 2001a; Canals et al. 2001b).

Glutathione was shown to be depleted in oxidative glutamate toxicity, previously (Tan et al. 2001a). Elevated extracellular glutamate blocks the cystine import via the System \( X_c^- \) glutamate/cystine antiporter and the subsequent decrease in cystine results in GSH depletion. The same results were obtained in our hands (Figure 4.5), leading to ROS accumulation in the cells, and inevitably to cell death.

Considering the similarities, signaling induced by serum-derived factors that support survival under normal circumstances might as well be interpreted as a death-promoting signal by the HT22 cells during oxidative glutamate toxicity.

5.3 Resistant HT22 cells differ from the parental cell line in their GPCR pattern

Next to growth factor receptors, belonging to the family of receptor tyrosine kinases (Schlessinger and Ullrich 1992), GPCRs are a family of candidate receptors that may account for the modulation of susceptibility in HT22 cells under cell culture conditions. Serum contains a vast amount of substances that are ligands of GPCRs and thus, activate G-protein dependent signaling cascades (Milligan 1987; Bogoyevitch et al. 1995; Wenzel et al. 2005). Activation or inactivation of different types of GPCRs had been reported to protect the cells in different models of neurodegeneration (Bond et al. 1998; Jolkkonen et al. 1999; Kimura et al. 2001). In addition, the activation of metabotropic glutamate receptor 1 and 5 (Sagara and Schubert 1998), the dopamine D4 receptor (Ishige et al. 2001), and stimulation of the GPCR-activated protein kinase C (Davis and Maher 1994; Maher 2001) were reported to protect HT22 cells and immature neurones from oxidative glutamate toxicity.
To investigate the role of GPCRs in glutamate-induced oxidative toxicity, the glutamate resistant cell line HT22CR, created by Dr. Jan Lewerenz in the group of Dr. Axel Methner, was used. Repetitive treatment with high doses of glutamate, followed by expansion of the surviving cells, led to the glutamate resistant cell line HT22CR. In line with previous results (Sagara and Schubert 1998), evoked resistance was not specific to glutamate as the selected cells were also resistant to cell death induction by direct exposure to H₂O₂ (Figure 4.7) or other stress-inducing agents like ceramide and tunicamycin (Figure 4.8 and 4.9).

As an initial step, the activation of signal transduction pathways involved in GPCR signaling was compared, using phosphorylation-specific antibodies (Figure 4.11). An increase in phosphorylated cAMP-response element binding protein (CREB) levels hinted towards an activation of Gₛ-coupled receptors, which has been described to confer neuroprotection in other systems (Walton et al. 1996; Lee et al. 2005; Papadia et al. 2005) and in HT22 cells (Lewerenz et al. 2003). A reduction in phosphorylation of extracellular-signal regulated protein kinases 1 and 2 (ERK1/2) confirmed this result, as ERK1/2 phosphorylation is known to occur downstream of receptor tyrosine kinase activation, through Gₛ-coupled mechanisms or via activation of protein kinase C (PKC) by the G₉₆-coupled receptors (Gutkind 1998a). The exact mechanism is still not known, as several studies have shown phosphorylation to occur through the βγ-subunits without PKC activation (Crespo et al. 1994; Koch et al. 1994). Nevertheless, these results were in line with previous findings on glutamate-induced oxidative toxicity (Maher 2001; Lewerenz et al. 2003).

As the observed activation of Gₛ-coupled signal transduction pathways could occur through the activation of protective GPCRs by their cognate ligands or an increase in the expression of GPCRs, the relative mRNA expression of 24 candidate receptors was quantified (Figure 4.13). These receptors were chosen for their roles in cell differentiation, survival, and apoptosis. Out of the 24 receptors, the receptors for the vasoactive intestinal peptide (VIP), VPAC₂, and the metabotropic glutamate receptor mGlu₁ were found to be upregulated in the resistant cells, while the angiotensin receptor AT2 was expressed to a higher extent in sensitive cells.

From these results, it was hypothesized that in glutamate-resistant cells protective GPCRs are upregulated, while detrimental receptors are downregulated.
5.4 VPAC$_2$ – a model neuroprotective receptor

The vasoactive intestinal peptide (VIP) is a well-known neuropeptide that has a wide distribution in peripheral and central nervous systems and a large spectrum of biological actions in mammals. It has been described to have effects in the digestive tract, cardiovascular system, airways, reproductive system, immune system, endocrine glands and brain (Rayan et al. 1991). Besides its short-term actions on exocrine secretions and hormone release, VIP has also been characterized as a growth regulator for fetuses (Gressens et al. 1993; Waschek 1995) and a regulator in the suppression of inflammation and immunomodulation (Delgado and Ganea 2001).

Following the cloning of the first VIP receptor (later termed VPAC$_1$) from a rat cDNA (Ishihara et al. 1992) other orthologues (Sreedharan et al. 1993; Couvineau et al. 1994), and a second VIP receptor (later termed as VPAC$_2$) were cloned (Lutz et al. 1993). Both receptors bind to the neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) with similar affinities, VIP having a slightly higher affinity than PACAP (Vaudry et al. 2000).

It was early identified that cellular action of VIP induces a robust increase in cyclic AMP production in target cells (Laburthe et al. 1978) and, it still represents the signaling pathway for VIP through the VPAC receptors. This increase in cyclic AMP production was shown to activate adenylyl cyclase, which further activated protein kinase A (Shreeve et al. 2000). Furthermore, it was clearly demonstrated that both of these receptors couple mainly to stimulatory type G-proteins (Gs) when transmitting a signal (Luo et al. 1999; Shreeve et al. 2000).

To demonstrate the action of VIP in glutamate-induced oxidative toxicity, cell extracts from glutamate-sensitive cells were analyzed with or without the addition of VIP and glutamate (Figure 4.14). It was shown that by the addition of VIP and even glutamate, CREB phosphorylation could be increased while ERK1/2 phosphorylation was decreased. This was similar to the phosphorylation pattern observed in HT22CR cells. Interestingly, adding both VIP and glutamate substantiated this effect, suggesting a synergistic activity.

Because Bcl-2 expression is regulated by the transcription factor cyclic AMP response element-binding protein (CREB) (Pugazhenthi et al. 2000; Freeland et al. 2001), Bcl-2
expression levels were also checked. As expected, the addition of VIP and/or glutamate increased Bcl-2 expression in HT22 cells, underlining the synergistic activity of glutamate and VIP. According to these results, it was hypothesized that VPAC$_2$ activation through VIP would lead to Bcl-2 activation, which in turn would result in a protective effect in glutamate-induced oxidative stress.

In order to test the hypothesis, HT22 cells were transfected with Bcl-2 or VPAC$_2$ (Figure 4.15 and 4.16). Bcl-2 transfection not only increased the viability of the cells under glutamate, but also resulted in higher glutathione levels in the cell. This result was in line with previous work done (Pugazhenthi et al. 2003). Furthermore, the same results, though to a lesser extent, were observed in the VPAC$_2$ transfected cells. Although an increase of glutathione levels through VIP had been described (Offen et al. 2000), and protection mediated by VIP is a well-known phenomenon (Brown 2000; Steingart et al. 2000), the mechanism of protection was not characterized. Studies done on the VIP receptors and neuroprotection, suggested a protection through the VPAC$_1$ receptor, as in the case of serum-withdrawal (Gutierrez-Canas et al. 2003), but not the VPAC$_2$ receptor.

In conclusion, it was clearly shown that VPAC$_2$ activation leads to neuroprotection and functions at least partially over the Bcl-2 pathway.

5.5 Resistance conferred by the Upregulated Orphan Receptors

The difference in GPCR patterns between glutamate-sensitive and resistant HT22 cells, led to the investigation of orphan receptors in mouse, with the aim of finding new receptors that could lead to neuroprotection. By using the same method, three receptors were found to be upregulated in glutamate-resistant HT22 cells: mGPR15, mGPR39, and mP2Y9 (Figure 4.17). P2Y9 receptor was later characterized by another group as the lysophosphatidic acid receptor 4 (LPA4), increasing the adenylate cyclase activity within the cell (Noguchi et al. 2003). A neuroprotection mediated by the LPA4 receptor has not been reported yet. As P2Y9 (LPA4) was de orphanized, studies were concentrated on mGPR15 and mGPR39.

As a first step, cells transfected with mGPR15, mGPR39, and hVPAC$_2$ were subjected to different concentrations of glutamate. While mGPR39 protected the cells even under high concentrations, mGPR15 had almost no effect at all. The positive control, hVPAC$_2$, had
the same effect as mGPR39, though surprisingly to a lesser extent (Figure 4.18). This result remained the same under hydrogen peroxide toxicity, but this time the only difference between mGPR39 and hVPAC2 was seen at 934 μM (Figure 4.19).

After observing protection against both glutamate and hydrogen peroxide, the transfected cells were used to investigate two main oxidative stress indicators: glutathione and ROS. A difference in cellular glutathione levels had already been shown in cells transfected with hVPAC2 and Bcl-2 (Figure 4.16). Cells transfected with Bcl-2 had the highest amount of cellular glutathione, followed by mGPR39- and hVPAC2-transfections with higher glutathione contents than mock-transfected cells (Figure 4.20). Interestingly, mGPR39 transfected cells had the lowest amount of ROS levels in the cells, while Bcl-2 and hVPAC2 transfected cells had higher values.

These differences could be due to the expression and activation requirements of GPCRs, as they have to be transported to the cell surface to become fully activated while Bcl-2 becomes active once it is expressed within the cell. Next, once activated both of the GPCRs have to function over some G-proteins and effectors, which takes longer than the effect observed by Bcl-2. Furthermore, the presence of an endogenous ligand in the medium would result in direct activation of the receptors and once expressed, hGPR39 was shown to be constitutively active (Holst et al. 2004).

As a next step, the expression of mGPR39 during mouse development was investigated. GPR39 was expressed predominantly in epithelial tissues, with a consistent intensity through different developmental stages (Figure 4.22). Starting as early as E12.5, the signals could be detected in tissues forming later the intestine, lungs, liver, kidneys, bladder, stomach, spinal cord, and the ribs. In the brain, cerebral cortex, hippocampus, cerebellum and amygdala were labelled at all stages of development. Later in development, signals could be detected in the trigeminal ganglion, cochlea and cochlear gang, as well as the olfactory bulb (not shown). Starting at E14, signals could also be detected in the retina and the epithelium surrounding the retina. These signals indicated a role for mGPR39 in various functions within the development, concentrating on the gastrointestinal tract.

While this work was in progress, mGPR39 was characterized as the receptor for obestatin (Zhang et al. 2005), a peptide opposing the effects of ghrelin, suppressing food intake, inhibiting jejunal contraction, and decreasing body-weight gain. This is in line with our findings, since mGPR39 is mainly expressed in the gastrointestinal tract in our studies.
Furthermore, oxidative stress is known to take a role in intestinal ischemia (Aw 1999), radiation enteritis, sepsis (Sener et al. 2005), inflammatory bowel disease and the promotion of gastric and colorectal cancer (Thomson et al. 1998; Zhou et al. 2005).

For a more determined investigation of the mechanism of protection mediated by mGPR39, HT22 cells stably overexpressing this receptor were generated.

5.6 HT22 cell lines stably overexpressing mGPR39

HT22 cells overexpressing mGPR39 were generated using a retroviral expression system. The cells generated expressed not only mGPR39 but also EGFP, since a construct containing EGFP was co-transfected as a marker for positive transfection. Positive clones were investigated for surface HA expression using FACS-Analysis and the best six clones were selected for further investigations. These clones were selected according to their HA-expression levels, with two clones with low-, mid- and high-level expression, respectively.

The selected clones were first subjected to glutamate and then to hydrogen peroxide (not shown). Between these six clones, clones 1 and 17 showed the least and most protection, respectively. As this result was in line with surface HA-expression levels, these two clones were selected for further analyzes. Before progressing with other experiments, these clones were tested for their surface mGPR39 expression levels by FACS analysis using a GPR39-specific antibody directed against the third extracellular loop, that became available recently (Figure 4.25). As expected and in line with HA-expression levels, clone 1 had less mGPR39 expressed on its surface when compared to clone 17, though both levels were much higher than in both HT22 and HT22CR. These clones showed also resistance against hydrogen peroxide (Figure 4.27), tunicamycin (Figure 4.28A) and ceramide (Figure 4.28B).

Although a significant difference between the cell viabilities of the clones could not be observed, both clones conferred resistance against different forms of oxidative stress inducers when compared to HT22 cells. Further experiments using these clones would not only identify the pathways responsible for the observed protections, but would also clarify the molecular bases of the induced stress forms. Alternatively, knockout cells could be generated with the aim of clarifying the signaling pathways involved in mGPR39 signaling.
5.7 Implications for a role in diseases

In addition to HT22CR cells, mGPR39 was found to be upregulated in various tumor cell lines (Bayer Pharmaceuticals, personal communication), and in the retina during development (Figure 4.24D). Using PCR and FACS-Analysis, the presence of mGPR39 in the retinal pigment epithelium was proven (Figure 4.29A-B). As RPE cells contain the pigments required for vision, they can fluoresce under certain wavelengths. This was also the case in our hands, as freshly prepared mouse RPE cells without any antibodies or dyes fluoresced to a certain extent. Addition of a GPR39-specific antibody increased the fluorescence, albeit slightly.

As mGPR39 protected HT22 cells against oxidative stress, it was postulated that it could have a similar function in RPE, as a defence mechanism against age-related macular degeneration (AMD). Moreover, its upregulation in various tumor cell lines indicates an important role in cancer. Future investigations concerning the role of mGPR39 in this context could be of importance for the identification of new pathways and drugs.
6 Summary

The aim of this study was to demonstrate a protective effect against oxidative glutamate toxicity by G-protein coupled receptors, and to characterize the mechanisms of protection mediated by the orphan mouse GPCR, GPR39.

Cell selection for resistance is widely used to characterize cellular mechanisms of defence against toxicity or forms of oxidative stress. Therefore, HT22CR cells which are chronically resistant to glutamate toxicity were used for comparisons to the parental HT22 cells line. Medium conditioned by confluent HT22 or HT22CR cells protected low-density cultures, even under high concentrations of glutamate. Under serum-free conditions, the glutamate susceptibility was low, increasing serum concentrations prominently exacerbated cell death, indicating that serum-derived death promoting factors modulate the susceptibility to oxidative glutamate toxicity.

To investigate whether G-protein coupled receptors are involved in the propagation of this modulation, cellular pathways activated through GPCRs were compared between sensitive and resistant cells. An activation of the cAMP pathway was observed, monitoring the phosphorylation state of distinct members of different signaling cascades. The activation was most probably due to Gs stimulation which was confirmed by CRE induced EGFP activation. This finding led to the investigation of GPCRs related to protection and apoptosis. The highest upregulation was observed for the vasointestinal peptide (VIP) receptor, VPAC2. Stimulating cells with VIP increased the observed protective effects against glutamate in the cells, and resulted in Bcl-2 activation, similar to the activation observed in HT22CR cells. Further investigations led us to identify VPAC2 as a GPCR to take a role in protection against oxidative stress, which we then used as a positive control in glutamate-induced oxidative toxicity.

Following the identification of VPAC2’s role in glutamate-induced oxidative stress, 82 known mouse orphan GPCRs were investigated. Three different receptors were found to be repetitively upregulated in glutamate resistant cells. Only one of the receptors, mGPR39, yielded a protection. This receptor turned out to be protective against different forms of oxidative stress like glutamate, H₂O₂, ceramide and tunicamycin. Analysis of the expression pattern of mGPR39 during development yielded clear signals in the
gastrointestinal tract and epithelial tissues as well as the retina. For a more detailed analysis, stable cell lines overexpressing mGPR39 were generated. Two of these new cell lines, conferring the least and most protection against glutamate, were resistant against all oxidative stress inducers used in this study, thus demonstrating a protective effect of the orphan receptor mGPR39.

Expression analyzes and preliminary experiments indicated a role for mGPR39 in various diseases like age-related macular degeneration (AMD) and cancer, though the exact roles and mechanisms have yet to be elucidated.

Taken together, this work provides further insight into the mechanisms of protection against glutamate-induced oxidative stress, in particular into the mechanisms mediated by G-protein coupled receptors. The results further support the merit and veracity of continuing this line of investigation with the aim to someday alleviate problematic conditions such as Parkinson’s Disease and Alzheimer’s Dementia.
7 Literature


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## 8 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
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<tr>
<td>Aβ</td>
<td>amyloid β protein</td>
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<td>APP</td>
<td>amyloid β precursor protein</td>
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<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAK</td>
<td>cAMP-activated kinase</td>
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<tr>
<td>cAMP</td>
<td>adenosine-3’,5’-cyclic monophosphate</td>
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<tr>
<td>cGMP</td>
<td>guanosine -3’,5’-cyclic monophosphate</td>
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<tr>
<td>CMF</td>
<td>5-chloromethylfluorescein</td>
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<tr>
<td>Cpm</td>
<td>counts per minute</td>
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<td>dATP</td>
<td>2’-deoxyadenosine 5’-triphosphate</td>
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<td>dCTP</td>
<td>2’-deoxycytidine 5’-triphosphate</td>
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<td>dTTP</td>
<td>2′-deoxythymidine 5′-triphosphate</td>
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<td>DCFDA</td>
<td>dihydrodichlorofluorescein diacetate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified minimal essential medium</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DL-Dithiothreitol</td>
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<td><em>E. coli</em></td>
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<td>EDTA</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>GSSG</td>
<td>oxidised glutathione</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl)-piperazine-N’-(2-ethanesulfonic acid)</td>
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<td>HO·</td>
<td>hydroxyl radical</td>
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<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HT22CR</td>
<td>chronically resistant HT22 cells</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>MAP2</td>
<td>micortuble-assiciated protein 2</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>methylthiazoltetrazolium</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide radical</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>3PDGH</td>
<td>3-phosphoglycerate dehydrogenase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinosite-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13- acetate</td>
</tr>
<tr>
<td>PSL</td>
<td>photostimulated luminescence</td>
</tr>
<tr>
<td>rcu</td>
<td>randomly chosen units</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium / sodium citrate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl phosphate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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</table>
9 Appendix I

9.1 Publications Related To This Work

9.2 Acknowledgements

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9.3 Curriculum Vitae

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October 2002 – October 2005 Graduiertenkolleg “Neurale Signaltransduktion und deren pathologische Störungen”
9.4 Erklärung nach §4 der Promotionsordnung

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Mert Sahin