Allosteric Activation and Genetic Antagonism of Metabotropic Glutamate Receptor Subtype 7 (mGluR7): Implications for Stress-Related Physiology and Behavior

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1. Acknowledgement

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2. Abbreviations

ACTH	adrenocorticotropic hormone
AMN082	N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride
AMPA	(RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albmine
cAMP	cyclic adenosine-monophosphate
CHO cells	chinese hamster ovary cells
CNS	central nervous system
CPPG	(RS)-*α*-cyclopropyl-4-phosphonophenylglycine
CRF	corticotrophin-releasing factor
CRFR1	corticotrophin-releasing factor receptor type 1
CTA	conditioned taste aversion
FST	forced swim test
GABA	γ-aminobutyric acid
GPCRs	G protein-coupled receptors
GR	glucocorticoid receptor
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
GTPγ[^{35}S]	guanosine 5'[gamma-^{35}S] thiotriphosphate
hmGluR	human metabotropic glutamate receptor
HPA	hypothalamic-pituitary-adrenal
5-HT	5-hydroxytryptamine (serotonin)
5-HT$_{1A}$R	5-hydroxytryptamine receptor subtype 1A
IBMX	3-isobutyl-methylxanthine
i.c.v.	intracerebroventricularly
iGluRs	ionotropic glutamate receptors
KA	kainate
L-AP$_4$	L-amino-4-phosphonobutyric acid
L-SOP	L-serine-*O*-phosphate
LY341495	(2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid
[^{3}H]LY341495	(2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-[2,7-^{3}H]xanth-9-yl) propanoic acid
mGluR	metabotropic glutamate receptor
MR	mineralocorticoid receptor
MSOP	(RS)-*α*-Methylserine-O-phosphate
NMDA	N-methyl-D-aspartate
PVN	paraventricular nucleus
rmGluR	rat metabotropic glutamate receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SIH	stress-induced hyperthermia
siRNAs	short interfering RNAs
SPA	scintillation proximity assay
TST	tail-suspension test

3. Summary

Metabotropic glutamate receptor subtypes (mGluR1 to -8) act as pre- and postsynaptic regulators of neurotransmission in the central nervous system. Regulation of neurotransmission via metabotropic glutamate receptors has recently been implicated in the pathophysiology of anxiety and stress-related disorders including depression. Among metabotropic glutamate receptor subtypes, the group III metabotropic glutamate receptor subtype 7 (mGluR7) shows the highest evolutionary conservation (Flor et al., 1997; Makoff et al., 1996), which suggests that this receptor could play an important physiological role. Cryan et al. (2003) have demonstrated that mice with a targeted deletion of the gene for mGluR7 ($mGluR7^{-/-}$) show antidepressant and anxiolytic-like effects in a variety of stress-related paradigms, including the forced swim stress and the stress-induced hyperthermia tests. Furthermore, the same group has recently developed mGluR7 knockdown using siRNA, which further supported the critical role of mGluR7 in anxiety- and stress-related behaviors (Thakker et al., 2005). Since the hypothalamic-pituitary-adrenal (HPA) axis regulates stress responses, it was investigated in this thesis whether the levels of selected mRNA transcripts and endocrine hormones were altered in mGluR7 deficient mice in the HPA axis. Over all, $mGluR7^{-/-}$ mice showed only moderately lower serum levels of corticosterone and adrenocorticotropic hormone (ACTH) compared to $mGluR7^{+/+}$ mice. However, strong evidence has been found for up-regulation of glucocorticoid receptor (GR)-dependent feedback suppression of the HPA axis in mice with mGluR7 deficiency: (i) mRNA transcripts of GR were significantly higher in the hippocampus of $mGluR7^{-/-}$ animals, (ii) similar increases were seen for $5\text{-}HT_{1A}$ receptor transcripts which are thought to be directly controlled by the GR transcription factor and finally (iii) $mGluR7^{-/-}$ mice showed elevated sensitivity to dexamethasone-induced suppression of serum corticosterone when compared with $mGluR7^{+/+}$ animals. These results indicate that mGluR7 deficiency causes dysregulation of HPA axis parameters which may account, at least in part, for the phenotype of $mGluR7^{-/-}$ mice in animal models for anxiety and stress-related disorders. In addition, the data given here show that protein levels of brain-derived neurotrophic factor (BDNF) are elevated in the hippocampus of $mGluR7^{-/-}$ mice which will be discussed at the latter part of this thesis in the context of the stress-resistant phenotype found in those animals. It can be concluded that genetic ablation of mGluR7 in mice interferes at multiple sites in the neuronal circuitry and molecular pathways implicated in anxiety and stress-related disorders.

Taken together, studies with mGluR7 mutant mice and gene knockdown methods in combination with behavioral paradigms and the neurochemical studies described above, have strongly indicated that mGluR7-selective ligands could be of interest for psychiatric research. To date, there were no pharmacological tools selective for mGluR7. The present study shows the characterization of the first mGluR7-selective agonist: AMN082 (N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride), which directly activates receptor signaling via an allosteric site in the transmembrane domain, while orthosteric agonists such as L-AP_4 and L-glutamate bind to the extracellular region. At transfected mammalian cells expressing mGluR7, AMN082 potently inhibits cAMP accumulation and stimulates GTPγS binding (EC_{50}-values: 64-290 nM) with agonist efficacies comparable to L-AP_4 and superior to L-glutamate. AMN082 (≤10 μM) failed to show appreciable activating or inhibitory effects at other mGluR subtypes and selected ionotropic GluRs. Chimeric receptor studies position the binding site of AMN082 in the transmembrane region of mGluR7 and it has been demonstrated that this novel allosteric agonist has little, if any, effect on the potency of orthosteric ligands. To my knowledge, this study provides the first evidence for full agonist activity mediated by the heptahelical domain of family 3 G protein-coupled receptors (GPCRs with mGluR-like structure), which opens up new drug discovery opportunities. Further, AMN082 is orally active, penetrates the blood-brain barrier, and elevates the plasma stress hormones corticosterone and ACTH in an mGluR7-dependent fashion. Interestingly, it has been found that AMN082 induced partial internalization of mGluR7 in the hippocampal region, which might lead to functional antagonist activity at mGluR7 when tested *in vivo*.

The present work suggests that modulation of mGluR7 function may interfere at various levels in the physiology of anxiety and stress and that mGluR7-selective ligands may provide a novel strategy for the treatment of stress-related disorders.

4. Introduction

4.1. Metabotropic glutamate receptors (mGluRs)

Glutamate elicits fast excitatory synaptic responses in the central nervous system (CNS) by activating ligand-gated cation channels termed ionotropic glutamate receptors (iGluRs) and plays an important role in a wide variety of CNS functions. In addition, in the mid to late 1980s, studies were conducted that provided the first evidence for the existence of glutamate receptors that are not ligand-gated cation channels but are coupled to effector system through GTP-binding proteins (Sladeczek et al., 1985; Sugiyama et al., 1987), termed metabotropic glutamate receptors (mGluRs). The wide diversity and heterogeneous distribution of mGluR subtypes could provide an opportunity for development of pharmacological agents that selectively target specific CNS systems to achieve a therapeutic effect.

4.1.1. mGluR subtypes and classification

mGluRs are G protein coupled receptors functioning via second messenger pathways to modulate activity of glutamatergic circuits. To date, eight mGluR subtypes (designated mGluR1 to mGluR8) have been cloned from mammalian brain. These mGluRs are traditionally classified into three groups based on their sequence homology, signal transduction mechanisms, and agonist pharmacology (Fig. 4.1.; Conn, 2003). The detailed descriptions about pharmacological agents are reviewed in Conn (2003) and Schoepp et al., (1999). Group I mGluRs include mGluR1 and mGluR5, which couple primarily to Gq, increases in phosphoinositide hydrolysis and are selectively activated by 3, 5-dihydroxyphenylglycine (DHPG) and L-quisqualate. Group II mGluRs include mGluR2 and mGluR3, which couple to Gi/o and adenylyl-cyclase inhibition followed by the decrease of cAMP production. This group is selectively activated by (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) and (2S,1'S,2'S)-2-(dicarboxycyclopropyl)glycine (DCG-IV). Group III mGluRs, which also negatively couple via Gi/o to adenylyl-cyclase and inhibit cAMP formation, include mGluR4, mGluR6, mGluR7 and mGluR8, and are selectively activated by L-amino-4-phosphonobutyric acid (L-AP_4), L-serine-*O*-phosphate (L-SOP) and phenylglycine derivatives phosphonophenylglycine (PPG).

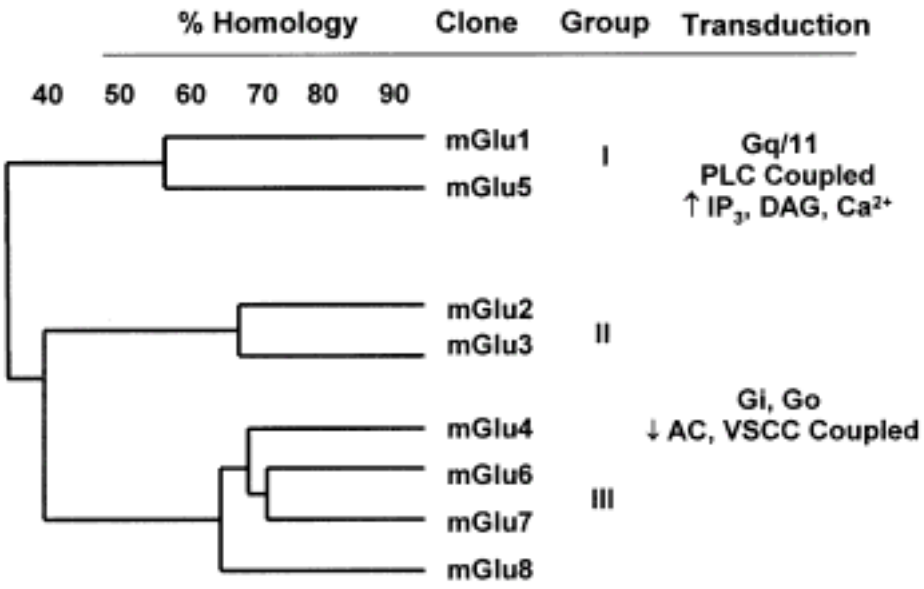

Figure 4.1. **Classification of mGluR family (taken from Conn, 2003).**
The mGluRs are divided into three major groups, on the basis of sequence homology, pharmacological profile, and second messenger coupling.

4.1.2. mGluR structure

mGluRs are members of the G protein coupled receptor (GPCR) family 3 which also includes the γ-aminobutyric acid $(GABA)_B$, pheromone, Ca^{2+}-sensing and taste receptors (reviewed in Pin et al., 2003). The family 3 GPCRs possess a large bi-lobed extracellular N-terminal domain analogous to the "Venus' flytrap" model, which has been demonstrated by both site-directed mutagenesis and X-ray crystallography. This N-terminal bi-lobed domain is linked via an extracellular cystein-rich region to the seven transmembrane domain which is the common structural domain of all known GPCRs. The "Venus' flytrap" domain contains the L-glutamate binding site and induces a conformational change that results in the closure of the flytrap and propagation of the signal through the transmembrane domain (Fig. 4.2.; Spooren et al., 2003). The C-terminus is intracellular and plays an important role in the regulation of receptor activity and targeting through interaction with proteins including calmodulin, the Homer proteins and PICK1 (Dev et al., 2001; El Far and Betz, 2002; Pin et al., 2003). mGluRs are dimeric proteins and functional mGluRs are thought to comprise homodimers stabilized by not only an intersubunit disulfide bond but also hydrophobic interactions. The dimeric ligand-binding region of mGluR adopts multiple conformations, where the activated forms of the dimer are in equilibrium with other states (Kunishima et al., 2000). When mGluRs are not activated, the bi-lobed extracellular domains of the homodimer exist far from each other, which constitutes an open conformation. Binding of an agonist makes the dimer closer and it adopts a closed conformation. Recently it was shown that while a single molecule agonist binding

to one of the homodimer is sufficient to partially activate the receptor, full receptor activation requires agonist binding to both sides of the homodimer (Kniazeff et al., 2004). In addition, Suzuki et al., (2004) has demonstrated the negative co-operativity of glutamate binding in the dimeric mGluRs.

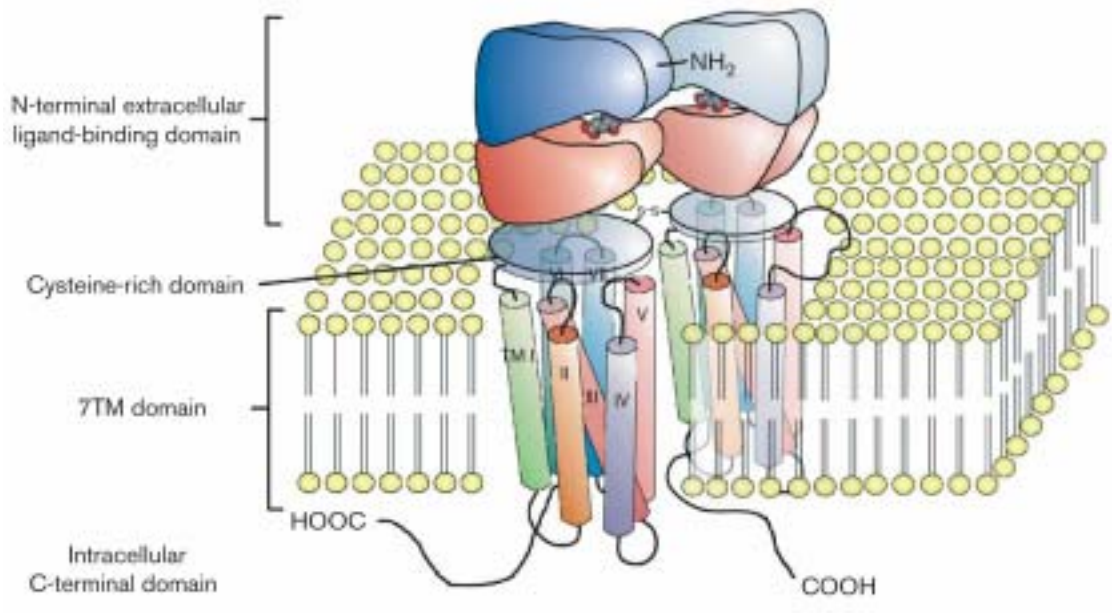

Figure 4.2. **A schematic representation of the dimeric prototypical form of mGluRs (taken from Spooren et al., 2003).**
The large N-terminal extracellular domain contains the L-glutamate binding site. The homodimer is stabilized by non-covalent binding and a disulfide bond. Upon agonist binding, the flytrap closes and the agonist is thought to stabilize the closed conformation by making bonds with residues of both lobes.

4.1.3. mGluR localization and function

4.1.3.1. Group I mGluRs

Distribution of immunoreactivity for mGluR1 was observed extensively throughout the rodent brain (reviewed in Spooren et al., 2003), with especially abundant levels in the olfactory bulb, the thalamus, the cerebellum and certain regions of the hippocampus. In the hippocampus, mGluR1 was strongly expressed in dendritic fields of the dentate gyrus and the CA3 area, but not in the CA1 region. mGluR5 was highly expressed in the accessory olfactory bulb, the hippocampus, the input regions of the basal ganglia (striatum and nucleus accumbens) and the lateral septum. Group I mGluRs are mainly localized to somatodendritic domains of neurons and have shown to be expressed preferentially at the periphery of the postsynaptic densities of asymmetrical synapses (Lujan et al., 1996), although there is some anatomical and biochemical evidence for presynaptic localization (van den Pol, 1995). Within the CNS, Group I mGluRs play an important role in regulating fast synaptic transmission mediated by glutamate, and are involved in synaptic plasticity

phenomena, including long term potentiation (LTP) and long term depression (LTD). For example, mice lacking mGluR1 or mGluR5 have been reported to have impaired cerebellar LTD and hippocampal LTP (Aiba et al., 1994; Conquet et al., 1994) or hippocampal CA1 LTP (Lu et al., 1997), respectively. In addition, an mGluR5 specific antagonist caused impaired LTP in the lateral amygdala (Rodrigues et al., 2002). Furthermore, group I mGluRs have been shown to interact with NMDA receptors bi-directionally (Alagarsamy et al., 2005; Heidinger et al., 2002).

4.1.3.2. Group II mGluRs

mGluR2 is expressed largely in extrasynaptic sites remote from the active zone in neuronal terminals. Moderate to high expression of this receptor is found in the neocortex, various limbic cortical regions including the hippocampus and the amygdala (Ohishi et al., 1998; reviewed in Spooren et al., 2003). Immunoreactivity for mGluR3 was found extensively in the rodent brain with intense levels in the neocortex, the hippocampus and the lateral and the basolateral amygdaloid nuclei. mGluR3 is localized in both pre- and postsynaptic elements of neurons, as well as more widely in glial cells (Tamaru et al., 2001). The vast majority of mGluR3 at presynaptic site was found in extrasynaptic membrane. Therefore it is conceivable that group II mGluRs might be activated by spillover glutamate from distant synapses on the same or other presynaptic elements (Vogt and Nicoll, 1999) and function as sensors for glutamate spill over. As mentioned above, Group II mGluRs are coupled to the inhibition of the cAMP cascade in neuronal and glial cells, but also linked to rapid-onset regulation of various channels including calcium channels and G protein coupled inwardly rectifying K^+ channels depending on neuronal cell types (reviewed by Anwyl, 1999).

4.1.3.3. Group III mGluRs

Strong mGluR4 immunoreactivity was observed in the cerebellum and in the globus pallidus, although it was generally weak in the hippocampus. Electron microscopic studies of mGluR4 revealed presynaptic localization, as well as exclusive postsynaptic localization in the retina (reviewed in Shigemoto and Mizuno, 2000). It has been reported that mice lacking mGluR4 had impaired cerebellar synaptic plasticity and motor performance (Pekhletski et al., 1996). mGluR6 expression is restricted to the postsynaptic, dendritic part of retinal ON bipolar cells. Knockout animals of this receptor have been reported to have visual acutely deficits (Masu et al., 1995; Takao et al., 2000). Distributions of two alternative splice forms of mGluR7 (mGluR7a and mGluR7b) were examined in the CNS by using variant-specific antibodies. The distribution of mGluR7b is more limited than mGluR7a which is widely distributed throughout the brain with a high level of

expression in the locus coeruleus and the limbic regions. mGluR7b is relatively highly expressed in the limbic regions and the globus pallidus. mGluR7 is exclusively detected in the presynaptic active zone in glutamatergic terminals and is believed to function as an autoreceptor activated by glutamate released from the active zone where it is located. In addition, mGluR7 is also highly expressed in some GABAergic terminals (Somogyi et al., 2003) and therefore heterosynaptic interaction should be present. Moreover, compared to other mGluR subtypes, mGluR7 has a much lower affinity for glutamate (mM) which is indeed thought to be the concentration range reached at presynaptic release sites of glutamate; consistent with the localization profile of this receptor. mGluR8 shows a much more restricted distribution compared to that of mGluR4 or mGluR7. Expression of mGluR8 was observed in the olfactory system, the neocortex, the limbic cortex including the hippocampus and the amygdala. Electron microscopically, mGluR8 was largely observed on the axon terminals. Especially in several regions of the hippocampus it was found in the active zone of both asymmetrical and symmetrical synapses where mGluR8 may regulate glutamate release as an autoreceptor or GABA release heterosynaptically (Ferraguti et al., 2005).

4.1.4. The potential role of mGluRs in nervous system disorders: focus on Group III mGluRs

Because of their ubiquitous distribution, mGluRs can contribute to a wide variety of functions in the CNS. Because of the wide diversity and heterogeneous distribution of mGluR subtypes, there is an opportunity for developing highly selective drugs to affect specific CNS functions. Because the focus of this thesis is mGluR7, only disorders in which group III mGluRs are thought to be involved are addressed. Since group III mGluRs with the exception of mGluR6 which is exclusively expressed in the retina are predominantly located presynaptically, activation of these receptors might predominantly result in a reduction of glutamatergic activity, or even GABAergic transmission as heteroautoreceptors. It has therefore been hypothesized that group III mGluRs might be targets for following disorders even though there are few pharmacological tools with the receptor subtype selectivity ideal for testing the significance of the subtypes of group III mGluRs.

4.1.4.1. Epilepsy

Recent studies pointed to mGluRs as potential targets for the treatment of epilepsy. Group I mGluR agonists were shown to be convulsants, while the group II and III mGluR agonists showed anticonvulsant effects (Moldrich et al., 2003). For example, group III mGluR agonists (reviewed in Yang, 2005), L-AP_4, L-SOP, PPG and (S)-3,4-dicarboxyphenylglycine [(S)-3,4-DCPG] blocked seizures in several rodent models and the prolonged anticonvulsant effect of L-SOP has been

reported to be possibly induced by mGluR4 and/or mGluR7 up-regulation (Yip et al., 2001). Furthermore, an mGluR4 selective agonist protected against several kinds of seizures following its intracerebroventricular (i.c.v.) administration (Chapman et al., 2001), although Snead et al., (2000) showed that the mGluR4 knockout mice were markedly resistant to $GABA_AR$ antagonists-induced absence seizures. Taken together, the group III mGluRs can most probably counteract the epileptic activity possibly via regulating enhanced glutamatergic excitability observed in epilepsy.

4.1.4.2. Parkinson's disease

Many recent studies have revealed that members of the mGluR family are differentially distributed and function in several basal ganglia nuclei which regulate motor function and which is involved in the pathology of the movement disorder, Parkinson's disease. In acute and chronic models of Parkinson's disease, L-AP_4 produced therapeutic benefits with decreased GABAergic transmission at the striatopallidal synapse where increased GABAergic inhibition is thought to play an important role in motor dysfunction (Valenti et al., 2003). Consistent with this, mGluR4 allosteric modulation with mGluR4 selective potentiators also produced antiparkinsonian effect in a rodent model of Parkinson's disease (Marino et al., 2003).

4.1.4.3. Anxiety and Stress-related disorders

Various mGluR subtypes specifically modulate excitability within brain structures thought to be involved in anxiety and stress-related illness. Besides the anxiolytic-like effects of Group I antagonists and Group II agonists, recent works point to the potential roles of group III mGluRs in psychiatric disorders (reviewed in Swanson et al., 2005). For instance, intrahippocampal injection of L-SOP showed anxiolytic-like effects in the Vogel conflict drinking test and i.c.v. injection of PPG demonstrated antidepressant-like stress-resistant action in the forced swim test (Palucha et al., 2004). In addition, knockout animal studies indicate the significance of the group III mGluRs to anxiety and stress-related pathways. mGluR7 knockout mice have shown the impairment of two distinct amygdala-dependent behavioral paradigms; the fear-mediated freezing response and the conditioned taste aversion test, without altering their pain threshold nor abnormalities in taste preference (Masugi et al., 1999). Furthermore, these knockout animals have demonstrated anxiolytic-like and stress-resistant phenotype in the elevated plus maze, the staircase test, the light-dark box test, the stress-induced hyperthermia model, the tail suspension test and the forced swim test (Cryan et al., 2003). By contrast, mGluR8 knockout mice have been shown to increase measures of anxiety-like behavior in the open field and the elevated plus maze (Duvoisin et al.,

2005). Taken together, it appears that group III mGluRs have various roles in anxiety and stress-related disorders.

4.2. Psychiatric disorders: focus on anxiety and depression

Psychiatric disorders are serious and debilitating medical conditions with high levels of morbidity and mortality. Depression and anxiety represent some of the most common health problems worldwide (reviewed in Cryan and Holmes, 2005). Of greater concern is that suicide, which is one of the devastating consequences of mental disruption, is now the third largest cause of death among young adults in Western countries and was indeed the largest cause of death in 2004 among Japanese young people from twenty to forty-five years of age.

4.2.1. Molecular changes: focus on HPA axis

All living organisms tend to maintain a dynamic equilibrium called homeostasis (reviewed in Stratakis and Chrousos, 1995). Under stressful situations, this equilibrium is often disturbed and our bodies try to rebuild the homeostasis with some stress responses. These stress responses should be activated rapidly when they are needed and should be efficiently terminated when the stress-causing situation ends. If the stress responses are inappropriate or immoderately and prolonged, mental illnesses may result. The stress responses are composed of many molecular and cellular changes, and the activation of the hypothalamic – pituitary – adrenal (HPA) axis plays a pivotal role. Dysregulation of the HPA axis is thought to play a central role in the pathophysiology of anxiety and depressive disorders (Barden, 2004; de Kloet et al., 2005). During stress, the synthesis of corticotrophin-releasing factor (CRF) in the hypothalamic paraventricular nucleus (PVN) increases and when CRF is released and reaches the anterior pituitary gland, it promotes the release of adrenocorticotropic hormone (ACTH), which, in turn, induces the synthesis and release of glucocorticoids (primarily corticosterone in rodents and cortisol in humans) from the adrenal cortex. CRF is also known to play a role in the activation of the sympathetic nervous system. Glucocorticoid effects are mediated by two types of nuclear receptors; the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), the latter having less affinity to glucocorticoid than MR. Whereas disturbance of homeostasis is prevented by MR-mediated processes, its recovery is facilitated via GR that requires stress levels of glucocorticoids and it appears that the main function of GR in the brain is to suppress stress-induced hyperactivity of the HPA axis at the level of the PVN, the anterior pituitary, but also at the hippocampal level (reviewed in Barden, 2004).

Since MR and GR are nuclear receptors, they can regulate the transcription of genes. In the later recovery phase, GR monomers are capable of interacting with transcription factors and repress stress-induced responses (De Kloet, 2004).

In addition, accumulating data suggest that the serotonin (5-HT) system and the HPA axis have complex inter-relationships (reviewed in Porter et al., 2004), which is consistent with the clear understanding that dysfunction of serotonergic neurotransmission is associated with several mood disorders including depression and anxiety. In particular, the 5-HT_{1A} receptor has received a great deal of attention in recent years because this receptor is very susceptible to modulation by stress and HPA axis activation and is well known to play an important role in the pathophysiology of mood disorders (reviewed in Cryan and Leonard, 2000).

Furthermore, recent evidences point also to an important role for the neurotrophin brain-derived neurotrophic factor (BDNF) in both the pathophysiology of mood disorders and in the therapeutic effects of antidepressants (reviewed in Hashimoto et al., 2004). Pre-clinical studies have shown that the infusion of BDNF into the midbrain has an antidepressant-like influence on animal models of depression and clinical studies have demonstrated that serum levels of BDNF in drug-naive patients with depressive disorders are significantly decreased when compared with normal controls.

4.2.2. Stress models in rodents

Exposure to excessive stress has been thought to be one of the main factors to make individuals susceptible to depression and the inability to cope effectively with stress is often regarded as a hallmark in depressed patients. Therefore, many rodent models for assessing depression-like stress-related behavior are based on this aspect and involve exposure to stressful situations (reviewed in Cryan and Holmes, 2005). Among those, probably the most studied and known model is the forced swim test (FST). In the FST, rodents are placed in an inescapable cylinder filled with tepid water. They will initially try to escape with vigorous movements, but then within minutes they will develop immobile postures. Another well-known model is the tail-suspension test (TST), in which mice hung upside-down by their tails will exhibit passive immobility in response to this inescapable stress after minutes of fruitless struggling. However, the TST has also been reported to have wide strain differences (Liu and Gershenfeld, 2001) and is often used in addition to the FST. The other reason why these tests are regarded as rodent models for depression in humans is that administration of various clinically effective antidepressants to rodents before the test leads to longer periods of escape-oriented behaviors compared to vehicle-treated rodents. Therefore these animal models have also been used as rapid screening assays for searching new antidepressants.

Anxiety models were also developed based on the effects of clinically effective anxiolytics. However, it should be noted that the state of anxiety itself is indeed a normal response that everybody has, as distinct from depression. The hallmark of anxiety is marked, persistent and excessive or unreasonable fear which is enough to affect everyday life (reviewed in Cryan and Holmes, 2005). Many animal models of anxiety disorders are therefore linked to the natural behavioral patterns of rodents; small rodents have an innate aversion to exposed and brightly open areas presumably because of their instincts to hide from enemies, on the other hand, they are also foraging species. Based on the conflicts between the tendency to explore a novel environment and aversion to bright spaces, many behavioral tests are regarded as models of anxiety in humans, such as the elevated plus maze, the elevated zero maze, the open field test, the light-dark box test and the staircase test. Another innate response of rodents in stress-related behavior is the increase in core body temperature, which is another hallmark of the stress response in mammals. The stress-induced hyperthermia (SIH) test which quantifies the degree of hyperthermia elicited by a short mild stress is technically easier to be done compared to other models, since measuring rectal temperature itself can be the stressor. Clinical anxiolytics reduced the degree of SIH and the innate aversion in ethological tests.

Furthermore, since cognitive disturbances are also seen in patients with depression or anxiety disorders, rodent models for studying emotional cognition have been also developed (reviewed in Cryan and Holmes, 2005). Pavlovian fear-conditioning tasks are one of the representative models; rodents are exposed to a harmless neutral stimulus associated with an innate aversive stimulus and after these repeated pairings, they develop fear responses only with the harmless neutral stimulus. Another conditioned learning test is conditioned taste aversion (CTA). In this test, rodents learn the association of a novel flavor with delayed malaise, such as that induced by lithium chloride and after a conditioning trial, they reduce their preference of the flavored foods associated with that aversive response.

Although we should be careful to translate animal models into human disorders like most of the other research tools, they can be useful in preclinical psychiatric research.

4.3. Behavioral effects of mGluR7 mutant animals in stress-related paradigms

mGluR7 is the most highly conserved receptor of all mGluR subtypes across different mammalian species (Flor et al., 1997; Makoff et al., 1996). Therefore, it might be conceivable that this receptor plays an important role in mammals. Since mGluR7 is abundant in brain regions that are known to be critical for the manifestation of anxiolysis and antidepressant action, such as the hippocampus,

the amygdala, and the locus coeruleus (Kinoshita et al., 1998), this receptor has attracted attention in psychiatric research. However, since there were no pharmacological tools selective for mGluR7, research on mGluR7 to date has been done with mGluR7 gene-mutated animals.

4.3.1. mGluR7 knockout animal studies

Mice lacking mGluR7 have deficits in amygdala-dependent behaviors (fear response and conditioned taste aversion), but show no alterations in locomotor activity nor pain sensitivity (Masugi et al., 1999). Electrophysiological analysis in mGluR7 knockout ($mGluR7^{-/-}$) mice further suggests that this receptor modulates short-term synaptic plasticity in the hippocampus (Bushell et al., 2002). Moreover, mGluR7 ablation in mice has been demonstrated to be associated with changes in animal behavioral paradigms and predictive of antidepressant and anxiolytic actions which suggest that drugs acting at mGluR7 may provide novel treatments for psychiatric disorders such as depression and anxiety (Cryan et al., 2003). These tests including the FST (Fig. 4.3.A.), the TST (Fig. 4.3.B.), the light-dark box test (Fig. 4.4.A.), and the SIH (Fig. 4.4.B.), all involve observing the animal's response to a novel stressful situation. The data suggest that mGluR7 may play a role in the stress response to aversive stimuli.

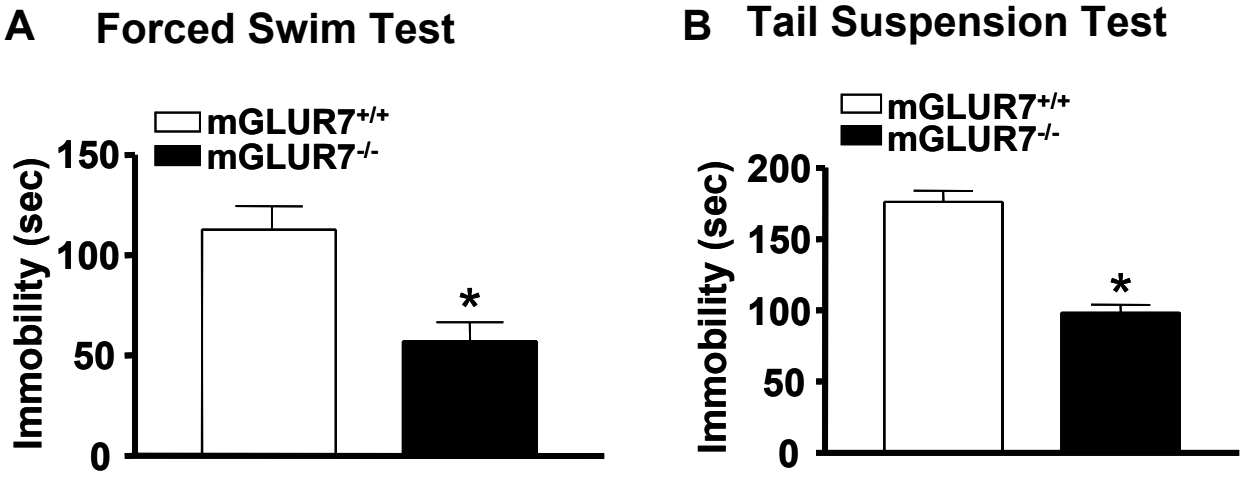

Figure 4.3. **Antidepressant-like activity in $mGluR7^{-/-}$ mice (taken from Cryan et al., 2003).**
(A) $mGluR7^{-/-}$ mice had a much lower immobility score than its littermate $mGluR7^{+/+}$ mice in the forced swim test. (B) Similarly, $mGluR7^{-/-}$ mice had a much lower immobility score than $mGluR7^{+/+}$ mice in the tail suspension test. *$p < 0.05$, significantly different from $mGluR7^{+/+}$ mice.

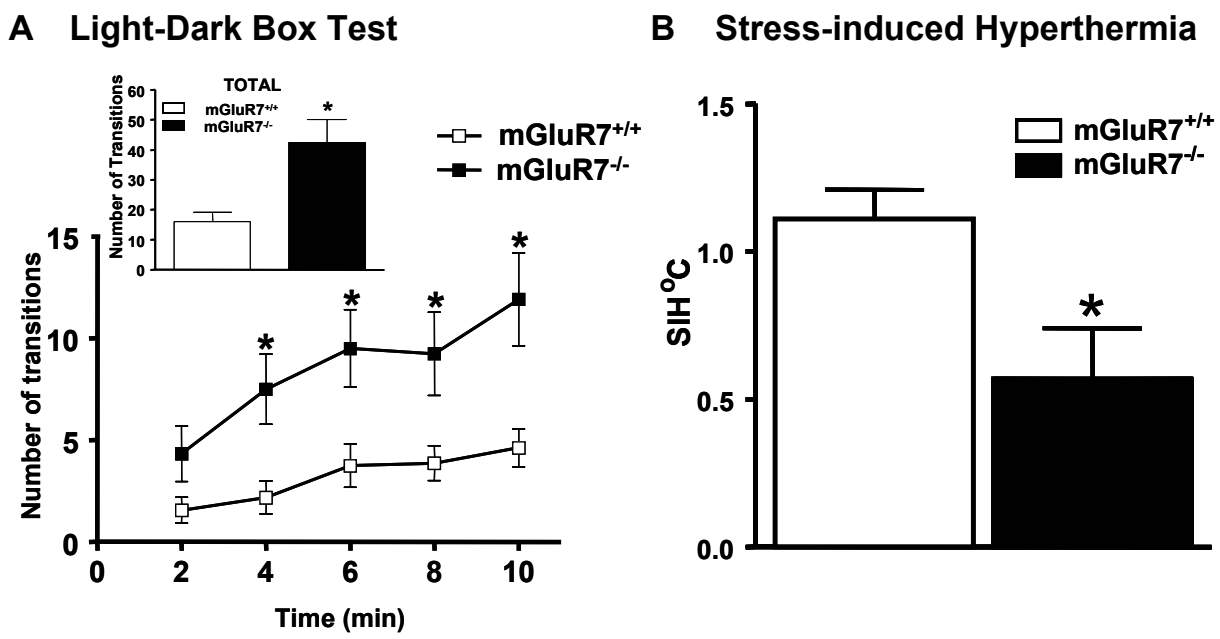

Figure 4.4. **Anxiolytic-like activity in mGluR7$^{-/-}$ mice (taken from Cryan et al., 2003).**
(A) mGluR7$^{-/-}$ mice had a markedly higher number of transitions between the dark and light compartments throughout the 10 min of the test compared to its littermate mGluR7$^{+/+}$ mice in the light-dark box test. (B) mGluR7$^{-/-}$ mice had a much lower stress-induced hyperthermia (SIH) than mGluR7$^{+/+}$ mice. *$p < 0.05$, significantly different from mGluR7$^{+/+}$ mice.

4.3.2. mGluR7 knockdown animal studies

In studies with gene knockout animals we always have to consider whether the readouts come from the direct consequences of genetic ablation or developmental compensations. To lessen the possibility of developmental compensations, there are several emerging techniques. One of those is the RNA interference (RNAi) technique. RNAi is a cellular surveillance mechanism found in nature, which responds to double-strand RNA (dsRNA) by destroying cytoplasmic mRNAs containing sequences homologous to the dsRNA trigger (Fire et al., 1998). However, when dsRNA is introduced in mammalian cells, it can induce an undesirable interferon response leading to cell death. Short interfering RNAs (siRNAs) that mimic the cleavage products of dsRNA in mammalian cells produce sequence-specific gene-silencing in these cells without such an undesirable response (Caplen et al., 2001). Thakker et al., (2004) established siRNA-induced knockdown in adult brain *in vivo*. Recently, by using this method, Cryan and his colleagues have developed and tested mGluR7 knockdown mice in several behavior models, including the light-dark box test and the SIH (unpublished data; but see Thakker et al., 2005). The knockdown of mGluR7 in adult mice brains results in the stress-resistant phenotype very similar to that observed in mGluR7 knockout animals. Interestingly, the level of mGluR7 knockdown was only significant in a few brain regions such as

the amygdala and the hippocampus. These results indicate that moderate knockdown of mGluR7 results in reduced anxiety-like and stress-related behaviors confirming the phenotype observed in mGluR7 knockout mice and further supporting an important role of mGluR7 in psychiatric diseases.

4.4. Scientific questions addressed in this thesis

Given that mGluR7 knockout mice have a phenotype that is indicative of an altered behavioral response to stress, which is further supported by mGluR7 siRNA knockdown studies the hypothesis was that mGluR7 affects stress-related systems at different levels. Since the neurophysiological mechanisms underlying the phenotype of mGluR7 knockout mice were not yet clarified, this thesis explores representative molecular targets and physiological mechanisms that are involved in stress regulation of the mammalian brain.

Moreover, in spite of cumulative reports for the importance of mGluR7 the research regarding mGluR7 still lags behind that of other mGluR subtypes because of the lack of selective pharmacological tools. From this point of view, it would be very desirable to have mGluR7-selective pharmacological tools. Since mGluRs can play a role in the fine tuning of neurotransmission and might be better targets with less side-effects compared to the targets of current treatments for psychiatric disorders, the discovery of mGluR subtype selective tools might result in scientific and possibly clinical advances. The second part of this thesis characterizes the first mGluR7 selective agonist.

5. Materials and Methods

5.1. Animals

mGluR7$^{-/-}$ mice were generated as described previously from E14 (129/Ola) embryonic stem cells (Sansig et al., 2001). The breeding, genotyping and housing animals were done by a central animal facility at Novartis. Larger age-matched groups of mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice were generated using a specific pathogen-free (SPF) breeding colony of 30 F14, B6-mGluR7$^{+/-}$ males mating with 60 - 90 F14, B6-mGluR7$^{+/-}$ females. All the mice in the studies reported here carried wild-type or mutant mGluR7 alleles on a fourteenth generation C57BL/6 genetic background. Mice were weaned at the age of 3 weeks, when tail biopsies were also taken for genotyping. Subsequently, genotyped mice were moved to a non-SPF conventional facility in which male animals were used for experiments at the ages of 3 - 9 month. Housing was at room temperature, in a 12 h light:dark cycle, with lights on at 6:00 a.m. Male animals were used in all experiments. Food pellets and tap water were available *ad libitum* unless stated otherwise. Following decapitation of mice (mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice in a randomized order), bloods were collected and brains were immediately removed and frozen on dry-ice and stored at –80 °C. All animal experiments were subject to institutional review and conducted in accordance with the Veterinary Authority of Basel-Stadt, Switzerland.

5.2. *in vivo* AMN082 administration

Male mGluR7$^{-/-}$ or littermate mGluR7$^{+/+}$ mice (C57BL/6 genetic background) were injected orally (p.o.) with either vehicle or 1 - 6 mg/kg N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride (AMN082; synthesized by Joachim Nozulak and Silvio Ofner within Novartis Pharma AG). One hour later, those mice were investigated for each purpose.

5.3. Blood sample collection

5.3.1. Blood sample collection for basal and stress-induced conditions

In order to investigate the effects of mGluR7$^{-/-}$ mice on basal corticosterone and ACTH levels, male mGluR7$^{-/-}$ (n = 10 per gender) and littermate mGluR7$^{+/+}$ (n = 10 per gender) mice were decapitated and the blood was collected. Two batches of mice were sacrificed, one between 7:00 and 8:00 a.m.

and the other between 5:00 and 5:30 p.m. For the stress-induced hormone levels, male mGluR7$^{-/-}$ and littermate mGluR7$^{+/+}$ mice were subjected to swim stress (6 min) into plexiglass cylinders (24 cm tall x 21 cm in internal diameter) filled with water (23 – 25 °C). Mice were decapitated 5 min or 90 min after the stress session (n = 10 per genotype and per time point) in another room and the blood was collected between 8:00 and 11:00 a.m. Simultaneously, non-stressed male mice (n = 10 per genotype) were sacrificed, which constituted control groups. All of the processes mentioned above were carried out by Hugo Buerki and Cedric Mombereau.

5.3.2. Blood sample collection following Dexamethasone suppression test

The Dexamethasone suppression test was carried out essentially as described by Groenink and colleagues (2002). Male mGluR7$^{-/-}$ and littermate mGluR7$^{+/+}$ mice were injected subcutaneously (SC) with saline, 0.03 or 0.1 mg/kg dexamethasone-21-phosphate disodium salt (Fluka, Buchs, Switzerland). Six hours later, mice were decapitated rapidly (within 30 sec after first touching the cage) in an adjacent room, and trunk blood was collected (n = 10 per genotype). All of the processes mentioned above were carried out by Hugo Buerki and Cedric Mombereau.

5.3.3. Blood sample collection following AMN082 injection

AMN082 administration was done as mentioned above. One hour later, mice were decapitated rapidly (within 30 seconds after first touching the cage; mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice in a randomized order) and trunk blood was collected ($n \geq 9$ per genotype).

5.4. Hormone measurement

Plasma corticosterone and ACTH concentrations were measured using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA, USA). The inter- and intra-assay coefficients of variability for ACTH and corticosterone were similar to the manufacturer's reported values with a detection limit of 5 - 10 pg/ml.

5.5. Extraction of BDNF from tissues

Various brain regions were dissected, weighed, collected on dry ice, and stored at –80 °C, or dissected as soon as the frozen brains started to thaw and weighed. The extraction of BDNF was performed basically as described previously (Kolbeck et al., 1999) and briefly mentioned as below.

20 - 40 vol/wt of extraction buffer [0.05 M sodium acetate, pH 4.0, 1.0 M sodium chloride, 1 % (vol/wt) BSA (A-9418 of Sigma, Buchs, Switzerland), 1 % Triton X-100, protease inhibitor cocktail tablets (Cat.No. 1 836 153, Roche, Rotkreuz, Switzerland)] was added and the tissues sonicated to homogeneity. The homogenates were kept on ice for 1 h and the sonication repeated three times. The homogenates were centrifuged (15 min, 10^5 *g*), the supernatants (first extraction) collected, and the pellets resuspended in 20 - 40 volumes of extraction buffer. After another centrifugation (15 min, 10^5 *g*), the supernatants were collected (second extraction) and the pellets resuspended in 20 - 40 volumes of extraction buffer. After another centrifugation (15 min, 10^5 *g*), the supernatants were collected again (third extraction).

5.6. BDNF immunoassay

Quantification of BDNF from tissue extracts was carried out essentially as described by Kolbeck et al. (1999). Plates (MaxiSorp plates, Nunc, Rosklide, Denmark) were coated with carbonate buffer, pH 9.7 containing 400 ng per well of biotinylated first anti-BDNF antibody (courtesy of Yves A. Barde, University of Basel, Switzerland) overnight at 4 °C and incubated with blocking buffer [Phosphate-buffered saline (PBS), 2 % BSA, 0.1 % Triton X-100] for 1 h at room temperature. Standard amounts of BDNF were diluted in extraction buffer (0.1 - 6.25 ng/ml), and 25 µl per well of each concentration was applied. 25 µl per well of the extraction buffer alone (negative control) was used to check background activity. Similarly, 25 µl per well of different dilutions of the tissue-extract supernatants was added to the wells. Subsequently, 175 µl per well of incubation buffer [0.1 M potassium/sodium phosphate, pH 8.0, 1 % BSA, protease inhibitor cocktail tablets (Cat.No. 1 836 153, Roche, Rotkreuz, Switzerland)] containing 100 mU/ml peroxidase (POD)-conjugated anti-BDNF antibody (courtesy of Yves A. Barde, University of Basel, Switzerland) was applied and incubated overnight at 4 °C. Plates were washed with washing buffer [Phosphate-buffered saline (PBS), 0.1 % Triton X-100] 3 times and incubated with 200 µl per well of the BM blue POD substrate (Roche, Rotkreuz, Switzerland) for 30 min at room temperature. The reaction was stopped with 50 µl of 1 M sulfonic acid per well, and the intensity of the reaction product was measured in a luminometer at 490 nm. BDNF levels in tissue extracts were determined by comparison with the standard curve.

5.7. “Real-Time” Reverse Transcriptase (RT) PCR assays

Brain regions per mice were disrupted (2 x 2 minutes at 20 Hz) by using a Mixer Mill MM 300 (Retsch GmbH & Co. KG, Haan, Germany). Total RNA was isolated from disrupted brain tissue using the commercially available “Absolutely RNA™” RT-PCR Miniprep Kit (Stratagene, Amsterdam, The Netherlands). The quantity of RNA was determined by optical density (OD) measurement using a NanoDrop®ND-1000 Spectrophotometer. To remove traces of genomic DNA contamination, an aliquot of 400 ng total RNA was digested with RNAse-free DNAse I (RNAse-Free DNase Set, Qiagen, Hilden, Germany). The enzyme was inactivated by addition of EDTA and by heating up to 65 °C. DNAse I-treated total RNA was reverse transcribed into cDNA by random priming using StrataScript™ Reverse Transcriptase (Stratagene, Amsterdam, The Netherlands) at 42 °C for 60 minutes. Primers and probes were designed with the aid of the Primer Express software (Version 1.0; Applied Biosystems, Foster City, CA, USA) by Erika Lötscher and Genevieve Kuntzelmann. The probes were labeled at the 5’ end with the reporter fluorophore 6-carboxyfluorescein (FAM) and at the 3’ end with the quencher 6-carboxytetramethyl-rhodamine (TAMRA). Primers and probes were synthesized commercially (Microsynth, Balgach, Switzerland). The sequences of the primer pairs and probes were as shown in the table of sequences (below). “Real-Time” RT PCR (TaqMan®) assays were performed using the qPCR™ Mastermix (Eurogentec, Seraing, Belgium) on an ABI Prism® 7700 sequence Detection System (Applied Biosystems, Foster City, CA, USA). In all assays for the target genes, primers and probes were used at 300 nM and 175 nM, respectively. Assays for the internal control gene 18S rRNA were performed by using the “18S Genomic Endogenous Control Kit” (Eurogentec, Seraing, Belgium). Relative levels of the target mRNA are reported after normalization to 18S rRNA, which was also detected by “Real-Time” RT PCR. The normalized raw data, representing the average values of two independent determinations, were analyzed by using the “comparative C_T method”, as described in details by Livak and Schmittgen, (2001).

Table of Sequences: Primers and probes used in "Real-Time" RT PCR assays for assessing relative expression profiles of target genes

Gene product	GenBank/RefSeq Accession Number	Sequences of primer and probe
BDNF	BC034862	5'-CCA TAA GGA CGC GGA CTT GT-3' 5'-GAG GCT CCA AAG GCA CTT GA-3' 5' FAM-CAC TTC CCG GGT GAT GCT CAG CA-TAMRA 3'
CRF	AY128673	5'-AGC AGT TAG CTC AGC AAG CTC A-3' 5'-GGC CAA GCG CAA CAT TTC-3' 5' FAM-TCC CGA TAA TCT CCA TCA GTT TCC TGT TGC T-TAMRA 3'
CRF1	X27305	5'-TCC GGT GCC TGA GAA ACA TC-3' 5'-CGT GGC GTT GCG TAG GA-3' 5' FAM-TCC ACT GGA ACC TCA TCT CGG CTT TC-TAMRA 3'
GR	X04435	5'-CGG GAC CAC CTC CCA AA-3' 5'-CCC CAT AAT GGC ATA CCG AA-3' 5' FAM-TCT GCC TGG TGT GCT CCG ATG AAG-TAMRA 3'
5-HT$_{1A}$	NH012585	5'-GCT CAT GCT GGT TCT CTA CGG-3' 5'-GAC AGT CTT GCG GAT TCG GA-3' 5' FAM- CGC ATC TTC AGA GCC GCA CGC-TAMRA 3'
mGluR7	NM000844	5'-GCG CTC GAA CAG TCG CTT A-3' 5'-CGC ACG TCG GAG GTG TC-3' 5' FAM-TTT CGT CCA GGC GCT CAT CCA GA-TAMRA 3'
MR	XM356093	5'-TGG ATG TGG TTG GAT GTA GGG-3' 5'-TCT ACC TGT TGC AGC GCT TG-3' 5' FAM-CCA CGA CAA CCC TGC TGC GGA G-TAMRA 3'

5.8. Stable cell lines

Generation, culture and pharmacological characterization of stable cell lines for mGluR1b, mGluR2, mGluR3, mGluR4, mGluR5a, mGluR6, rmGluR7a, hmGluR7b, NMDAR1A/2A, NMDA1A/2B and GluR3i have been described (Flor et al., 1995; Flor et al., 1997; Gasparini et al., 1999a; Gasparini et al., 1999b; Knopfel et al., 1995; Maj et al., 2003). Cell lines for rmGluR7a and rmGluR2 were generous gifts from Shigetada Nakanishi at Kyoto University, Japan.

5.9. Construction of chimeric receptors and corresponding cell lines

cDNAs encoding wild-type hmGluR6 and hmGluR7b were described previously (Flor et al., 1997; Weng et al., 1997). cDNAs encoding chimeric hmGluR6/7b and hmGluR7/6 proteins were constructed by Natacha Stoehr, using a PCR overlap extension approach (Horton et al., 1989): the mGluR6/7b construct contains 570 amino acids derived from the N-terminal extracellular region of mGluR6 and the remaining C-terminal portion of mGluR7b, comprising the entire transmembrane region; mGluR7/6 is essentially the reverse chimera with the fusion point at amino acid 575. The four constructs were used to generate stably transfected cell lines by Flp recombinase-mediated integration (Flp-In^{TM} T-REx^{TM} Core Kit; Invitrogen Ltd., Paisley, UK) in Chinese Hamster Ovary (CHO)-C4 cells (Novartis Pharma AG, Basel, Switzerland), which contain a single Flp recognition target (FRT)-site; the identical host was used for all the four new cell lines.

5.10. Membrane preparation and GTPγ[^{35}S] binding assays

Membranes were prepared from transfected cells using essentially the protocol described previously (Lazareno and Birdsall, 1993; Gasparini et al., 1999b). The culture of transfected mGluR2-, mGluR3-, mGluR4-, mGluR6-, mGluR7a-, mGluR7b-, mGluR6/7b-, or mGluR7/6-expressing cells was expanded to seed 30 - 60 cells per 15 cm dishes. The cells were harvested when they reached 80 - 95% confluency followed by homogenization in buffer A (20 mM HEPES, 10 mM EDTA, pH 7.4) using a Polytron and centrifugation at 39'000 g at 4 °C for 20 min. The pellet was resuspended in buffer B (20 mM HEPES, 0.1 mM EDTA, pH 7.4), homogenized using a Polytron and re-centrifuged at 39'000 g at 4 °C for 20 min. The pellet was resuspended in buffer B at a protein concentration of 2 to 3 mg/ml, aliquoted, snap frozen on dry ice and stored at -80 °C. Membrane fractions were diluted in assay buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM EGTA,

20 μM GDP, pH 8.0), homogenized briefly using a Polytron and incubated for 10 min at 30 °C. Following that pre-incubation, assay-mixtures were prepared in 96-well microtiter plates. After optimization of various parameters, the composition of the assay mixtures in a final volume of 200 μl per well was as follows: 20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM EGTA, 20 μM GDP, pH 8.0, 15 - 35 μg membrane protein (pre-treated as described above), 1.5 mg wheat germ agglutinin scintillation proximity assay (WGA SPA) beads (Amersham, Freiburg, Germany), 0.05 - 0.2 nM GTPγ[^{35}S] (Amersham, Freiburg, Germany), and the test compounds (agonists and/or antagonists) at the appropriate concentrations. Non-specific binding was measured in the presence of unlabelled GTPγS (Sigma, Buchs, Switzerland) in excess (10 μM). The samples were incubated at room temperature for 40 - 60 min (with shaking) before the WGA SPA beads were sedimented by centrifugation at 200 g for 10 min at room temperature. The plates were then counted in a Packard TopCount (Packard, Zürich, Switzerland). L-Glutamate (L-Glu), DL-2-amino-4-phosphonobutyric acid (DL-AP_4), L-(+)-2-amino-4-phosphonobutyric acid (L-AP_4), (RS)-*α*-Methylserine-O-phosphate (MSOP), (RS)-*α*-Cyclopropyl-4-phosphonophenylglycine (CPPG), (2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), and L-Serine-O-phosphate (L-SOP) were purchased from Tocris (Bristol, UK). (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-[2,7-^{3}H]xanth-9-yl) propanoic acid ([^{3}H]LY341495) was purchased from Amersham Biosciences (Freiburg, Germany).

5.11. Second messenger assays

5.11.1. Cyclic AMP assay

Measurements of cyclic AMP (cAMP) accumulation were performed essentially as previously described (Flor et al., 1995; Flor et al., 1997) using cAMP SPA direct screening assay system, code RPA 559 (Amersham Biosciences, Freiburg, Germany) in CHO cells stably expressing individual mGluR subtypes. Briefly, CHO cells expressing mGluR2 and mGluR7 were seeded in 96-well plates at a density of 6.5 - 8.5 × 10^3 cells per well and grown for 1 day until a 40 - 70 % confluent layer of cells was obtained. Cells were incubated for 20 min in HBS containing 1 mM IBMX. After washing cells with HBS, they were incubated with fresh HBS containing 30 μM forskolin, 1 mM IBMX and the test agents for 20 min. The reaction was stopped by aspirating off the drug containing medium. The cAMP produced by the cells was released by adding 1 % solution of dodecyltrimethylammonium bromide per well and the samples were shaken at room temperature for 5 min. Then the immunoreagent solution containing equal volumes of [^{125}I]cAMP tracer, antiserum

and SPA anti-rabbit reagent in lysis buffer was placed in a final volume of 200 µl per well. A standard curve was constructed in the range of 0.2 - 25.6 pmol/well of cAMP for each plate. The samples were incubated at room temperature for 15 - 20 hours before the SPA beads were sedimented by centrifugation at 900 rpm for 6 min at room temperature. The plates were then counted in a Packard TopCount (Packard Instrument Company, Meriden, U.S.A.). The cAMP levels of the test wells were calculated relative to the standard curve.

5.11.2. Measurement of [^{3}H] Inositol Phosphate Formation

Clonal cell lines expressing hmGluR1b or hmGluR5a receptors were seeded in 24-well tissue culture plates. Cells were labeled to equilibrium with 2 µCi/ml myo-[^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO) for 20 h in Dulbecco's modified Eagle's medium, washed twice in Krebs-Henseleit buffer (Sigma, Buchs, Switzerland), and incubated for 30 min at room temperature. Subsequently, cells were washed in buffer containing 10 mM LiCl and incubated in the same medium for 20 min at 37°C. After aspiration of medium, compounds were added to triplicate wells in the presence or in the absence of L-Glutamate in buffer containing 10 mM LiCl. Inositol phosphate formation was measured essentially as described by Seuwen et al., (1988). In brief, the reaction was stopped after an incubation of 20 min at 37°C by aspiration of the medium and lysis of the cells with 0.75 ml of ice-cold 10 mM formic acid (pH 3). After 30 min the extract was diluted into 2 ml of 5 mM NH_3 solution (yielding a final pH of 8 - 9) and applied to a column containing DOWEX-1 × 8 (Fluka, Buchs, Switzerland). After flow-through of the extract, columns were washed with 10 ml of H_2O and 6 ml of 5 mM sodium tetraborate, 60 mM sodium formate, respectively. Inositol monophosphates were eluted with 6 ml of 100 mM formic acid, 200 mM anmonium formate. The eluted samples were counted 7 h after addition of 15 ml Irgasave Plus scintillation cocktail (Packard, Zürich, Switzerland). Each data point represents triplicate measurements expressed as mean ± S.E.M.

5.11.3. Calcium measurements

Functional activation of ionotrophic glutamate receptors was assayed by means of fluorometric measurements of changes in intracellular free calcium concentration $[Ca^{2+}]_i$ as described by Flor et al., (1996) based on the calcium sensitive dye fura-2 (Grynkiewicz et al., 1985). Briefly, cells were grown on glass cover slips. On the day of the assay, the cover slips were incubated at room temperature for 60 min in a HEPES buffered saline (Life Technologies/GibcoBRL, Basel,

Switzerland) containing 10 µg/ml fura-2/AM (Molecular Probes Inc., Eugene, OR, USA), 5 µl/ml DMSO and 0.5 % pluronic (Molecular Probes Inc., Eugene, OR, USA). Glass cover slips carrying the dye loaded cells were mounted into a perfused cuvette in a fluorescence spectrophotometer (Hitachi F - 4500; Hitachi, Tokyo, Japan). Changes in $[Ca^{2+}]_i$ were monitored by measuring the ratio of fura-2 fluorescence (510 nm) excited alternatively (1.6 Hz) at 340 and 380 nm. Thus, an increase of the fluorescent response ratio F_{340}/F_{380} corresponds to an increase in $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). To test specificity of agonist activation of NMDA or AMPA receptors, D-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid, CGP40116 (provided by Novartis Pharma AG) or 6-cyano-7-nitroquinoxaline-2, 3-dione, CNQX (Tocris Cookson Ltd., Bristol, UK) was applied respectively.

5.12. [^{3}H]LY341495 binding assay

This radioligand binding assay was done by Yamamoto R, as follows. Membrane fractions of CHO cells stably expressing mGluR7a (see above) were diluted in assay buffer (10 mM KH_2PO_4, 100 mM KBr, pH 7.6), homogenized briefly using a Polytron and incubated for 10 min at 30 °C. Assay-mixtures were prepared in 96-well microtiter plates. The composition of the assay mixtures in a final volume of 200 µl per well was as follows: 10 mM KH_2PO_4, 100 mM KBr, pH 7.6, 50 µg pre-treated membrane protein, 1.5 mg WGA SPA beads, 10 nM (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-[2,7-^{3}H]xanth-9-yl) propanoic acid, [^{3}H]LY341495 (Amersham Biosciences, Freiburg, Germany) and the test compounds at the appropriate concentrations. Non-specific binding was measured in the presence of 1 mM L-SOP (Tocris, Bristol, UK). The samples were incubated for 60 min at room temperature (with shaking), before being counted in a Packard TopCount (Packard, Zürich, Switzerland). Data were analyzed using nonlinear regression in the GraphPad Prism program (Graphpad Software Inc., San Diego, USA.). IC_{50}s were converted into K_is using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

5.13. Electron microscopy studies

Wild-type mice (10 – 12 weeks, C57BL/6 genetic background) were injected either vehicle or 6 mg/kg AMN082 as described above (see Materials and Methods 5.2.). One hour later, these mice were anesthetized by pentobarbital (60 mg/kg, i.p.) and then perfused transcardially with PB containing 4 % paraformaldehyde, 0.05 % glutaraldehyde, and 0.2 % picric acid. The brains were

cut into 50 µm-thick frontal sections on a vibratome (Microslicer, Dosaka, Kyoto, Japan). The sections were placed in PB containing 25 % sucrose and 10 % glycerol for 30 minutes, and then freeze–thawed in liquid nitrogen. The sections were immunostained with the affinity – purified antibody (courtesy of Ryuichi Shigemoto, National Institute for Physiological Sciences, Okazaki, Japan) against mGluR7a or mGluR7b as follows. They were incubated sequentially with (1) 20% normal goat serum (NGS) diluted in 50 mM Tris buffer (TB) containing 0.9% NaCl [Tris-buffered saline (TBS)] for 1 hr, (2) incubation buffer (PBS containing 0.1 % BSA) for 10 minutes, 2 times, (3) 1.0 µg/ml rabbit anti-mGluR7a IgG or 1.0 µg/ml rabbit anti-mGluR7b IgG in incubation buffer at 4 °C for 24 hours, (4) goat anti-rabbit (Fab fragment, diluted 1:100) coupled to 0.8 nm gold (Nanoprobes, Stony Brook, NY) made up in incubation buffer. After washes in incubation buffer and PBS, sections were washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes) for 5 - 10 min. After washes in PB, the sections were treated with 1% OsO_4 in PB for 40 min, washed in PB and double-distilled water, and then contrasted in 1% uranyl acetate for 25 min. They were dehydrated in a series of ethanol and propylene oxide and flat embedded in epoxy resin (Durcupan ACM; Fluka, Buchs, Switzerland). After polymerization, sections were cut at 50 - 75 nm thickness by Ryuichi Shigemoto and Sachiko Yamada, using an ultramicrotome (Reichert Ultracut E; Leica, Vienna, Austria). Ultrathin sections were analyzed in an EM208S transmission electron microscope (Philips, Netherlands) with Kodak electron microscope films 4489 (Kodak, USA). These analyses were conducted in a blind trial. Then films were developed into papers (Fujiboro WP FM2; Fuji photo film, Tokyo, Japan) using with FSP-1435 B/W paper processor (FC Manufacturing, Itami, Japan) and 450M-D classic enlarger (fujimoto photo industrial, Osaka, Japan).

5.14. Statistical analysis

Data are reported as means ± S.E.M. Values of mGluR7$^{-/-}$ mice or AMN082 injected mice were compared with those of mGluR7$^{+/+}$ mice or vehicle injected mice respectively by using the two-tailed Student's *t* test. Otherwise, Dunnett's *t* test was used as mentioned in detail in each figure legends. The level of significance was set at $p < 0.05$.

6. Results

6.1. Neurophysiological analysis of mGluR7 deficient mice

6.1.1. Analysis of transcript levels of stress-related genes

In an attempt to address the physiological basis of the behavioral phenotype of mGluR7$^{-/-}$ mice, which showed reduced anxiety- and depression-like features, gene expression levels associated with the stress system of the brain were analyzed. The genes chosen were those encoding GR, MR, CRF, CRF receptor type 1 (CRFR1) and the 5-HT$_{1A}$ receptor. Fig. 6.1.1. shows expression levels of mGluR7 and these five stress-related genes in the hippocampus, the hypothalamus and the prefrontal cortex of mGluR7$^{+/+}$ and mGluR7$^{-/-}$ male mice. As expected, there was no detectable level of mGluR7 mRNA either in the hippocampus, in the hypothalamus or in the prefrontal cortex of mGluR7$^{-/-}$ mice. In the hippocampus, mGluR7$^{-/-}$ mice showed an increased GR level compared to mGluR7$^{+/+}$ mice, which was highly significant in p.m. sacrificed animals (p = 0.007; Fig. 6.1.1.D) but did not reach statistical significance in a.m. sacrificed animals (p = 0.093; Fig. 6.1.1. A). 5-HT$_{1A}$ receptor (5-HT$_{1A}$R) levels were increased in the hippocampus and in the prefrontal cortex of both a.m. (p = 0.002 and 0.016, respectively) and p.m. (p = 0.002 and 0.038, respectively) sacrificed mGluR7$^{-/-}$ mice, when compared to mGluR7$^{+/+}$ mice (Fig. 6.1.1.A, C, D, F). The elevated levels of 5-HT$_{1A}$ receptor mRNA in the hypothalamus of mGluR7$^{-/-}$ mice did not quite reach the level of statistical significance (p = 0.055 and 0.064; for a.m. and p.m. sacrificed animals respectively; Fig. 6.1.1.B, E). Expression levels of MR, CRF, and CRFR1 remained unchanged in the hippocampus, the hypothalamus and the prefrontal cortex between the two genotypes.

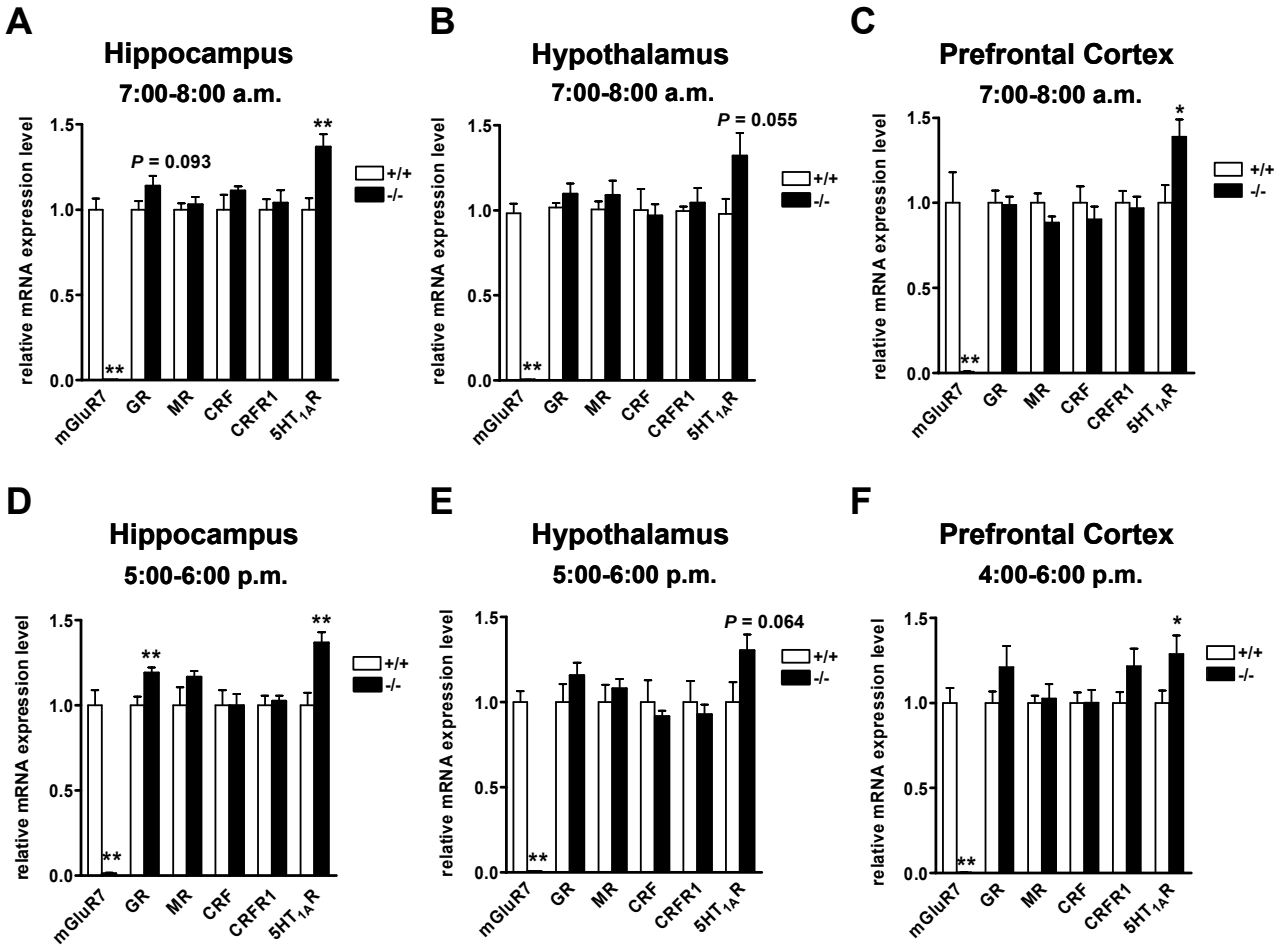

Figure 6.1.1. **Transcript levels related to the stress system in the brain (Mitsukawa et al., 2006).**

Relative transcript levels of mGluR7 and stress-related genes in the hippocampus (A, D), the hypothalamus (B, E) and the prefrontal cortex (C, F) of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice. Sample collection was performed in the morning (7:00 - 8:00 a.m.; A, B, C) and in the afternoon (4:00 - 6:00 p.m.; D, E, F). All values are normalized to the expression level of the reference gene 18S rRNA. The mRNA levels of mGluR7$^{+/+}$ mice are set arbitrarily to the expression level of 1.0. Expression levels in mGluR7$^{-/-}$ mice (filled columns) are shown relative to mGluR7$^{+/+}$ animals (opened columns). The data, displayed as means ± S.E.M., are the average values of two independent determinations (A, B, D, E) or the average of the triplicate experiment (C, F). Each bar represents a group of 7 - 12 animals. Statistical analysis was done by Student's t test. ** $p < 0.01$, * $p < 0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice.

6.1.2. Basal conditions of stress hormone levels

Basal levels of serum corticosterone and ACTH concentrations were measured in mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice. These analyses were conducted in both male and female mice. Although mGluR7$^{-/-}$ male mice showed lower levels of both corticosterone and ACTH, especially in the morning (Fig. 6.1.2.), this did not reach statistical significance presumably due to the large variation between animals. mGluR7$^{-/-}$ female mice also showed lower levels of these stress hormones and the ACTH concentration of p.m. sacrificed mGluR7$^{-/-}$ mice was significantly decreased, compared to that of mGluR7$^{+/+}$ mice (Fig. 6.1.3.). Body weight and adrenal gland weight of mGluR7$^{-/-}$ mice were

somewhat lower than those of mGluR7$^{+/+}$ mice; however adrenal gland weight normalized to body weight showed no difference between mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Table 6.1.; provided by Conrad Gentsch).

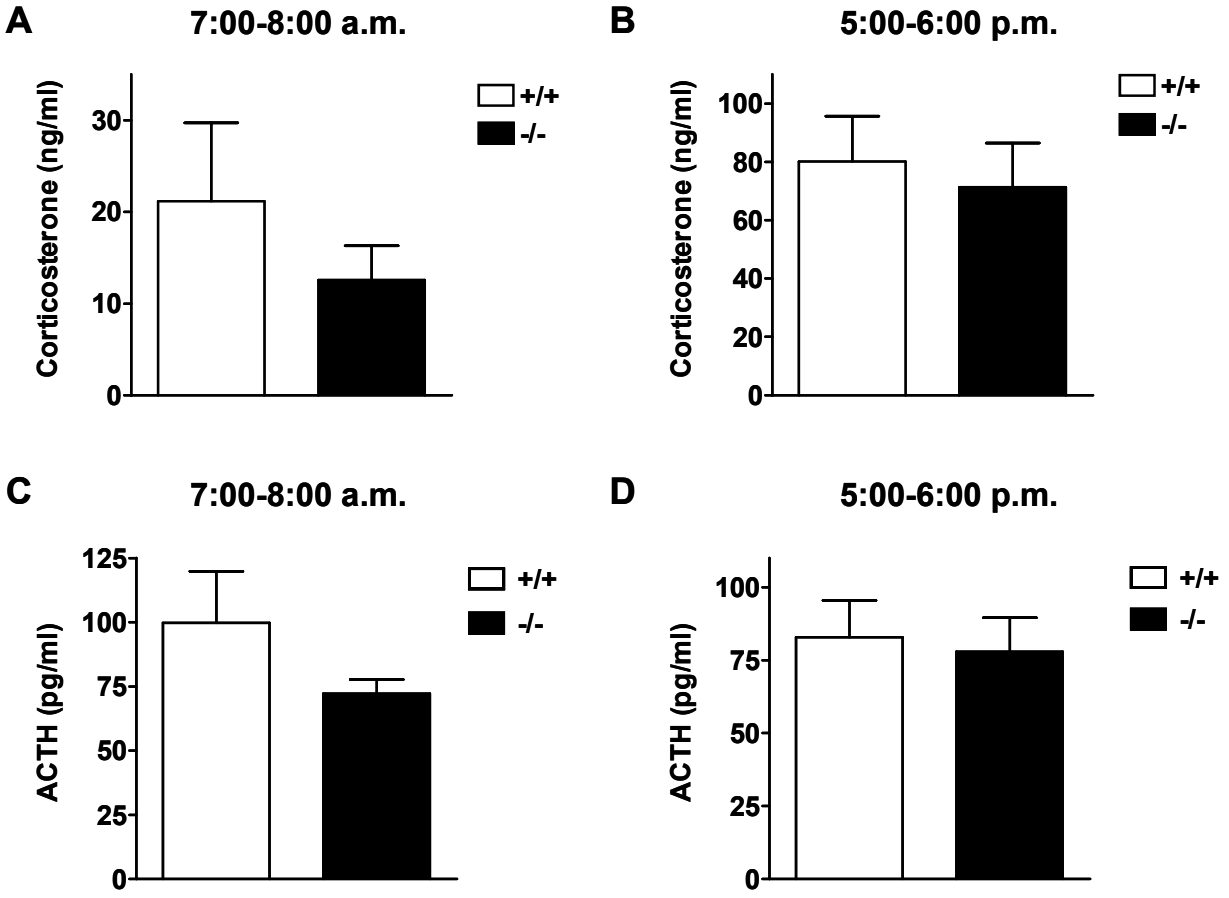

Figure 6.1.2. **Basal serum stress hormone levels in male mice (Mitsukawa et al., 2006).**
Basal plasma corticosterone (A, B) and ACTH (C, D) concentrations of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (n = 8 - 10 per group). Sample collection was performed in the morning (7:00 - 8:00 a.m.; A, C) and in the afternoon (5:00 - 6:00 p.m.; B, D). All bars represent means ± S.E.M. Statistical analysis was done by Student's t test. No mGluR7$^{-/-}$ group showed significant difference when compared with the mGluR7$^{+/+}$ group ($p > 0.05$).

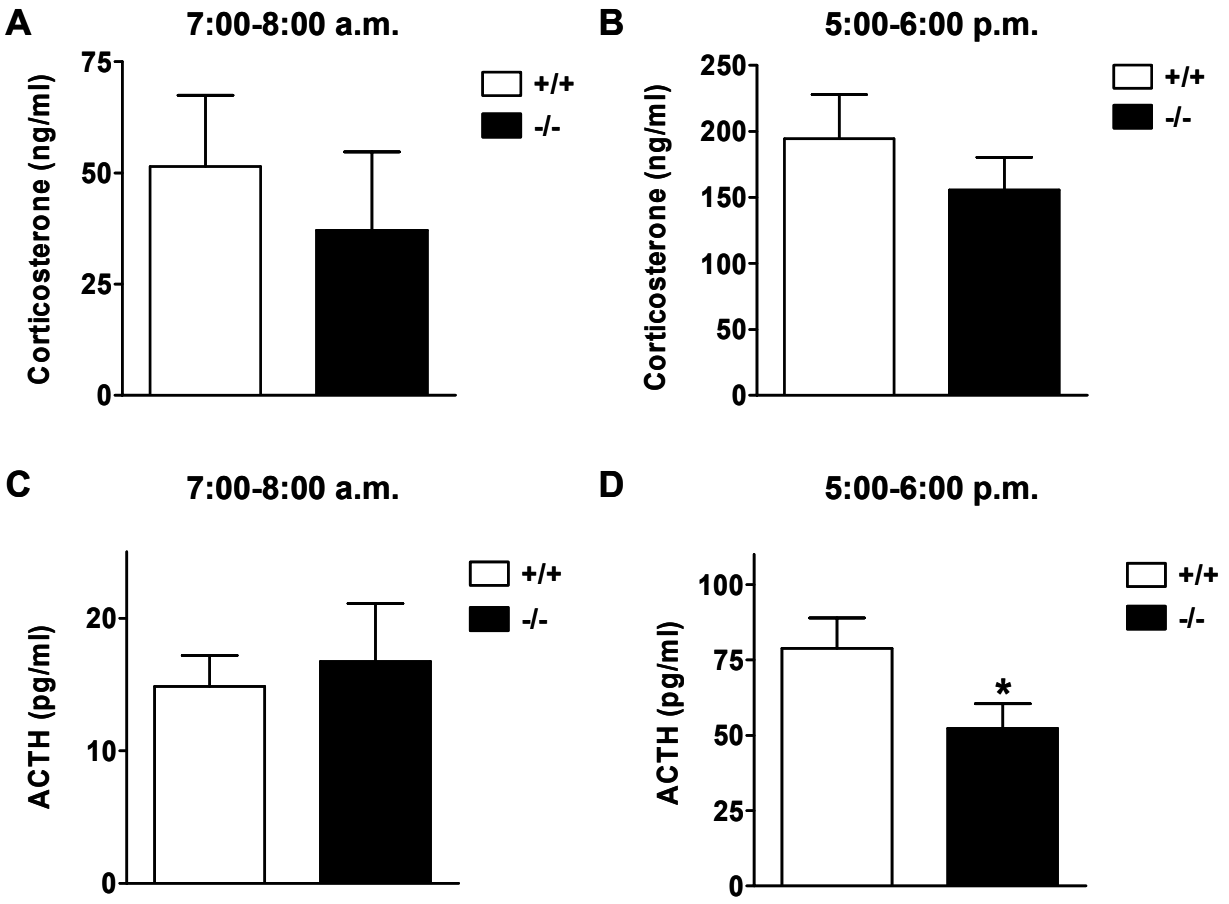

Figure 6.1.3. **Basal serum stress hormone levels in female mice.**
Basal plasma corticosterone (A, B) and ACTH (C, D) concentrations of female mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (n = 8 - 10 per group). Sample collection was performed in the morning (7:00 - 8:00 a.m.; A, C) and in the afternoon (5:00 - 6:00 p.m.; B, D). All bars represent means ± S.E.M. Statistical analysis was done by Student's t test. No mGluR7$^{-/-}$ group showed significant difference when compared with the mGluR7$^{+/+}$ group ($p > 0.05$).

	genotype	***Body weight*** **(g)**	***Adrenal gland weight***		***n***
			mg	**mg/10 g BW**	
male	**+/+**	**30.3 ± 0.6**	**3.2 ± 0.3**	**1.0 ± 0.1**	**8**
	-/-	**26.6 ± 1.1**	**2.7 ± 0.3**	**1.0 ± 0.1**	**8**
female	**+/+**	**24.4 ± 0.8**	**5.7 ± 0.5**	**2.4 ± 0.2**	**10**
	-/-	**22.5 ± 0.7**	**5.6 ± 0.5**	**2.5 ± 0.2**	**10**

Table 6.1. **Body weight and adrenal gland weight of mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Mitsukawa et al., 2006).**
Results represent means ± S.E.M. BW; body weight . This table was provided by Conrad Gentsch.

6.1.3. Effects of swim stress on stress hormone levels

In a next experiment, the levels of corticosterone and ACTH in response to swim stress were tested with mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (10 male animals each). When blood samples were taken between 8:00 and 11:00 a.m., mGluR7$^{-/-}$ mice showed lower levels of corticosterone (Fig. 6.1.4.A) and ACTH (Fig. 6.1.4.D) compared to mGluR7$^{+/+}$ mice (corticosterone; 9.55 ± 1.86 ng/ml vs. 27.57 ± 6.86 ng/ml, p = 0.029, ACTH; 33.65 ± 3.00 ng/ml vs. 75.55 ± 18.78 ng/ml, p = 0.054). These basal levels without swim stress showed less animal to animal variation than data of Fig. 6.1.2. Immediately after swim stress, mGluR7$^{-/-}$ mice showed an increase in the levels of corticosterone (Fig. 6.1.4.B) and ACTH (Fig. 6.1.4.E) comparable to mGluR7$^{+/+}$ mice, and there was no difference between the two genotypes. In addition, at the point of 90 min after swim stress, the stress-induced increase of corticosterone and ACTH levels were recovered at least partially in both genotypes (Fig. 6.1.4.C, F), however the levels of both stress hormones in mGluR7$^{-/-}$ mice showed a trend lower than in mGluR7$^{+/+}$ mice, which may lead to the suggestion that mGluR7$^{-/-}$ mice may affect the recovery of the hormonal system from the stress, although these differences did not reach statistical significance.

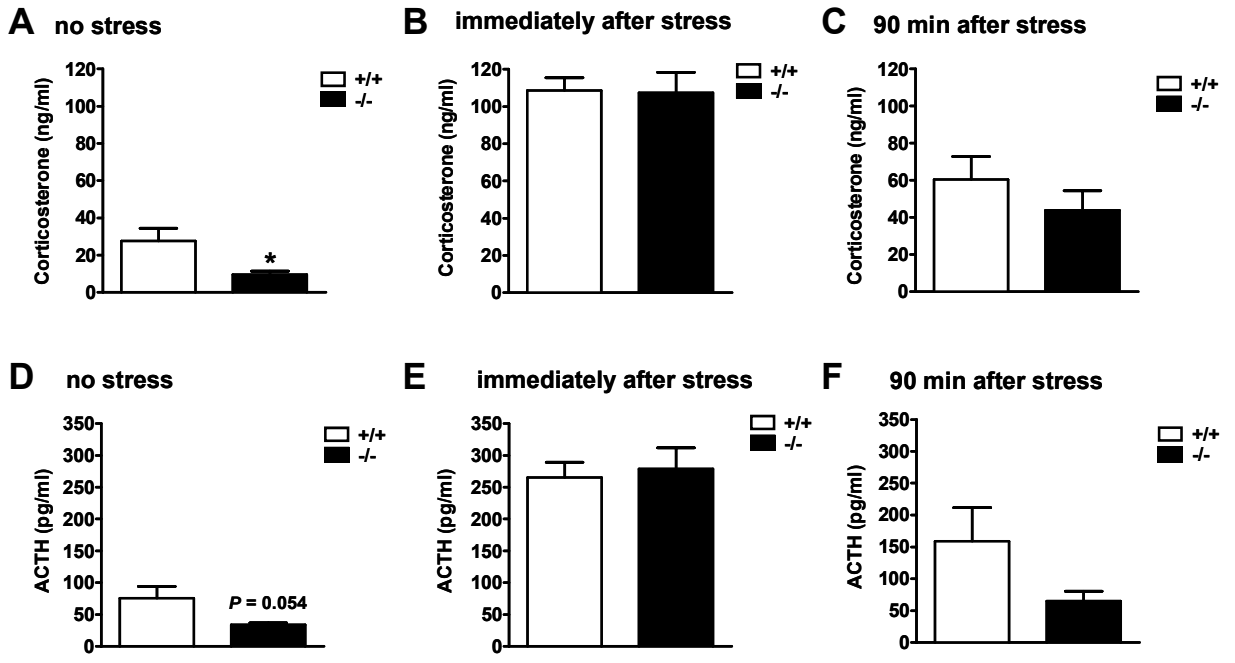

Figure 6.1.4. **Basal and swim stress-modified levels of serum stress hormone levels in male mice (Mitsukawa et al., 2006).**
Baseline and swim stress-modified levels of plasma corticosterone (A, B, C) and ACTH (D, E, F) concentrations of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (n = 9 - 10 per group). The duration of swim stress was 6 min. Sample collection was performed between 8:00 and 11:00 a.m.. All bars represent means ± S.E.M. Statistical analysis was done by Student's t test. * $p < 0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice.

6.1.4. Dexamethasone suppression test

The levels of plasma corticosterone and ACTH were also examined when mice were injected with the synthetic glucocorticoid, dexamethasone, to investigate the GR-mediated negative feedback system of the HPA axis. Fig. 6.1.5. illustrates plasma concentrations of corticosterone and ACTH in mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice injected with saline, 0.03 or 0.1 mg/kg dexamethasone subcutaneously (SC). Plasma corticosterone concentrations of vehicle-treated mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice were not different at afternoon time points (2:00 – 3:00 p.m.; Fig. 6.1.5.A).
Administration of 0.03 mg/kg dexamethasone reduced plasma corticosterone level in mGluR7$^{-/-}$ mice (Fig. 6.1.5.B) and there was a significant difference between these two genotypes ($p = 0.041$). The injection of 0.1 mg/kg dexamethasone showed a suppression of corticosterone levels both in mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Fig. 6.1.5.C) compared to vehicle-treated animals of both genotypes (Fig. 6.1.5.A) and there was no significant difference anymore between the two genotypes. On the other hand, at the same time points (2:00 – 3:00 p.m.), the plasma ACTH level of vehicle-treated mGluR7$^{-/-}$ mice was significantly less than that of mGluR7$^{+/+}$ mice (Fig. 6.1.5.D; $p = 0.022$). Administration of 0.03 mg/kg dexamethasone resulted in reduced plasma ACTH levels in mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice (Fig. 6.1.5.E); ACTH was lower in mGluR7$^{-/-}$ mice than in mGluR7$^{+/+}$ mice, although this was not quite statistically significant ($p = 0.061$). With the injection of 0.1 mg/kg dexamethasone, both mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice showed suppressed ACTH levels (Fig. 6.1.5.F) and there was no significant difference between the genotypes.

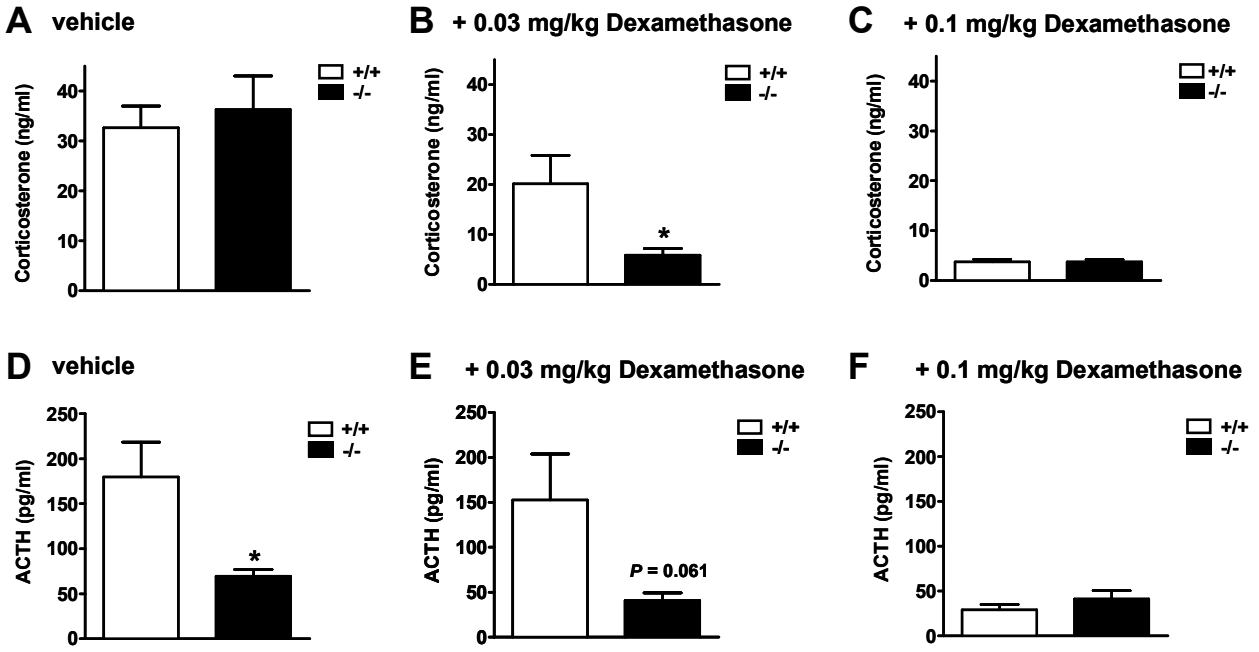

Figure 6.1.5. **Basal and swim stress-modified levels of serum stress hormone levels in male mice (Mitsukawa et al., 2006).**
Baseline and dexamethasone-inhibited corticosterone (A, B, C) and ACTH (D, E, F) concentrations of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (n = 8 - 10 per group). Sample collection was performed between 2:00 and 3:00 p.m., 6 hr after subcutaneous (SC) injection of dexamethasone. All bars represent means ± S.E.M. Statistical analysis was done by Student's t test. * $p < 0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice.

6.1.5. Expression levels of BDNF transcript and protein

Next, the investigation as to whether BDNF levels are changed in mGluR7$^{-/-}$ mice was conducted by using RT-PCR and ELISA assays for mRNA and protein level determination, respectively. Sample collection was done at the same time point as Fig.6.1.1. In a.m. sacrificed animals, there was no difference in either mRNA or protein level at a preliminary experiment between mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (data not shown). In p.m. sacrificed animals (4:00 - 6:00 p.m.), there was no difference in BDNF mRNA either in the hippocampus, in the hypothalamus, or in the prefrontal cortex between mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (Fig. 6.1.6.A). However, mGluR7$^{-/-}$ mice showed a significantly increased level of BDNF protein in the hippocampus as compared to mGluR7$^{+/+}$ mice ($p = 0.022$), although the prefrontal cortex showed no difference (Fig. 6.1.6.B) which demonstrates that there is a level of regional specificity to the increases in BDNF. This result in the hippocampus was confirmed by repeating the experiment independently with 9 - 12 mice per group in tissue that was freshly dissected (Fig. 6.1.6.C). The same differences between the two genotypes ($p = 0.001$) were obtained, which demonstrates that mGluR7$^{-/-}$ mice show a 20 - 25 % increase of BDNF protein level in the hippocampus as compared to mGluR7$^{+/+}$ animals.

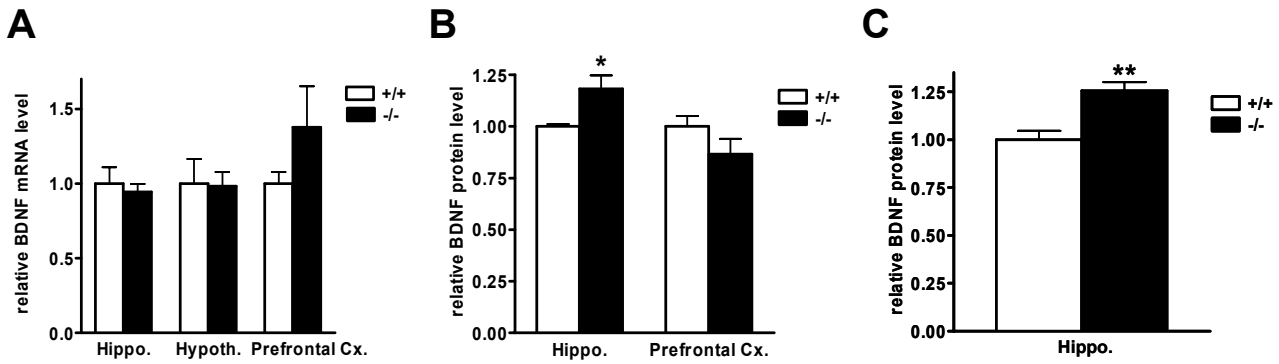

***Figure 6.1.6. Basal and swim stress-modified levels of serum stress hormone levels in male mice* (Mitsukawa et al., 2006).**
Relative BDNF transcript levels in the hippocampus, the hypothalamus and the prefrontal cortex (A) and protein levels in the hippocampus (B, C) and the prefrontal cortex (B) of male mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice (n = 8 - 12 per group). Sample collection was performed in the afternoon (4:00 - 6:00 p.m.) and various regions were dissected either from frozen/freshly thawed brains (A, B) or fresh brains (C). The relative transcript levels are normalized to the expression level of the reference gene 18S rRNA. The mRNA data, displayed as means ± S.E.M., are the average value of two independent determinations in the hippocampus and the hypothalamus, or the average value of the triplicate experiment in the prefrontal cortex (n = 8 - 11 animals per group). Expression levels in mGluR7$^{-/-}$ mice (filled columns) are shown relative to those in mGluR7$^{+/+}$ mice (opened columns) that are set arbitrarily to the expression level of 1.0. Statistical analysis was done by Student's t test. ** $p < 0.01$, * $p < 0.05$; groups that differed significantly from mGluR7$^{+/+}$ mice. Hypoth.; hypothalamus, Prefrontal Cx.; prefrontal cortex

6.2. Characterization of the first selective mGluR7 agonist, AMN082

6.2.1. Effect of AMN082 on cloned mGluR7

As described and demonstrated above, cumulative data has supported an important physiological role of mGluR7. However, there was no previously known pharmacological tool selective for mGluR7. It was therefore an aim in this thesis to identify and characterize mGluR7 selective pharmacological tools. Here, I would like to disclose the characterization of the first selective mGluR7 agonist, N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride, AMN082. This compound was originally obtained a patent by Ciba-Geigy AG with its property of alleviation of breathing difficulties (Karl et al., 1982) and was therefore in chemical libraries at Novartis (Costa et al., 2002). In the year 2001 – 2002, approximately 200,000 compounds were screened with mGluR7-expressing cell lines by high throughput random screening of chemical libraries at Novartis. Among those, AMN082 was initially identified as a compound with similar responses to

L-AP_4. The chemical structure of AMN082 (Fig. 6.2.1.B) is completely unrelated to the known mGluR7 ligands which are all derived from the L-glutamate back-bone such as L-AP_4 (Fig. 6.2.1.A; reviewed in Yang, 2005)

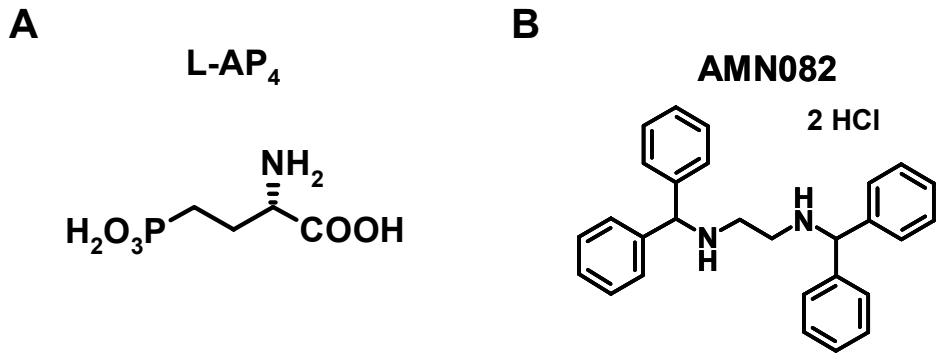

Figure 6.2.1. Chemical structures of L-AP_4 and AMN082.

To investigate stimulating effects of AMN082, stimulation of GTPγ[^{35}S] binding was measured. This stimulation of GTPγ[^{35}S] binding is widely used to assess functional activity of G protein coupled receptors (Lazareno and Birdsall, 1993; Kowal et al., 1998; Urwyler et al., 2001). The novel compound, AMN082, itself induced the stimulation of GTPγ[^{35}S] binding significantly in CHO cells stably expressing human mGluR7b (hmGluR7b), without any orthosteric ligands (Fig. 6.2.2.A). In addition, when tested in the presence of the lower concentration (200 μM) and the higher concentration (saturating concentration; 4 mM) of DL-AP_4, GTPγ[^{35}S] binding was markedly enhanced by AMN082 (Fig. 6.2.2.A). However, no such activity with AMN082 was seen in CHO cells stably expressing hmGluR2 (Fig. 6.2.2.B). This was also the case in CHO cells stably expressing rat mGluR7a (rmGluR7a) or rmGluR2 (Fig. 6.2.2.C, D). AMN082 itself stimulated GTPγ[^{35}S] binding comparable to or even greater than the saturating activity of DL- AP_4.

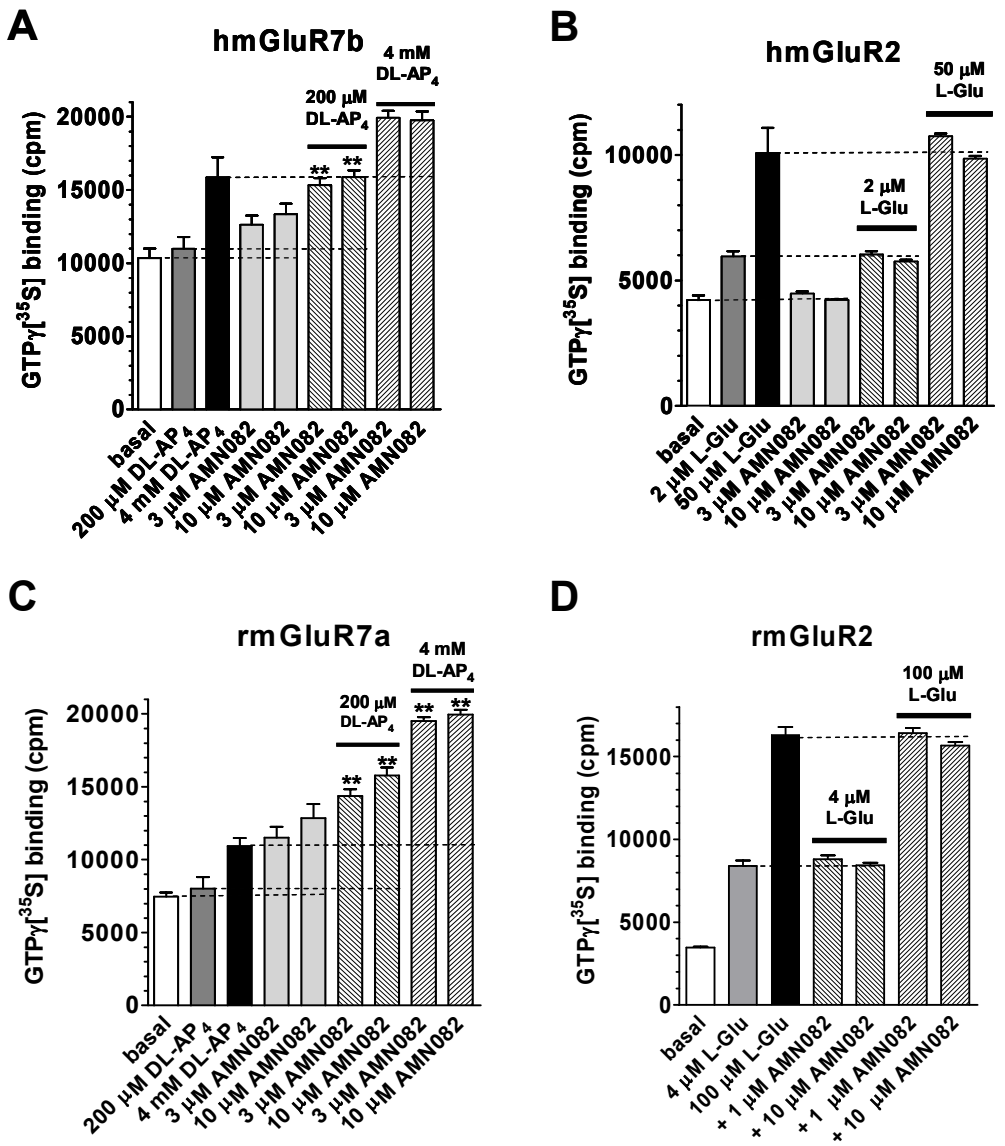

Figure 6.2.2. mGluR7-selectivity of AMN082 in GTPγ[^{35}S] binding assays.
Robust stimulation of both mGluR7 splice variants was demonstrated by GTPγ[^{35}S] binding (A, C), although no stimulation of GTPγ[^{35}S] binding by AMN082 was detected in CHO cells stably expressing both human (h)- and rat (r)- mGluR2 (B, D). In A and C, All six groups that contained AMN082 showed significantly increased level of GTPγ[^{35}S] binding compared with the respective controls ($p < 0.01$ for each of those six groups; Dunnett's *t* test; symbols are not shown). **, More than additive increases ($p < 0.01$) vs. the combined effect of 200 µM DL- AP_4 or 4 mM DL- AP_4 and the respective concentration of AMN082, respectively; Dunnett's *t* test.

As shown in Fig.6.2.3.A, the saturating concentration of 4 mM DL-AP_4 produced approximately 50 % inhibition of forscolin-induced cAMP accumulation in CHO cells stably expressing the hmGluR7b. AMN082 elicited a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in hmGluR7b-expressing CHO cells (Fig. 6.2.3.C, EC_{50} = 64 ± 32 nM), comparable in efficacy to a saturating concentration of the orthosteric group III mGluR agonist, 4mM DL-AP_4 (Fig. 6.2.3.C, dotted line). Up to 3 µM, there was no effect of AMN082 in the same assay conducted with hmGluR2-expressing CHO cells (Fig. 6.2.3.B). Again the same can be said in CHO

cells stably expressing rmGluR7a or rmGluR2 (Fig. 6.2.3.D - F), as follows. The saturating concentration of 4 mM DL-AP4 produced approximately 50 % inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably expressing rmGluR7a. AMN082 was found to potently inhibit forskolin-stimulated cAMP accumulation in rmGluR7a-expressing cells, producing 30 to 50 % inhibition at a maximally effective concentration of 3 μM and an EC_{50} value of 94 ± 48 nM (Fig. 6.2.3.D, F). In contrast, AMN082 showed no inhibition of forskolin-stimulated cAMP accumulation in rmGluR2-expressing cells at concentrations of up to 3 μM (Fig. 6.2.3.E).

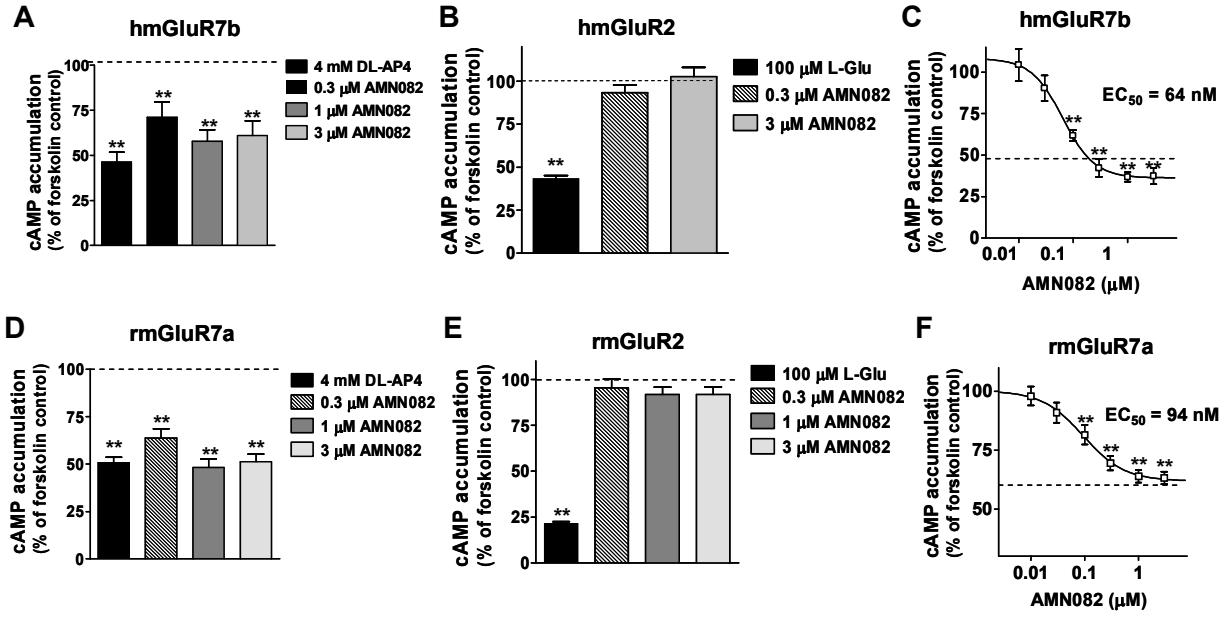

Figure 6.2.3. Agonist effects of AMN082 in CHO cells stably expressing mGluR7 but not mGluR2.
Forskolin (30 μM) stimulated cAMP levels (taken as control) and AMN082 inhibited this forskolin-stimulated cAMP accumulation in CHO cells expressing either hmGluR7b (A, C) or rmGluR7a (D, F) in a concentration dependent manner, although AMN082 showed no effects on CHO-mGluR2 cells (B, E). Results are expressed as % of control and were pooled from at least four measurements obtained in two independent experiments, and expressed as means with S.E.M. **, $p < 0.01$ vs. control; Dunnett's *t* test.

6.2.2. Selectivity profiling of AMN082

The activity of AMN082 was addressed at all eight known mGluRs and at three selected ionotropic receptors. Fig.6.2.4. shows the effects of AMN082 on group II and III mGluRs except mGluR2 and mGluR7 which were already shown in Fig 6.2.2. While GTPγ[^{35}S] binding was enhanced by AMN082 on hmGluR7b- and rmGluR7a-expressing membranes (Fig 6.2.2.), little or no stimulating effect was seen on membranes from hmGluR3-, hmGluR4-, hmGluR6-, and hmGluR8a-expressing

cells, although the overall signal quality on hmGluR8 was relatively small. No GTPγ[^{35}S] binding stimulation in untransfected CHO cells was confirmed.

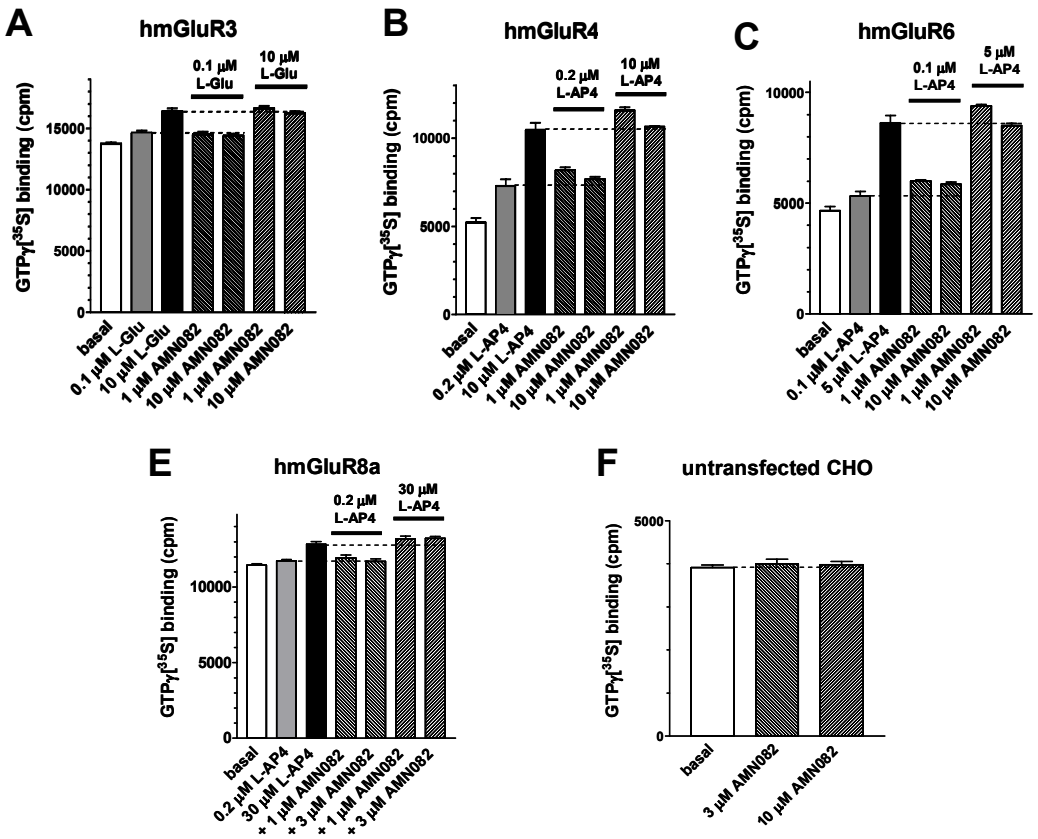

Figure 6.2.4. Effects of AMN082 across group II and III mGluR subtypes.
AMN082 had little or no effect on the agonist-induced stimulation of GTPγ[^{35}S] binding via mGluR3, mGluR4, mGluR6 or mGluR8a expressed in CHO cells, nor in untransfected CHO cells. The data shown are taken from a typical experiment, performed in triplicate, and expressed as mean counts per minute (cpm) values with S.E.M.

Measurements of phosphoinositol (PI) hydrolysis were done to address whether AMN082 activates group I mGluR subtypes (Fig.6.2.5.). There were no effects of AMN082 in the absence and in the presence of the lower and the maximal concentration of L-glutamate at either hmGluR1b- or hmGluR5a-expressing cells, suggesting that AMN082 has neither agonist-like, positive modulatory nor negative modulatory activities at these cells.

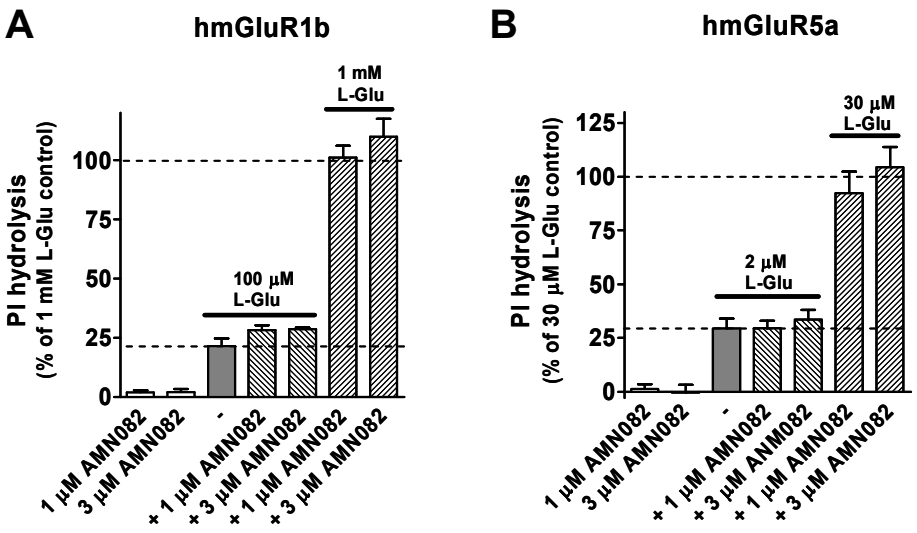

Figure 6.2.5. Effects of AMN082 on group I mGluRs.
AMN082 had no effect on mGluR1b (A) nor mGluR5a (B) in the absence and in the presence of a low (100 µM for mGluR1b and 2 µM for mGluR5a) and a high (1 mM for mGluR1b and 30 µM for mGluR5a) concentration of L-Glu. IP accumulation was measured in CHO cells stably expressed mGluR1b or mGluR5a that were incubated with AMN082 in the absence and in the presence of L-Glu. Data represent the mean ± S.E.M. of at least three independent experiments and all values were normalized to the control stimulation of respective concentrations (1 mM for mGluR1b, and 30 µM for mGluR5a, set to 100%)

Furthermore, the characterization was extended to ionotropic receptors. To address functional agonist and antagonist activity of AMN082 at cloned human NMDARs (hNMDARs), cytoplasmic calcium determinations were employed with stable cell lines transfected with hNMDAR1a/2A and hNMDA1a/2B subunit combinations (Fig.6.2.6.). In the presence and in the absence of L-glutamate, AMN082 showed no effect on the cytoplasmic calcium ion concentrations in these cell lines, even when it was also pre-applied. The representative calcium signal wave is shown in Fig. 6.2.6.A. D-cycloserine (DCS), a partial agonist at the strychnine-insensitive glycine-recognition site on the NMDAR complex, increased calcium signal amplitude only in hNMDAR1a/2A transfected cells (Fig.6.2.6.B), but not in hNMDAR1a/2B transfected cells (Fig.6.2.6.C). In both cell lines transfected hNMDAR1a/2A and hNMDAR1a/2B subunit combinations, a competitive NMDAR antagonist D-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid, CGP40116 (Fagg et al., 1990) completely inhibited L-glutamate induced response.

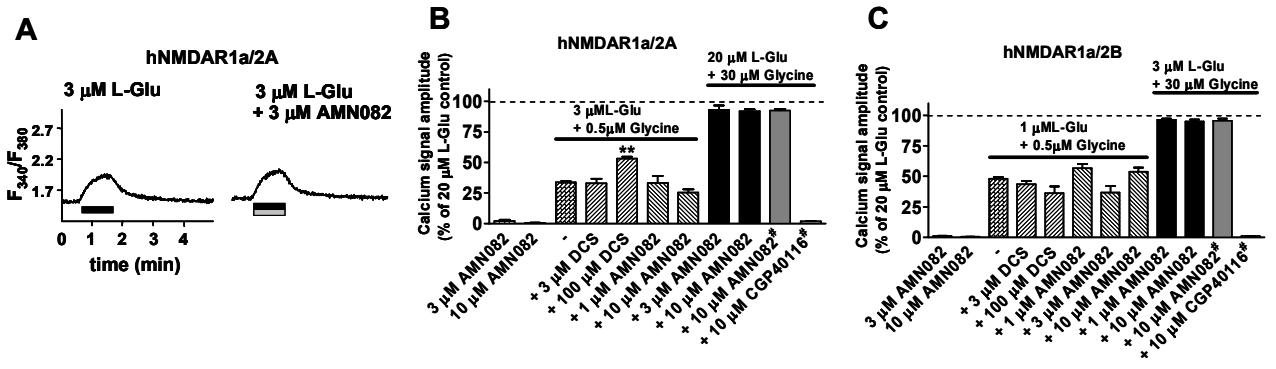

Figure 6.2.6. Effects of AMN082 on NMDA-type ionotrophic glutamate receptors.
Elevation of cytoplasmic calcium ion concentrations, $[Ca^{2+}]_i$, in Ltk$^-$ cells expressing cloned hNMDA receptors (subunit combination 1a/2A and 1a/2B). (A) Increase in the fluorescent response ratio F_{340}/F_{380} corresponds to an elevation in $[Ca^{2+}]_i$. Two consecutive calcium measurements are shown: application of L-Glu alone, followed by co-application of L-Glu and AMN082. The duration of the applications is indicated by bars (black: 3 μM L-glutamate; grey: 3 μM AMN082). (B, C) Each column represents calcium signals (mean values ± S.E.M.) of three independent experiments in either NMDAR1a/2A (B) or NMDA1a/2B (C) transfected cells. AMN082 had no effects on the cytoplasmic calcium ion concentrations, $[Ca^{2+}]_i$, in the absence and in the presence of low and high concentrations of L-Glu. L-Glu induced response was inhibited completely by the competitive NMDA antagonist, CGP 40116. 100 μM D-cycloserine (DCS) increased 3 μM L-Glu induced calcium signal in NMDA1a/2A transfected cells (B). The response of saturated concentration of L-Glu (20 μM for NMDA1a/2A and 3 μM for NMDA1a/2B) was taken as control and set to 100%. #; Groups in which drugs were pre- and co-applied with L-Glu. ** $p < 0.005$; Groups that differed significantly from 3 μM L-Glu (Dunnett's *t* test).

In addition, the profile of AMN082 was characterized against stable cell lines expressing human AMPA receptor GluR3 flip splice variant (hGluR3i) by the same method as hNMDARs; intracellular calcium measurements (Fig.6.2.7.). There was no change of calcium signal amplitude by AMN082 application either in the presence or in the absence of L-glutamate stimulation. Pre-application of AMN082 showed no change, either. L-glutamate induced response was completely inhibited by a selective AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2, 3-dione, CNQX (Honore et al., 1988).

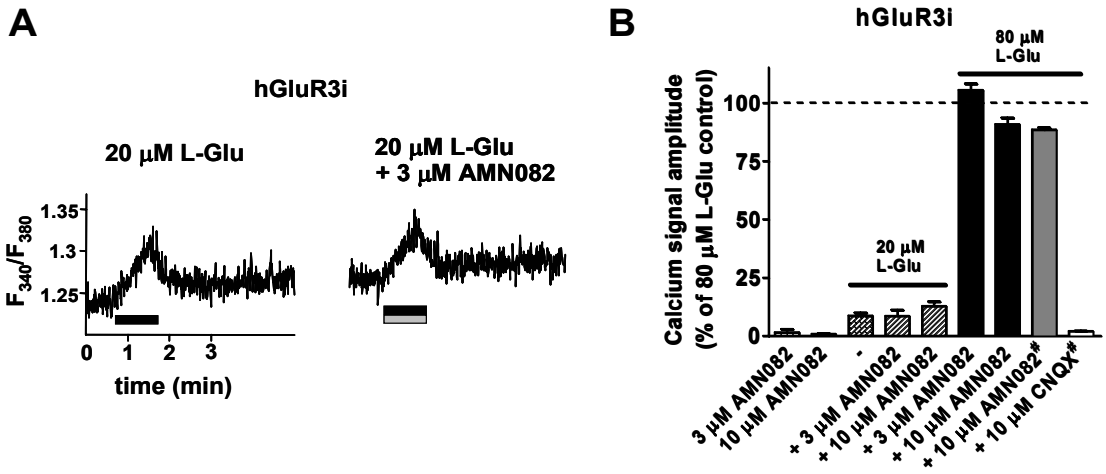

Figure 6.2.7. Effects of AMN082 on AMPA-type ionotrophic glutamate receptors.
Elevation of cytoplasmic calcium ion concentrations, $[Ca^{2+}]_i$, in HEK cells expressing cloned hAMPA receptor, GluR3 flip splice variant (GluR3i). (A) Increase in the fluorescent response ratio F_{340}/F_{380} corresponds to an elevation in $[Ca^{2+}]_i$. Two consecutive calcium measurements are shown: application of L-Glu alone, followed by co-application of L-Glu and AMN082. The duration of the applications is indicated by bars (black: 20 μM L-Glu; grey: 3 μM AMN082). (B) Each column represents calcium signals (mean values ± S.E.M.) of three independent experiments. AMN082 had no effects on $[Ca^{2+}]_i$, in the absence and in the presence of low (20 μM) and high (80 μM) concentrations of L-Glu. L-Glu induced response was inhibited completely by the AMPA receptor antagonist, CNQX. The response of 80 μM L-Glu was taken as control and set to 100%. #; Groups in which drugs were pre- and co-applied with L-Glu.

Table 6.2.1. shows the summary of the effects of AMN082 on all eight mGluRs and on three ionotropic GluRs. The activating effect of AMN082 (up to 10 μM) was selectively seen at mGluR7a and -7b with large efficacies of 70 - 140 % (relative to maximal DL-AP_4 effects), although AMN082 elicited little or no stimulating effects at mGluR1b, mGluR2-, mGluR3-, mGluR4-, mGluR5a, mGluR6-, mGluR8a-, NMDAR1a/2A-, NMDAR1a/2B-, or GluR3i-expressing cells.

Receptor subtype	*Functional assay*	*% Activation induced by AMN082; concentration*	*% Inhibition induced by AMN082; concentration*
mGluR7a	**GTPγ[^{35}S] binding**	**105 – 140 %; 3 μM, 10 μM**	**n.e.; ≤ 10 μM**
mGluR7b	**GTPγ[^{35}S] binding**	**70 – 90 %; 3 μM, 10 μM**	**n.e.; ≤ 10 μM**
mGluR1b	PI hydrolysis	5 – 15 %; 1 – 10 μM	n.e.; ≤ 10 μM
mGluR2	GTPγ[^{35}S] binding	n.e.; ≤ 10 μM	n.e.; ≤ 10 μM
mGluR3	GTPγ[^{35}S] binding	n.e.; ≤ 10 μM	n.e.; ≤ 10 μM
mGluR4	GTPγ[^{35}S] binding	6 – 18 %; 1 – 10 μM	n.e.; ≤ 10 μM
mGluR5a	PI hydrolysis	n.e.; ≤ 3 μM	n.e.; ≤ 3 μM
mGluR6	GTPγ[^{35}S] binding	n.e.; ≤ 10 μM	n.e.; ≤ 10 μM
mGluR8a	GTPγ[^{35}S] binding	10 – 20 %; 1 – 10 μM	n.e.; ≤ 10 μM
NMDA1a/2A	Cytoplasmic Ca^{2+}	n.e.; ≤ 10 μM	n.e.; ≤ 10 μM
NMDA1a/2B	Cytoplasmic Ca^{2+}	n.e.; ≤ 10 μM	n.e.; ≤ 10 μM
GluR3 (flip)	Cytoplasmic Ca^{2+}	n.e.; ≤ 10 μM	n.e.; ≤ 10 μM

***Table 6.2.1. Summary table of mGluR7-selectivity of AMN082 across glutamate receptor subtypes* (Mitsukawa et al., 2005).**
Data from 2 - 4 independent experiments were averaged; n.e., no effect.

6.2.3. AMN082 directly interacts with the heptahelical region of mGluR7

Concentration-response curves for AMN082, DL-AP_4 and L-glutamate are compared in Fig. 6.2.8. AMN082 is far more potent than the orthosteric ligands, DL-AP_4 and L-glutamate; the EC_{50}-values (95 % confidence intervals) are 260 nM (200; 360), 540 µM (440; 670) and 700 µM (580; 850), respectively.

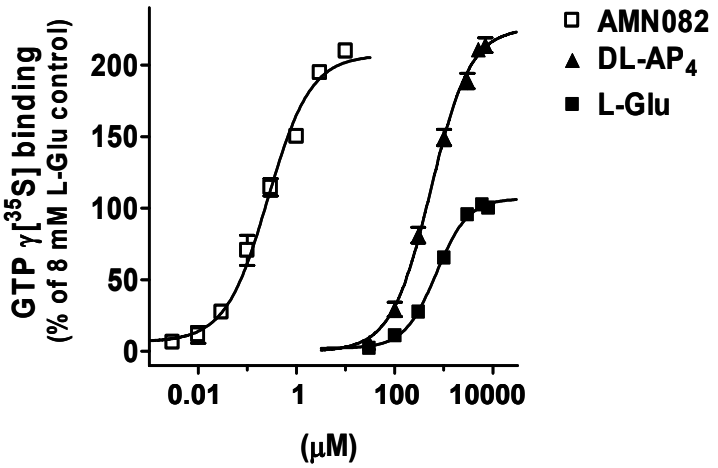

Figure 6.2.8. Concentration-response curves for AMN082, DL-AP_4 and L-Glu **(Mitsukawa et al., 2005).** At least three independent experiments were pooled and all values were normalized to the control stimulation of 8 mM L-Glu (set to 100%). Means with S.E.M. are shown.

Stimulation of GTPγ[^{35}S] binding was performed to evaluate further the pharmacological properties and mechanism of action of AMN082. AMN082 (3 µM) produced 167 ± 8 % stimulation relative to the maximal agonist activity of L-glutamate (set to 100 %, Fig. 6.2.9.). The stimulating effects of AMN082 were almost additive with those of L-glutamate and DL-AP_4 (238 ± 11 % and 336 ± 19 %, respectively), while DL-AP_4 plus L-glutamate (both at maximally active concentrations) produced a smaller stimulation than DL-AP_4 alone (133 ± 4 % versus 216 ± 12 %, Fig. 6.2.9.).

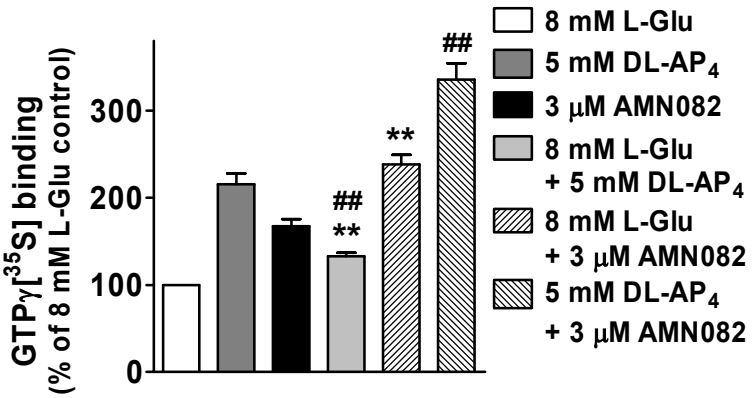

***Figure 6.2.9. Additive effects of AMN082 to orthosteric ligands on membranes from CHO-mGluR7b cells* (Mitsukawa et al., 2005).**
The indicated concentrations of AMN082, DL-AP_4, and L-Glu were applied alone and in combination with each other in GTPγ[^{35}S] binding experiments. Three independent experiments were combined and means with S.E.M. are shown. Statistics symbols are shown for co-application of drugs as follows: **, $P < 0.01$ vs. 8 mM L-Glu (control); ##, $P < 0.01$ vs. 5 mM DL-AP_4; Dunnett's *t* test.

Next, the group III mGluR-selective antagonists MSOP and CPPG (Thomas et al., 1996; Toms et al., 1996) were tested against concentration-response curves of AMN082 conducted in the absence (Fig. 6.2.10.A) and presence of submaximal DL-AP_4 (Fig. 6.2.10.B): the DL-AP_4 component was completely abolished by the antagonists (Fig. 6.2.10.B) while there was no inhibition of the AMN082-stimulated GTPγ[^{35}S] binding (Fig. 6.2.10.A).

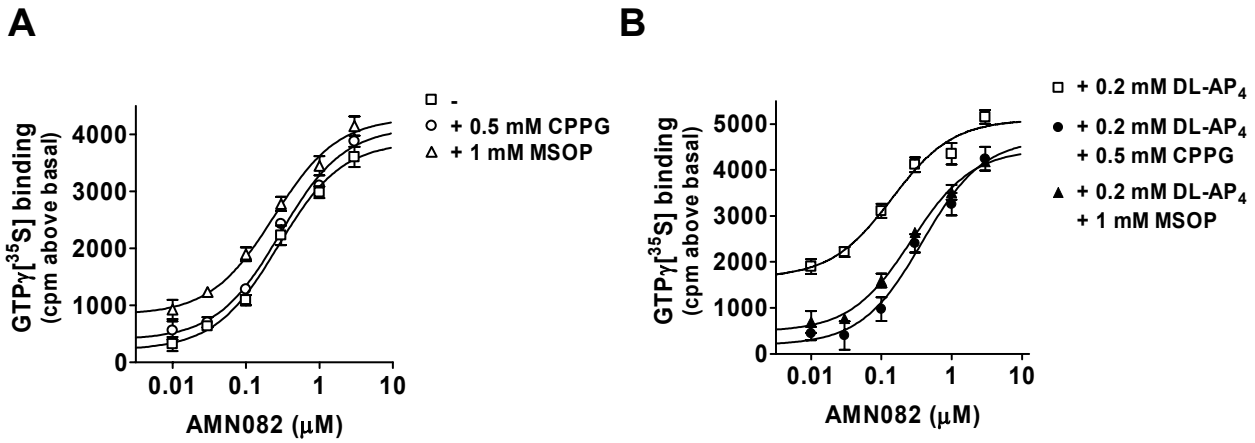

Figure 6.2.10. Lack of the effects of competitive group III mGluR antagonists on AMN082 stimulated GTPγ[^{35}S] binding.
Concentration-response curves of AMN082 in the absence (A) or in the presence (B) of 0.2 mM DL-AP_4 with and without CPPG or MSOP, using GTPγ[^{35}S] binding on CHO-mGluR7b membranes; mean data ± S.E.M. from a single representative experiment (n = 3).

To analyze a potential co-operativity between L-glutamate site ligands and AMN082, concentration-response curves for AMN082 at different fixed concentrations of L-glutamate (Fig. 6.2.11.A), and inversely, curves for L-glutamate versus fixed concentrations of AMN082 (Fig. 6.2.11.B) were conducted. The EC_{50} of AMN082 varied between 140 and 290 nM with largely overlapping 95 % confidence intervals (Fig. 6.2.11.A and Table 6.2.2.). Similarly, the EC_{50} of L-glutamate was consistently between 640 μM and 830 μM, irrespective of the added concentration of AMN082 (Fig. 6.2.11.B and Table 6.2.2.).

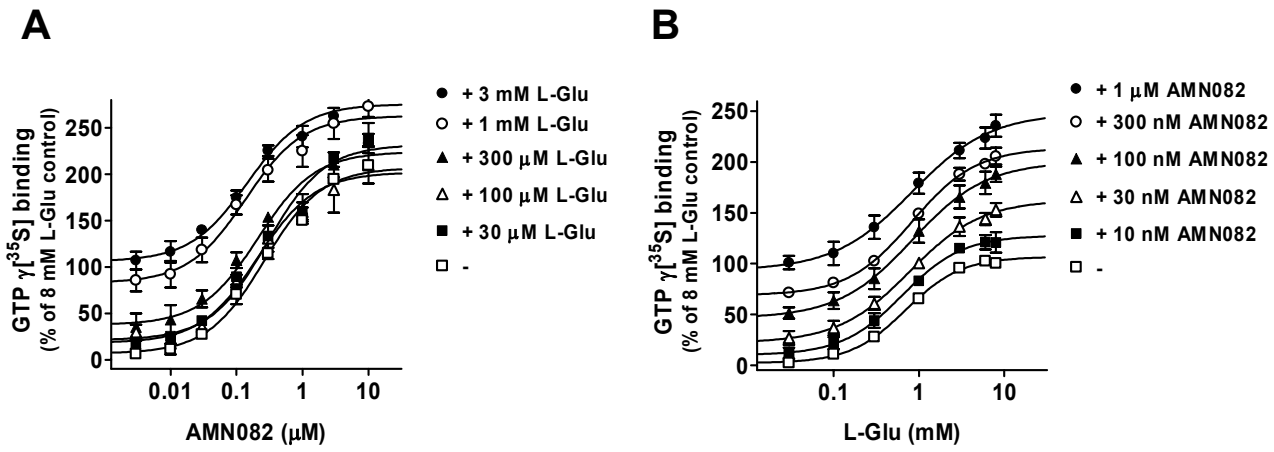

Figure 6.2.11. AMN082 and orthosteric L-glutamate (L-Glu) site ligands have little, if any, effect on each other's potency.
(A) Concentration-response curves for AMN082 in the absence and in the presence of different concentrations of L-Glu. Three independent experiments were pooled and all values were normalized to the control stimulation of 8 mM L-Glu (set to 100%); means with S.E.M. are shown. (B) Concentration-response curves for L-Glu in the absence and in the presence of different concentrations of AMN082. Four independent experiments were pooled and all values were normalized to control (as above).

L-Glu	*EC_{50} of AMN082 (95 % CI)*	*Maximal effect relative to 8 mM L-Glu control*	*n*
μM	*nM*	*%*	
0	260 (160; 450)	207 (187; 227)	3
30	290 (130; 650)	232 (199; 264)	3
100	210 (110; 390)	202 (181; 223)	3
300	210 (80; 570)	224 (191; 257)	3
1000	140 (70; 250)	263 (244; 281)	3
3000	150 (80; 270)	276 (259; 293)	3

AMN082	*EC_{50} of L-Glu (95 % CI)*	*Maximal effect relative to 8 mM L-Glu control*	*n*
nM	*μM*	*%*	
0	700 (580; 850)	107 (100; 114)	3
10	640 (410; 1000)	128 (110; 145)	3
30	780 (460; 1320)	163 (134; 191)	3
100	830 (400; 1760)	200 (155; 246)	3
300	830 (590; 1170)	214 (195; 233)	3
1000	830 (350; 1980)	248 (194; 302)	3

Table 6.2.2. Summary table of Fig.6.2.11 **(Mitsukawa et al., 2005).**
The calculated parameters from at least three separate experiments, parts of which are shown in Fig.6.2.11., indicated that L-Glu or AMN082 had little effect on AMN082 or L-Glu potency, respectively; CI, 95 % confidence interval.

To address whether binding of AMN082 to mGluR7 affects the binding affinity of ligands for the L-glutamate site, displacement of 10 nM [^{3}H]LY341495 (a competitive mGluR antagonist; Wright et al., 2000) binding was analyzed from membranes prepared from CHO cells stably expressing rmGluR7a (This was done by Rina Yamamoto; Master thesis in 2005 at School of Allied Health Sciences, Graduate School of Medicine, Osaka University, Osaka, Japan); up to 30 μM AMN082 showed no displacement of this radioligand; in contrast, 10 mM of L-AP$_4$, L-glutamate, or L-SOP abolished 100 % of specific binding (Fig.6.2.12.; provided by Rina Yamamoto). Moreover, [^{3}H]LY341495 displacement curves were conducted with L-SOP, L-AP$_4$ and L-glutamate, each in the absence and presence of 3 μM AMN082. The addition of AMN082 induced only a minor left-shift of each of the three curves; the calculated K_is with 95 % confidence intervals (in brackets in μM) were as follows: L-SOP, 45 μM (39; 50); L-SOP + 3 μM AMN082, 29 μM (24; 35); L-AP$_4$, 193 μM (165; 224); L-AP$_4$ + 3 μM AMN082, 176 μM (144; 216); L-glutamate, 624 μM (446; 870); L-glutamate + 3 μM AMN082, 524 μM (436; 631).

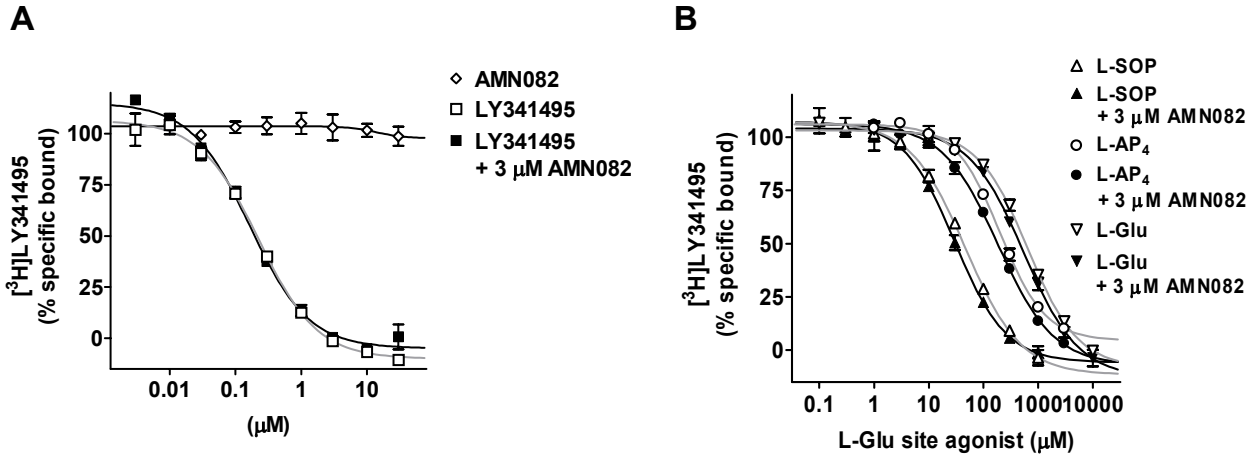

Figure 6.2.12. Displacement experiments for [3H]LY341495 binding to rmGluR7a membranes with AMN082 and known L-glutamate site ligands .
Assays with known L-glutamate site ligands were performed in the absence (open symbols) and in the presence (closed symbols) of 3 μM AMN082. (A) Effects of AMN082 or unlabelled-LY341495 alone or the combination of those are presented. In contrast to LY341495, AMN082 did not displace binding at concentrations of up to 30 μM. (B) Effects of L-SOP, L-AP4 and L-Glu are presented. All values represent % of total specific bound, and were pooled from at least six measurements obtained in three independent experiments, and expressed as means with S.E.M. Non-specific binding, defined by the amount bound in the presence of 1 mM L-SOP, was subtracted from all values. This figure was provided by Rina Yamamoto (Master thesis in 2005 at School of Allied Health Sciences, Graduate School of Medicine, Osaka University, Osaka, Japan).

Furthermore, to localize the binding site of AMN082 to one discrete region of the mGluR7 protein, GTPγ[^{35}S] binding assays were conducted with constructs of wild-type mGluR7b and mGluR6 as well as two chimeras: the mGluR6/7b construct contains the N-terminal extracellular region of mGluR6 and the C-terminal portion of mGluR7b comprising the entire transmembrane region; mGluR7/6 is the reverse chimera (See Materials and Methods 5.9.; Fig. 6.2.13.). The activity of AMN082 on mGluR6/7b and mGluR7/6 chimeras was very similar to wild-type mGluR7b and mGluR6, respectively: AMN082 stimulated mGluR7b and mGluR6/7b to 150 - 200 % relative to the maximal effect of DL-AP$_4$, but AMN082 produced only minor effects on mGluR6- and mGluR7/6-expressing membranes (10 - 25 % relative to maximal DL-AP$_4$ stimulation); Fig. 6.2.13.A - D. It is interesting to note that the stimulating effects of AMN082 and DL-AP$_4$ were more than additive on those mGluR7 expressing membranes but not on mGluR6/7b membranes (Fig. 6.2.13.A, C).

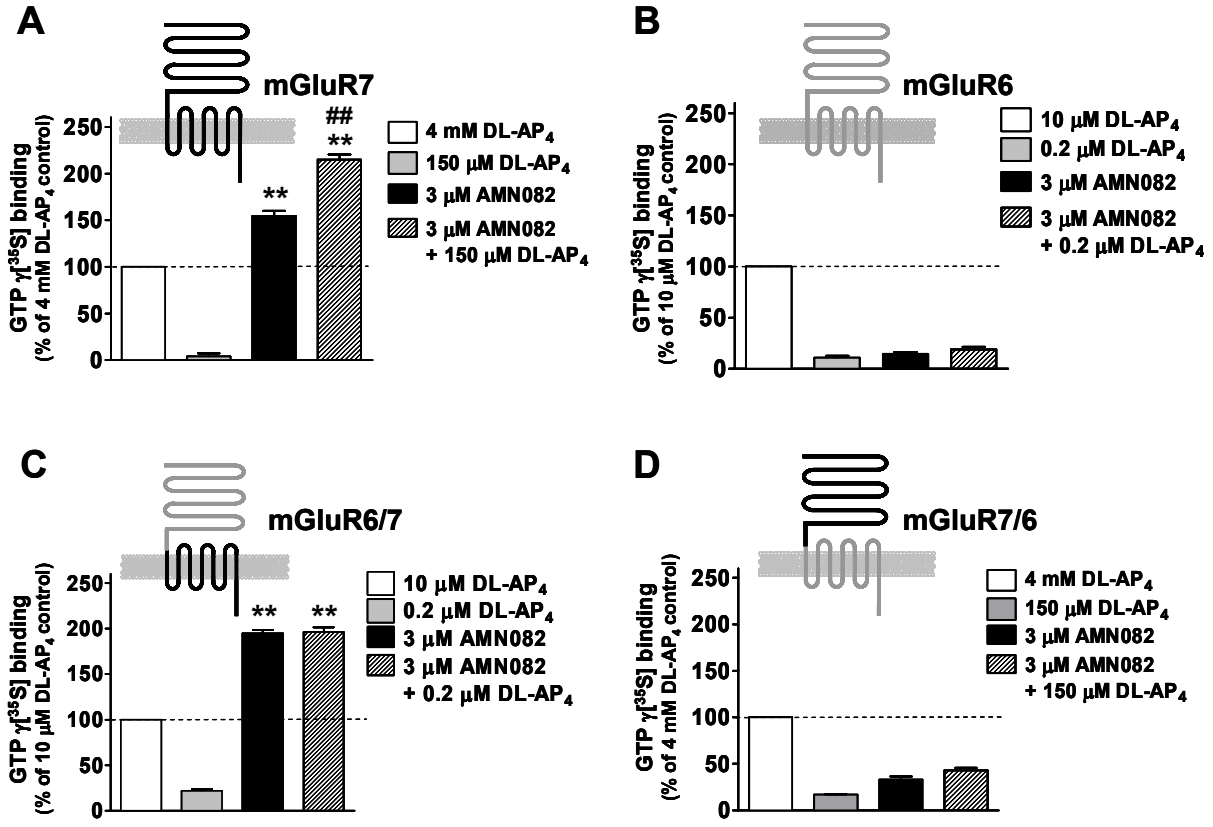

***Figure 6.2.13. AMN082 activates G protein signaling via a binding site in the transmembrane region of mGluR7* (Mitsukawa et al., 2005).**
Effect of AMN082 on GTPγ[^{35}S] binding in the absence and presence of DL-AP$_4$ using membranes from CHO-C4 cells stably expressing mGluR7b (A) or mGluR6 (B). (C, D) The effect of AMN082 on mGluR6/7b (C) and mGluR7/6 (D) chimeric receptor cell membranes is shown. cDNAs were constructed by Natacha Stoehr. All bars represent the mean ± S.E.M. of at least three independent experiments, conducted with n = 3 - 4, and all values were normalized to the control stimulation of the respective agonist concentrations (4 mM DL-AP$_4$ for mGluR7 and mGluR7/6, and 10 µM DL-AP$_4$ for mGluR6 and mGluR6/7b). **, significant increases versus maximal DL-AP$_4$ controls ($P < 0.01$); ##, $P < 0.01$ vs. additive effect of submaximal DL-AP$_4$ (gray bar) and 3 µM AMN082 (black bar); Dunnett's *t* test.

Concentration-response curves for AMN082 on mGluR6/7b and mGluR7/6 chimeras are compared in Fig. 6.2.14. AMN082 has much higher potency and efficacy on mGluR6/7b chimeras (Fig. 6.2.14.A) than those on mGluR7/6 chimeras (Fig. 6.2.14.B). In addition, initial attempts using truncated mutants with deleted extracellular domains were made, owing to Stoehr N and Pescott O, but no activation with DL-AP_4 or AMN082 was observed; it cannot be ruled out that their translated proteins were mis-folded or incorrectly inserted into membranes.

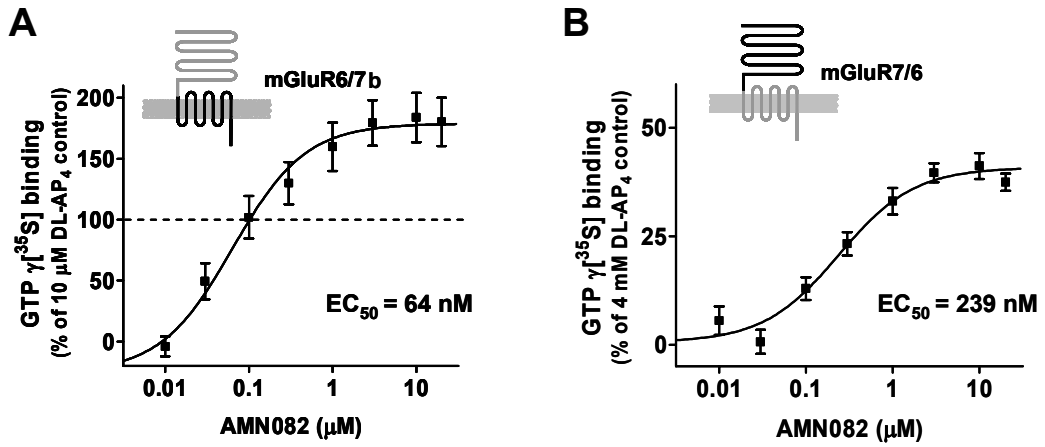

Figure 6.2.14. Considerably higher potency and efficacy of AMN082 on mGluR6/7b than on mGluR7/6. (A) A concentration-response curve for AMN082 in the GTPγ[^{35}S] binding assay on mGluR6/7b (A) or mGluR7/6 (B) chimeric receptor. Both efficacy and potency of AMN082 is much larger in mGluR6/7b chimeric receptor. Data represent the mean ± S.E.M. of at least three independent experiments and all values were normalized to the control stimulation of respective concentrations (4 mM for mGluR7/6, and 10 μM for mGluR6/7b, set to 100%).

6.2.4. *In vivo* activity of AMN082: modulation of stress hormones in an mGluR7-dependent fashion

The role of mGluR7 in stress-related behavior is well documented (Cryan et al., 2003; Masugi et al., 1999; Mitsukawa et al., 2006). Therefore, the effect of oral administration of AMN082 was analyzed on serum levels of the stress hormones corticosterone and ACTH. Fig. 6.12.15. shows that AMN082 readily passes the blood-brain barrier upon oral administration and increases plasma corticosterone in a dose-dependent manner in a wild-type mouse strain (C57BL/6). Next, mGluR7-deficient mice (mGluR7$^{-/-}$) and their wild-type littermates (mGluR7$^{+/+}$) were used. Again, oral administration of 6 mg/kg of AMN082 elicited a circa 200 % increase of plasma corticosterone in mGluR7$^{+/+}$ animals, but no such rise was observed in mGluR7$^{-/-}$ mice (Fig. 6.12.15.B). Similarly, blood-levels of ACTH were also increased to 200 %, one hour following oral AMN082 administration, in wild-type animals, but not in mGluR7-deficient mice (Fig. 6.12.15.C).

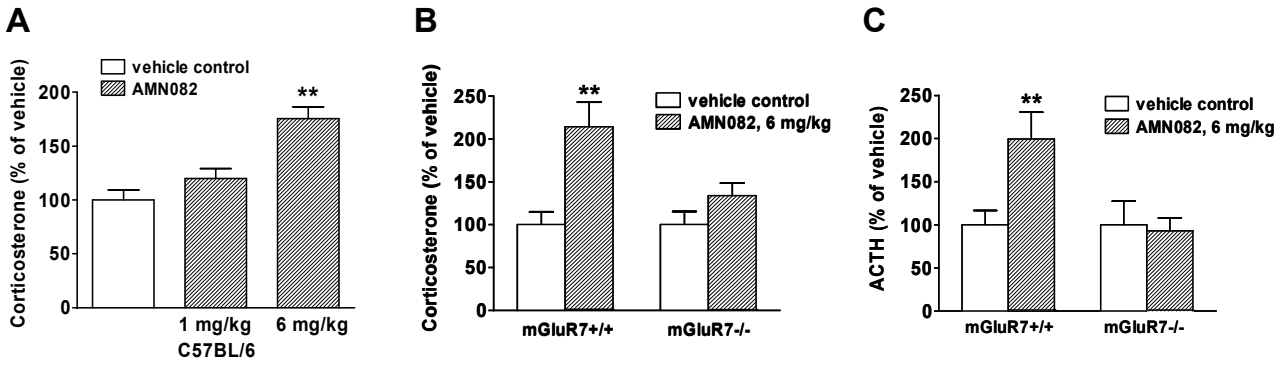

Figure 6.2.15. AMN082 induces stress hormone increases in an mGluR7-dependent fashion **(Mitsukawa et al., 2005).**
Vehicle or AMN082 were administered to mice orally (p.o.) and one hour later mice were decapitated and the blood was collected. Plasma corticosterone and ACTH levels were measured with radioimmunoassay kits. (A) Effect of 1 and 6 mg/kg AMN082 (p.o.) on plasma corticosterone level in naive C57BL/6 mice. (B) 6 mg/kg AMN082 (p.o.) increased plasma corticosterone level approximately to 200 % of vehicle control in mGluR7$^{+/+}$ mice, but not in mGluR7$^{-/-}$ mice. (C) AMN082 increased plasma ACTH level in mGluR7$^{+/+}$ mice to the same relative level as shown for corticosterone, but no change was observed in mGluR7$^{-/-}$ mice. Data were normalized to each vehicle control and represent the mean ± S.E.M. from 9 - 13 independent mice per group. **, $p < 0.01$ vs. vehicle control; two-way ANOVA followed by Fisher's post hoc test.

6.3. Internalization of mGluR7

It has recently been demonstrated that mGluR7 is internalized in response to L-AP$_4$ (Pelkey et al., 2005). It was therefore interesting to investigate whether AMN082 induces internalization of mGluR7. To test this possibility, immunoelectron microscopic studies were performed with antibodies against mGluR7a or mGluR7b (Shigemoto et al., 1997) in hippocampal sections of mouse brain slices treated orally with either vehicle or 6 mg/kg AMN082. In vehicle-treated sections, using pre-embedding immunogold labeling, mGluR7a (Fig. 6.3.1.A, left panel) and mGluR7b (Fig. 6.3.1.B, left panel) immunoreactivity consistently localized within active zones in presynaptic membranes making asymmetric synapses in the hippocampal CA3 as previously reported (Shigemoto et al, 1997; Pelkey et al., 2005). In contrast to these sections from vehicle-treated animals, immunogold particles for both mGluR7a (Fig. 6.3.1.A, right panel) and mGluR7b (Fig. 6.3.1.B, right panel) also often appeared far from the active zone deep within presynaptic areas making asymmetric synapses within the hippocampal CA3 of brain sections from AMN082-treated animals. To test specificity of the procedures for electron microscopy, the primary antibody was omitted. It was confirmed that under this condition, no selective labeling was observed (data not shown). In addition, some sections were proceeded with immunocytohistochemical methods without silver intensification. With these methods, no metal particles were detected (data not

shown). The observation of immunogold particles for mGluR7a and mGluR7b deep within mossy fiber boutons in AMN082-treated brain sections strongly suggests that mGluR7a and mGluR7b do indeed undergo internalization.

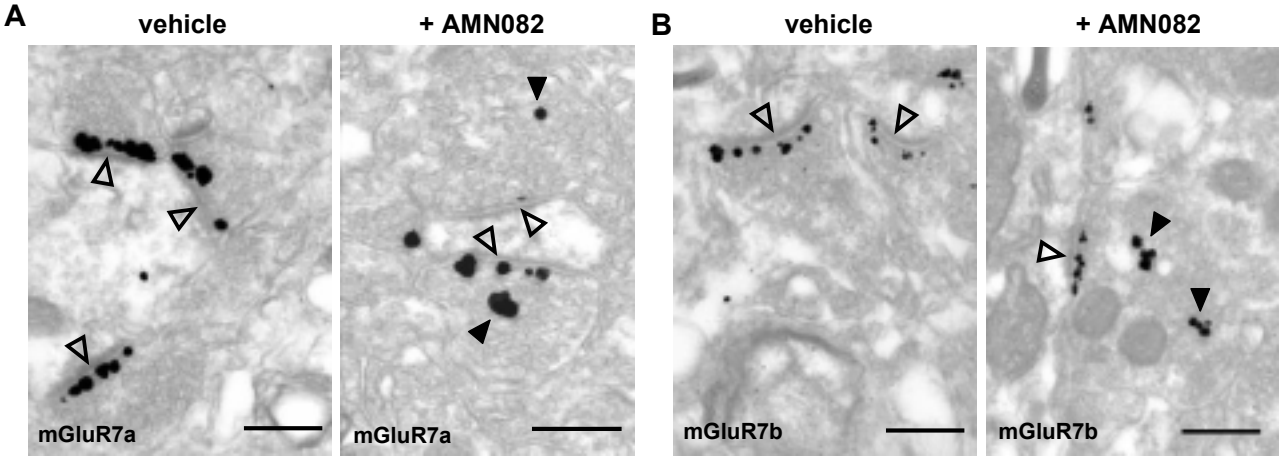

Figure 6.3.1. Representative electron micrographs showing mGluR7a and mGluR7b localization.
Pre-embedding immunogold labeling for mGluR7a and mGluR7b in CA3 after oral administration of 6 mg/kg AMN082 (A, B: right panel) or vehicle (A, B: left panel) is shown. Open arrowheads point at the synaptic cleft with post-synaptic density (asymmetrical synapses) and closed arrowheads point at immunoparticles indicating internalized mGluR7a (A) and mGluR7b (B). Scale bars are 0.3 µm.

Fig. 6.3.2. shows quantitative assessment from electron micrographs, representatives of which are shown in Fig. 6.3.1. For quantification, numbers of immunoparticles were counted which are localized within active zones and also those which located far from active zones deep within presynaptic areas. The following criteria for an active zone in electron micrographs were used; presynaptic areas within twice the length of synaptic cleft from the presynaptic membrane are regarded as an active zone. It was observed that acute 6 mg/kg AMN082 treatment induced mGluR7a internalization to 15.1 ± 1.7 % above the control level (vehicle-treated groups; 5.6 ± 1.1 %); Fig. 6.3.2.A. Similarly, mGluR7b was also internalized by AMN082 (12.5 ± 1.3 %) significantly more than the control level (vehicle-treated groups; 6.5 ± 0.1 %); Fig. 6.3.2.A. In addition, to test whether AMN082 changed the ratio of synapses which showed internalization of mGluR7, the number of synaptic profiles was evaluated based on whether they had internalized mGluR7 (Fig. 6.3.2.B). This approach showed that AMN082 indeed increased the ratio of synaptic profiles showing internalization of mGluR7a and mGluR7b compared to vehicle-treated controls (32.2 ± 0.3 % vs. 16.4 ± 3.1 % and 35.1 ± 3.3 % vs.22.4 ± 0.7 %, respectively). Therefore, acute AMN082 oral injection increased not only the total quantity of internalized mGluR7a and mGluR7b but also the number of synapses which have internalized mGluR7a and/or mGluR7b.

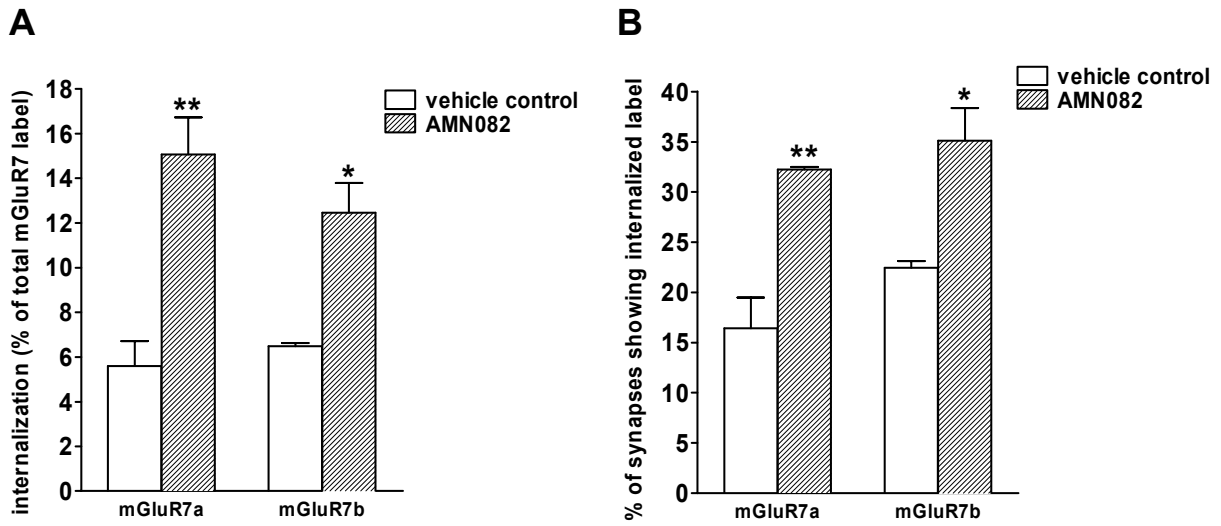

Figure 6.3.2. AMN082 increases internalization of mGluR7a and mGluR7b.
Quantitative analysis of electron micrographs from three independent treated mice per group (vehicle control and 6 mg/kg AMN082). AMN082 induces internalization with respect to two indicators; the ratio of internalized immunoparticles (A) and the ratio of synapses showing internalized particles (B). Results are expressed as means ± S.E.M., with 50 – 80 asymmetrical synapses per each group. ** $p < 0.05$ and * $p < 0.01$ vs. each vehicle control.

7. Discussion

7.1. Neurophysiological consequences of mGluR7 deficiency

The present study demonstrated that selective ablation of the group III mGluR subtype 7 is associated with specific changes in molecular targets that participate in the stress response and in psychopathological states. Thus, the findings here suggest that drugs acting at mGluR7 may provide new approaches to stress-related psychiatric disorders such as depression and anxiety.

mGluR7 ablation leads to increased levels of GR and the 5-HT_{1A}R in the hippocampus without altering MR, CRF or CRF1 mRNA levels. These transcripts were chosen for analysis because of their key regulatory role in the mammalian stress system (De Kloet et al, 2005). The finding that only GR and 5-HT_{1A} receptor transcript levels are altered in mGluR7-deficient mice implies a selective effect on the feedback system in the HPA-axis. The expression levels of both of these genes have previously been shown to be altered by stress, glucocorticoid and antidepressants (Hugin-Flores et al., 2004; Karandrea et al., 2002; Lopez et al., 1998; Okugawa et al., 1999; Seckl and Fink, 1992). Furthermore, mice with functional downregulation/ablation of these two receptors have altered behaviors in models of anxiety and depression (Boyle et al., 2005; Toth, 2003; Urani and Gass, 2003). The HPA axis activity is controlled by a feedback mechanism triggered mainly through the stimulation of GRs located in the cerebral cortex, the hippocampus, and the hypothalamus (Diorio et al., 1993; Feldman and Weidenfeld, 1999; Magarinos et al., 1987; Mizoguchi et al., 2003), and a reduced GR expression and function has been proposed to contribute to HPA axis alterations in depression (Holsboer and Barden, 1996; Pariante and Miller, 2001; Webster et al., 2002). It is also known that the expression and function of the 5-HT_{1A} receptor is regulated by glucocorticoids and GRs (Andrews et al., 2004; Chalmers et al., 1994; Froger et al., 2004; Hery et al., 2000; Laaris et al., 1995; Ou et al., 2001). Taken together these data suggest that there is a selective dysregulation of the stress response in mGluR7$^{-/-}$ mice which is opposite to that seen in some human psychopathological states, e.g. in some depressed patients.

Glutamatergic neurotransmission has long been implicated as an important regulator of the stress response (van den Pol et al, 1990; Brann, 1995). mGluRs in particular appear to play a crucial role in mediating neuroendocrine responses. Specifically, the nonselective mGluR agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate (ACPD), when administered i.c.v., induced a significant

increase in plasma corticosterone (Lang and Ajmal, 1995). Whereas, an involvement of group I and possibly group II mGluRs has been reported (Johnson et al, 2001; Bradbury et al, 2003; Scaccianoce et al, 2003), it has also been demonstrated that i.c.v. administration of the nonselective group III mGluR agonists L-AP_4 and L-SOP activates the HPA axis (Johnson et al, 2001). The current data showing both altered corticosterone levels and feedback regulation in mGluR7$^{-/-}$ mice suggest that mGluR7, at least in part, may modulate the increase in stress hormones induced by group III mGluR agonists. The mechanism underlying this increase is currently unclear. However, Johnson et al (2001), based on an earlier model of Tasker et al (1998), suggested that as group III mGluRs regulate the activity of GABA interneurones in the hypothalamus (Schrader and Tasker, 1997), agonists, such as L-AP_4 and L-SOP, might act by decreasing L-glutamate release from the hippocampal–hypothalamic tract. Thus, there would be a decreased tone driving GABAergic interneurones and a disinhibition on corticotrophin releasing factor (CRF)-containing paraventricular nucleus (PVN) neurons of the hypothalamus. Alternatively, it has been speculated that group III agonists might be acting directly on presynaptic terminals of the GABAergic interneurones and activate hetero-autoreceptors and thus group III mGluR stimulation could directly limit the degree of inhibitory tone that is on the PVN cells (Johnson et al., 2001; Tasker et al., 1998). Both scenarios might explain how the absence of mGluR7 leads to a state of neuroendocrine dysfunction as has been seen in the present studies which may subsequently induce an altered response in stress-related behaviors.

It is important to note that there are many brain regions other than the hippocampus, the hypothalamus and the prefrontal cortex, which contribute to stress-related pathology and future studies should focus on whether mGluR7 plays a role in regulating stress-relevant genes in structures such as the amygdala, dorsal raphe nucleus, and locus coeruleus.

To further examine the potential functional consequences of the changes in stress-related gene expression baseline and stress-induced concentrations of corticosterone and ACTH were assessed in mGluR7$^{-/-}$ mice. There was a strong trend toward a lower basal corticosterone and ACTH level which suggests that mice lacking mGluR7 have abnormal concentrations of circulating glucocorticoids. However, both genotypes responded equally, in terms of neuroendocrine activation, to swim stress insults which resulted in a marked increase in corticosterone and ACTH levels. It is possible that a ceiling response was observed and that a differential effect might be observed if a less severe stress were applied. However, the rationale for choosing this stressor was based on the fact that mGluR7$^{-/-}$ mice have an altered behavioral response in this model (Cryan et al., 2003).

To assess whether there is an altered GR-mediated negative feedback, studies with the synthetic glucocorticoid dexamethasone were conducted which at low doses acts as a GR agonist (Cole et al.,

2000; Groenink et al., 2002; Meijer et al., 1998). Plasma corticosterone concentrations of vehicle-treated mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice were not different at afternoon time points (2:00 – 3:00 p.m.; Fig. 6.1.5.A) in this test. This is probably due to circadian pattern in the levels of circulating glucocorticoids. Therefore, this further illustrates that caution needs to be taken regarding the time of day that sacrifice of animals is carried out for the assaying of stress hormones. On the other hand, the plasma ACTH level of vehicle-treated mGluR7$^{-/-}$ mice was significantly less than that of mGluR7$^{+/+}$ mice (Fig. 6.1.5.D). Difference between ACTH levels and corticosterone levels may reflect the different temporal patterns of these stress hormones. Full online analysis of these hormones maybe required to discriminate these differences in detail. Mice lacking mGluR7 show a clear hypersensitivity to dexamethasone at the lower dose tested (0.03 mg/kg). This suggests that there is indeed an altered functional negative feedback of the HPA axis. This enhanced suppression is opposite to that seen in many depressed patients who have a blunted hormonal response to dexamethasone (Carroll, 1982; Holsboer et al., 1980). This correlates well with the antidepressant-like response observed in behavioral studies with mGluR7$^{-/-}$ mice (Cryan et al., 2003). Despite its historical widespread use in clinical settings there are surprisingly few studies employing the dexamethasone suppression test in genetically modified mice (Cryan and Mombereau, 2004). Here it has been demonstrated that the dexamethasone suppression test can be a sensitive marker of HPA axis dysregulation. Interestingly, although mGluR7$^{-/-}$ mice have a dysregulated HPA axis, these alterations occur independently of any gross abnormality in adrenal weight with both genotypes having the same relative adrenal weight (Table 6.1.). Increases in relative adrenal weight have been observed following chronic stress (Watanabe et al., 1992) and in major depression (Nemeroff et al., 1992).

An increase in BDNF protein levels were observed in the hippocampus of mGluR7$^{-/-}$ mice in a reproducible fashion, which also correlates well with the antidepressant-like phenotype observed behaviorally. Intracerebral ventricular or intrahippocampal administration of BDNF has been shown to have an antidepressant-like effect in various animal tests which are often used for assessing antidepressant activity (Hoshaw et al., 2005; Shirayama et al., 2002; Siuciak et al., 1997). Furthermore, antidepressant medications have been shown to increase BDNF levels in the hippocampus (De Foubert et al., 2004; Nibuya et al., 1995; Russo-Neustadt et al., 2004; but see Altar et al., 2003; Coppell et al., 2003; Jacobsen and Mork, 2004). Further evidence for a role of BDNF in antidepressant action comes from studies using genetically modified mice with impaired levels of BDNF or its receptor TrK-B, which are resistant to the behavioral effects of antidepressants (Saarelainen et al., 2003). Emerging evidence further suggests that BDNF may play a key role in the pathophysiology of stress-related disorders. Lower hippocampal BDNF levels have

been found subsequent to various stressors such as prenatal stress (Fumagalli et al., 2004), footshock stress (Rasmusson et al., 2002), postnatal stress (MacQueen et al., 2003; Roceri et al., 2004) and restraint stress (Smith et al., 1995; Vaidya et al., 1997; Xu et al., 2004). Also of interest is the fact that intra-hippocampal administration of BDNF protects against stress-induced impairments in spatial learning and memory and LTP (Radecki et al., 2005). Mechanisms underlying how stress alters BDNF levels and how BDNF modifies behavior are not currently clear (Tapia-Arancibia et al., 2004). Interestingly, recent studies have shown that BDNF can have direct stimulating effects on the HPA axis suggesting that BDNF could be a stress-responsive intercellular messenger since when it is exogenously administered it acts as an essential factor in the activation and recruitment of hypothalamic CRF neurons (Givalois et al., 2004). However, the effects of chronic BDNF administration on the HPA axis are unknown. It is important to note, that the changes in protein levels of BDNF observed in mGluR7$^{-/-}$ mice occur independent of detectable changes at the mRNA levels. Reasons for this may in some way be due to the fact that whole tissue homogenate may dilute potential effects at the mRNA levels that occur only in specific subregions of the hippocampus, however this reasoning is unsatisfactory as it should also apply to regulation at the protein level. Moreover, in agreement with our findings, recent studies have also shown very disparate results between BDNF mRNA and protein levels in the same tissue following various antidepressant manipulations (De Foubert et al., 2004; Jacobsen and Mork, 2004). The reasons for such differential responses at mRNA and protein level are currently unclear and warrant further investigation. However BDNF is generated as pre-pro-BDNF protein, which is further processed until it is secreted as mature homodimeric protein into the extracellular space (reviewed in Lessmann et al., 2003) and therefore these processes possibly reflect regulation of BDNF at the translational level.

There has been an upsurge in the use of genetically modified mice to assess depression and anxiety related phenotypes (Cryan et al., 2002; Cryan and Mombereau, 2004). However, the majority of phenotypic analysis has focused specifically on behavioral readouts, such as immobility in the forced swim test, in isolation of other physiological and molecular biomarkers. The current data demonstrate the utility of adapting such an approach and exemplify how such analyses can complement behavioral studies. This is perhaps even more important for mice which have genetically modified levels of target proteins, such as in the case of mGluR7, where there are no complementary pharmacological tools available. However, it cannot be ruled out that the changes observed here may be secondary to alterations in other circuits, not yet identified, which are directly influenced by the null mutation of mGluR7. In conclusion, the current data demonstrate that ablation of mGluR7 results in specific changes in key regulators of the HPA axis, namely 5-HT$_{1A}$

receptors, GR, corticosterone, and in altered BDNF levels, in a direction opposite to that found in human depression or following chronic stress. These changes correlate with the previously identified anti-stress and antidepressant-like behavioral effects observed in mGluR7$^{-/-}$ mice. Furthermore, the current data suggest that selective mGluR7 ligands may provide a novel strategy for modifying stress-related disorders.

7.2. The first selective mGluR7 agonist

As discussed above, it was very desirable to identify mGluR7-selective ligands. This thesis presents the first selective mGluR7 agonist, AMN082. This compound acts via a novel site, is orally active, and modulates the *in vivo* levels of two stress hormones, corticosterone and ACTH in wild-type but not in mGluR7-deficient mice substantiating further a role for mGluR7 in stress physiology.

AMN082 elicits a full agonist response comparable to L-AP$_4$ in the absence of glutamate-site ligands, although L-glutamate and DL-AP$_4$ are likely to interact at the same receptor site and the activity of the full agonist DL-AP$_4$ appears to be inhibited by the partial agonist L-glutamate (Fig. 6.2.9.). The current data with chimeric receptors strongly suggests the localization of a novel allosteric agonist site within the heptahelical domain of mGluR7. Definitive proof for this allosteric site awaits the development of an appropriate radioligand which will enable determination of physico-chemical binding characteristics such as K_d, K_{on}, and K_{off}. However, the chimeric receptor data together with the absence of AMN082 activity at other G_i-coupled mGluRs expressed in identical host cells as used for mGluR7 functional studies strongly argues that the agonist activity of AMN082 results from a direct compound interaction with the mGluR7 protein. In particular, this mGluR7-dependent agonist activity is seen in both the G protein assay (GTPγS binding) and in second messenger determinations (cAMP) which drastically reduces the possibility of AMN082 interacting with downstream components of the intracellular signaling cascades. In GTPγS binding, the activities of AMN082 and L-glutamate-site ligands were close to additive using the previously described cell line for human mGluR7b (Flor et al., 1995; Flor et al., 1997; Gasparini et al., 1999a; Maj et al., 2003) in the presence of a saturating concentration of the orthosteric agonist (Fig. 6.2.2.A and Fig.6.2.9.). However, Fig.6.2.2.A showed more than additive increases in the presence of a submaximal concentration of DL- AP$_4$. Greater than additive effects of AMN082 and DL-AP$_4$ were also observed on membranes from the new human mGluR7b cell line prepared for the present chimera studies (Fig. 6.2.13), and although to a lesser extent on rat mGluR7a expressing membranes (Fig. 6.2.2.C). In addition, the % activation induced by AMN082 relative to DL-AP$_4$ differs between the three cell lines. Further studies are required to understand these differences; it

may be possible that the mGluR7 receptor number in relation to the expression level of endogenous G proteins is quite different between the cell lines; this may potentially mask allosteric potentiation in one case but allow it in the other case. It is also unclear at present whether the alternative C-termini of mGluR7a versus mGluR7b could contribute to these differences. AMN082 was not inhibited by competitive glutamate-site antagonists, and neither functional potency nor binding affinity of glutamate-site agonists were significantly affected by AMN082 (Fig. 6.2.10 – 6.2.12., Table 6.2.2.), strengthening the argument for a site of action different from the glutamate binding site. Accordingly, it is proposed that AMN082 binds to the heptahelical region of mGluR7 and favors directly the formation of the active receptor state, as postulated also for orthosteric agonists by the two-state model of receptor activation (Seifert and Wenzel-Seifert, 2003); orthosteric agonist binding seems to be not required for AMN082 interaction with mGluR7. Binding to the transmembrane region has also been postulated for the $GABA_B$ receptor modulator CGP7930, which was found to directly activate the receptor but with low efficacy (Binet et al., 2004). The mGluR5-positive modulator 3,3'-difluorobenzaldazine acted as a full agonist on mGluR5 deleted of its extracellular domain, but this compound did not act as agonist itself on the non-mutated full-length receptor (Goudet et al., 2004). A more recent mGluR5 positive modulator, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), displays partial agonist activity on wild-type mGluR5, and this agonist effect cannot be blocked by orthosteric antagonists (Kinney et al., 2005) which is similar to the findings with AMN082. In many ways, AMN082 is also similar to ectopic agonists of M1 muscarinic receptors such as 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) which shows 50 - 70 % efficacy relative to full agonists (Langmead et al., 2006; Spalding et al., 2002); however, the effect of AC-42 is blocked by atropine whereas AMN082 effects are not inhibited by competitive glutamate antagonists. The present study on AMN082 provides the first evidence for full agonist activity mediated by the heptahelical domain of a wild-type family 3 GPCR.

Heretofore, the synthesis of subtype-selective group III mGluR agonists that significantly penetrate the blood-brain barrier had not been successful, presumably because orthosteric receptor activation requires compounds with an α-amino acid moiety and a distal ionizable ω-phosphono group which makes such molecules too hydrophilic for passing biological membranes. In contrast, the allosteric agonist AMN082 is structurally completely unrelated to amino acids. Its physico-chemical properties allow oral administration resulting in good penetration of the blood-brain barrier. This opens up new drug discovery opportunities for harnessing pharmacological activity at its novel binding site.

A direct interaction of AMN082 with mGluR7 to elicit physiological efficacy *in vivo* can be assumed since AMN082 has been demonstrated to have direct mGluR7-selective activity *in vitro* and elevate plasma levels of the stress hormones corticosterone and ACTH in an mGluR7-dependent fashion, i.e. it does not occur in mGluR7-deficient ($mGluR7^{-/-}$) mice. Six minutes of swim stress elevated corticosterone and ACTH in both $mGluR7^{+/+}$ (wild-type) and $mGluR7^{-/-}$ mice to 400 - 600 % demonstrating that mGluR7-deficient mice are capable of rapid and robust stress hormone up-regulation just like wild-type mice (Fig.6.1.4.). However, the elevating effect of AMN082 on stress hormone levels is only seen in mice carrying the functional mGluR7 gene. L-glutamate has been implicated as a critical neurotransmitter in the regulation of neuroendocrine functions (Brann, 1995; van den Pol et al., 1990). While a component of glutamatergic control of neuroendocrine function is clearly ionotropic receptor-mediated (Farah et al., 1991; Yousef et al., 1994), another component appears to act via the G protein-coupled glutamate receptor family as already discussed above. The current data with AMN082 suggest that mGluR7, at least in part, modulates the increase in stress hormones. The mechanism underlying this increase is still unclear. However as already discussed in 7.1. it might be explained based on the implication by Johnson et al., (2001); group III mGluRs would lead to a decreased tone driving GABAergic interneurones in the hypothalamus and a disinhibition on CRF-containing PVN neurons of the hypothalamus.
In conclusion, the present study characterizes the first selective mGluR7 agonist AMN082 which acts via an allosteric site unknown before and can serve as an important new tool for further studying the role of mGluR7 in stress-related CNS disorders.

7.3. Internalization of mGluR7 and behavioral effects of AMN082

The results from this thesis also show that AMN082 induced internalization of mGluR7 in the hippocampal neurons one hour after a single oral administration which is in line with the recent report that a group III mGluR agonist L-AP_4 caused internalization of mGluR7 (Pelkey et al., 2005). This finding could be important for the effects of the compound *in vivo* since regulated endocytosis is central to the functional regulation of membrane receptors.
As described above, AMN082 showed mGluR7-selective agonistic effects *in vitro* on membranes and also elevated the levels of plasma stress hormones in an mGluR7-dependent fashion. However in contrast to these findings and also with the expectation from the mGluR7 knockout and knockdown phenotypes, AMN082 has shown anxiolytic-like effects in the SIH and the social exploration test one hour after oral administration (unpublished observation; but see Thakker et al., 2005). This discrepancy may be explained by the finding that mGluR7 is internalized by AMN082.

In addition, although the increase of internalized mGluR7 by AMN082 is small compared to the total numbers of mGluR7 (Fig. 6.3.2.), the stress models described above appear to be sensitive enough to this change which is further supported by the observations that 10 – 30 % knockdown of mGluR7 showed robust reduction of SIH (unpublished observation; but see Thakker et al., 2005).

The mechanism for internalization of mGluR7 by agonists remains to be determined. However, Lavezzari and her colleagues showed at the 35th Society for Neuroscience annual meeting (Lavezzari and Roche; poster No. 32.18.) that mGluR7 was internalized via a non-clathrin mediated pathway in response to L-AP_4 whereas mGluR1a subtype is known to be internalized via an arrestin- and clathrin-dependent pathway (Mundell et al., 2001; Pula et al., 2004). Lavezzari further showed that truncated mGluR7 with deleted C terminal tyrosine sites increased internalization, which was inhibited by a PKC activator. Moreover, they also showed that PKC inhibitor increased internalization of wild-type mGluR7, suggesting that mGluR7 phosphorylation was involved in regulating endocytosis. One possible mechanism for internalization of mGluR7 might involve the anchoring protein, protein kinase C interacting protein (PICK1). PICK1 is known to interact with a PDZ-binding motif located at the C terminus of mGluR7 and bind PKCα (Dev et al., 2001). Therefore, it can be hypothesized that PICK1 blocks the mGluR7 internalization, stabilizing mGluR7 on the surface through phosphorylation and that mGluR7 agonists change the conformation of the receptor causing dissociation or degradation of PICK1, which in turn leads to the mGluR7 internalization.

Together with the observations mentioned in this section, emerging studies have pointed to an important role of mGluR7 in stress responses and development of selective mGluR7 ligands allows us to progress further towards the elucidation of its role in psychiatric research. In this thesis, an anxiolytic-like and stress-resistant phenotype has been shown in both mGluR7 knockout and knockdown animals as well as in animals treated with AMN082 which induces internalization of mGluR7 using selected animal models; therefore, this thesis provides indirect evidence in favor of the use of mGluR7 antagonists as possible therapeutics for stress-related disorders.

8. References

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9. Appendix – Abstract pages of Publications from my thesis work.

Proc Natl Acad Sci U S A. 2005 Dec 20;102(51):18712-7.

A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo.

Mitsukawa K, **Yamamoto R**, **Ofner S**, **Nozulak J**, **Pescott O**, **Lukic S**, **Stoehr N**, **Mombereau C**, **Kuhn R**, **McAllister KH**, **van der Putten H**, **Cryan JF**, **Flor PJ**.

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Metabotropic glutamate receptor (mGluR) subtypes (mGluR1 to mGluR8) act as important pre- and postsynaptic regulators of neurotransmission in the CNS. These receptors consist of two domains, an extracellular region containing the orthosteric agonist site and a transmembrane heptahelical domain involved in G protein activation and recognition of several recently synthesized pharmacological modulators. The presynaptic receptor mGluR7 shows the highest evolutionary conservation within the family, but no selective pharmacological tool was known. Here we characterize an mGluR7-selective agonist, N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), which directly activates receptor signaling via an allosteric site in the transmembrane domain. At transfected mammalian cells expressing mGluR7, AMN082 potently inhibits cAMP accumulation and stimulates GTPgammaS binding (EC50-values, 64-290 nM) with agonist efficacies comparable with those of L-2-amino-4-phosphonobutyrate (L-AP4) and superior to those of L-glutamate. AMN082 (< or = 10 microM) failed to show appreciable activating or inhibitory effects at other mGluR subtypes and selected ionotropic GluRs. Chimeric receptor studies position the binding site of AMN082 in the transmembrane region of mGluR7, and we demonstrate that this allosteric agonist has little, if any, effect on the potency of orthosteric ligands. Here we provide evidence for full agonist activity mediated by the heptahelical domain of family 3 G protein-coupled receptors (which have mGluR-like structure) that may lead to drug development opportunities. Further, AMN082 is orally active, penetrates the blood-brain barrier, and elevates the plasma stress hormones corticosterone and corticotropin in an mGluR7-dependent fashion. Therefore, AMN082 is a valuable tool for unraveling the role of mGluR7 in stress-related CNS disorders.

PMID: 16339898 [PubMed - indexed for MEDLINE]

Neuropsychopharmacology. 2005 Oct 12; [Epub ahead of print]

Metabotropic Glutamate Receptor Subtype 7 Ablation Causes Dysregulation of the HPA Axis and Increases Hippocampal BDNF Protein Levels: Implications for Stress-Related Psychiatric Disorders.

Mitsukawa K, **Mombereau C**, **Lotscher E**, **Uzunov DP**, **van der Putten H**, **Flor PJ**, **Cryan JF**.

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Regulation of neurotransmission via group-III metabotropic glutamate receptors (mGluR4, -6, -7, and -8) has recently been implicated in the pathophysiology of affective disorders, such as major depression and anxiety. For instance, mice with a targeted deletion of the gene for mGluR7 (mGluR7(-/-)) showed antidepressant and anxiolytic-like effects in a variety of stress-related paradigms, including the forced swim stress and the stress-induced hyperthermia tests. Deletion of mGluR7 reduces also amygdala- and hippocampus-dependent conditioned fear and aversion responses. Since the hypothalamic-pituitary-adrenal (HPA) axis regulates the stress response we investigate whether parameters of the HPA axis at the levels of selected mRNA transcripts and endocrine hormones are altered in mGluR7-deficient mice. Over all, mGluR7(-/-) mice showed only moderately lower serum levels of corticosterone and ACTH compared with mGluR7(+/+) mice. More strikingly however, we found strong evidence for upregulated glucocorticoid receptor (GR)-dependent feedback suppression of the HPA axis in mice with mGluR7 deficiency: (i) mRNA transcripts of GR were significantly upregulated in the hippocampus of mGluR7(-/-) animals, (ii) similar increases were seen with 5-HT(1A) receptor transcripts, which are thought to be directly controlled by the transcription factor GR and finally (iii) mGluR7(-/-) mice showed elevated sensitivity to dexamethasone-induced suppression of serum corticosterone when compared with mGluR7(+/+) animals. These results indicate that mGluR7 deficiency causes dysregulation of HPA axis parameters, which may account, at least in part, for the phenotype of mGluR7(-/-) mice in animal models for anxiety and depression. In addition, we present evidence that protein levels of brain-derived neurotrophic factor are also elevated in the hippocampus of mGluR7(-/-) mice, which we discuss in the context of the antidepressant-like phenotype found in those animals. We conclude that genetic ablation of mGluR7 in mice interferes at multiple sites in the neuronal circuitry and molecular pathways implicated in affective disorders.Neuropsychopharmacology advance online publication, 12 October 2005; doi:10.1038/sj.npp.1300926.

PMID: 16237391 [PubMed - as supplied by publisher]

10. Curriculum Vitae

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Work Experience

2003 - 2006	Ph.D. Thesis Project, Neuroscience Research, Novartis Institutes for BioMedical Research, Basel, Switzerland Thesis Title: Allosteric Activation and Genetic Antagonism of Metabotropic Glutamate Receptor Subtype 7 (mGluR7): Implications for Stress-Related Physiology and Behavior
2001 - 2003	Master Thesis Project, Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan Thesis Title: Cell cycle activity-dependent and caspase-3 activation-independent mechanism of phenytoin-mediated neuronal death in cultured cerebellar granule cells
2000 - 2001	Practical Laboratory Training, Laboratory of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Japan - Pharmacological laboratory technique practice - Project in pharmacological analysis of Phenytoin-induced neuronal cell death
1999 - 2001	Practical Pharmacist training, The University of Tokyo Hospital, Japan

Technical Skills

Cell Culture	Primary culture (rat hippocampal neurons, cerebellar granule neurons and astrocytes); Culture of permanent cell lines
Functional Receptor Assays	Functional GTPγ^{35}S membrane binding assays, Cellular second messenger assays (cAMP, phosphoinositides (PI) hydrolysis assays, Intracellular Ca^{2+}-measurement)
Cell Survival/Death Assays	MTT assays, LDH (lactate dehydrogenase) assays, Caspase activity assays
Molecular Biology/ Biochemistry Techniques	Western blotting, RT-PCR, ELISA assays, FACS analysis, Immunocytochemistry
Morphological Techniques	Electron-microscopic analysis, Fluorescence-microscopic analysis
Animal Techniques/ Physiology	Dissection of mouse brain, Animal handling, Drug injection, Decapitation and blood sample collection, Plasma hormone level measurement with Radio Immuno Assay (RIA)
IT	Word, Excel, PowerPoint, GraphPad Prism, Sigma Plot

Languages

Japanese (mother tongue)
English (fluent)
German (basic)

Publications

Jeong-Ah Kim, Kayo Mitsukawa, Maki Kobayashi Yamada, Nobuyoshi Nishiyama, Norio Matsuki, and Yuji Ikegaya (2002). Cytoskeleton disruption causes apoptotic degeneration of dentate granule cells in hippocampal slice cultures. **Neuropharmacology** 42; 1109 - 1118.

Kayo Mitsukawa, Rina Yamamoto, Silvio Ofner, Joachim Nozulak, Oliver Pescott, Snezana Lukic, Natacha Stoehr, Cedric Mombereau, Rainer Kuhn, Kevin McAllister, Herman van der Putten, John F. Cryan and Peter J. Flor (2005). The First Selective mGluR7 Agonist AMN082, Activates Receptor Signaling via an Allosteric Site and Modulates Stress Parameters *In Vivo*., **PNAS** 102; 18712 – 18717.

Kayo Mitsukawa, Cedric Mombereau, Erika Lötscher, Doncho P. Uzunov, Herman van der Putten, Peter J. Flor and John F. Cryan (2006). Metabotropic Glutamate Receptor Subtype 7 Ablation Causes Dysregulation of the HPA Axis and Increases Hippocampal BDNF Protein Levels: Implications for Stress-Related Psychiatric Disorders. **Neuropsychopharmacology**, *in press*

Professional Presentations (posters and talks)

2005	Mitsukawa K., Yamamoto R., Nozulak J., Ofner S., Pescott O., Lukic S., Stoeher N., Urwyler S., Kuhn R., Herrling P., Cryan J. F. and Flor P. J., AMN082, the First Selective Metabotropic Glutamate Receptor Subtype 7 Agonist: Activation of Receptor Signaling via an Allosteric Site in the Transmembrane Domain: Poster presentation at *5th International Meeting on Metabotropic Glutamate Receptors* Mitsukawa K., Mombereau C., Lötscher E., Uzunov D. P., van der Putten H., Flor P. J. and Cryan J. F., Metabotropic Glutamate Receptor Subtype 7 Ablation Causes Dysregulation of the HPA Axis and Increases Hippocampal BDNF Protein Levels: Implications for Stress-Related Psychiatric Disorders: Oral presentation at *5th International Meeting on Metabotropic Glutamate Receptors* Mitsukawa K., Yamamoto R., Nozulak J., Ofner S., Pescott O., Lukic S., Stoeher N., Urwyler S., Kuhn R., Herrling P., Cryan J. F. and Flor P. J., AMN082, the First Selective Metabotropic Glutamate Receptor Subtype 7 Agonist: Activation of Receptor Signaling via an Allosteric Site in the Transmembrane Domain: Poster presentation at *35th Annual Meeting of Society For Neuroscience (SFN35th Annual Meeting)* Flor P. J., Mitsukawa K., Mombereau C., Lötscher E., Uzunov D. P., van der Putten H., and Cryan J. F., Metabotropic Glutamate Receptor Subtype 7 Ablation Causes Dysregulation of the HPA Axis and Increases Hippocampal BDNF Protein Levels: Implications for Stress-Related Psychiatric Disorders: Poster presentation at *35th Annual Meeting of Society For Neuroscience (SFN35th Annual Meeting)*:This was selected by the Public Information Committee for a Press Book.

2003	Mitsukawa K., Ikegaya Y., Yamada M. K., Matsuki N., Nishiyama N., Involvement of Cell Cycle Progression in Phenytoin-mediated Cerebellar Granule Cell Death: Poster presentation at *The 76th Annual Meeting of the Japanese Pharmacological Society* Nishiyama N., Mitsukawa K., Yamada M. K., Ikegaya Y., Matsuki N., Cell cycle activity-dependent and caspase-3 activation-independent mechanism of phenytoin-mediated neuronal death in cultured cerebellar granule cells: Poster presentation at *33rd Annual Meeting of Society For Neuroscience (SFN33th Annual Meeting)*
2002	Mitsukawa K., Yamada M. K., Ikegaya Y., Matsuki N., Nishiyama N., Pharmacological analysis of Phenytoin-induced cell death in cultured cerebellar granule cells: Oral presentation at *The 106th Kanto Regional Meeting of the Japanese Pharmacological Society*

Qualification/Licenses

1999	Radiation Protection Course, The University of Tokyo, Japan
2000	Animal Handling Course, The University of Tokyo, Japan
2001	Pharmacist, the Ministry of Health, Labor and Welfare, Japan.
2003	Radiation Protection Course, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

Membership

2002 - present	The Japanese Pharmacological Society
2005 - present	Society for Neuroscience

References

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