DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

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CHOLECYSTOKININ$_2$ RECEPTOR DEFICIENT MICE: CHANGES IN FUNCTION OF GABA-ERGIC SYSTEM

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ABBREVIATIONS

A71623	Boc-Trp-Lys(O-toluylaminocarbonyl)-Asp-(NMe)Phe-NH2
BC197	[c(Boc-D.Asp-Tyr(SO_3H)-Nle-D.Lys)-Trp-Nle-Asp-Phe-NH_2]
BC264	Tyr(SO3H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH2
B_{max}	the maximum specific binding
Boc-CCK-4	N-t-Boc-Trp-Met-Asp-Phe-amide
CCK	cholecystokinin
CCK_AR	cholecystokinin receptor of alimentary subtype
CCK_BR	cholecystokinin receptor of brain subtype
CCK_1R	CCK receptors displaying high affinity for sulphated form of CCK-8
CCK_2R	CCK receptors displaying high affinity for sulphated and desulphated forms of CCK-8, gastrin and CCK-4
CCK-4	cholecystokinin tetrapeptide
CCK-5	pentagastrin
CCK-8s	sulphated form of cholecystokinin octapeptide
CI988	(butanoic acid, 4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo[3.3.1. 13.7]dec-2-yloxy)carbonyl]amino]propyl] amino]-1-phenylethyl]amino-4]oxo[R-(R.R)]-N-methyl-D-glucamine
CNS	central nervous system
DAG	diacylglycerol
Des-CCK-8	desulphated cholecystokinin octapeptide
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
ECL cell	the gastrin-enterochromaffin-like
GABA	γ-aminobutyric acid
G_s	G protein, which stimulates adenylyl cyclase, the enzyme implicated in second messenger cyclic adenosine monophosphate (cAMP)
$G_{q/11}$	G protein, which activates enzyme phospholipase C leading to the production of two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3)
GV150013	N-(1-[1-adamantane-1-methyl]-2,4-dioxo-5-phenyl-2,3,4,5-tetrahydro-1H-1,5-benzodiazepin-3-yl)-N'-phenylurea
GW5823	2-[3-(1H-indazol-3-ylmethyl)-2,4-dioxo-5-phenyl-2,3,4,5-tetrahydrobenzo[b][1,4]diazepin-1-yl]-N-isopropyl-N-(methyoxyphenyl)acetamide
HPRT	hypoxanthineguanine phosphoribosyl transferase
5-HT	5-hydroxytryptamine
5-HTT	5-hydroxytryptamine transporter
IQM95333	(4αS,5R)-2-benzyl-5[N-(tert-butoxycarbonyl)-LTrp]amino-1,3-dioxoperhydropyrido[1,2-c]pyrimidine

JMV180	Boc-Tyr(SO3H)Ahx-Gly-Trp-Ahx-Asp2phenylethyl ester
K_d	the equilibrium dissociation constant
L365260	3r(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl)urea
L740093	N-([3R]-5-[3-azabicyclo{3.2.2}nonan-3-yl]-2,3-dihydro-1-methyl-2-oxo-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl)urea
L-DOPA	L-3,4-dihydroxyphenylalanine
LY262691	trans-N-(4-bromophenyl)-3-oxo-4,5-diphenyl-1-pyrrazolidine-carboxamide(3.3.1.13,7)
LY288513	trans-N-(4-bromophenyl)-3-oxo4, 5-diphenyl-1-pyrazolidine-carboxamide
MAP kinase	mitogen-activated protein kinase
mRNA	messenger RiboNucleicAcid
PD140548	N-(α-methyl-N-[{tricyclo(3.3.1.13,7)dec-2-yloxy}carbonyl]-L-Trp)-D-3-(phenylmethyl)-β-Ala
PLC	phospholipase C
PLA_2	phospholipase A_2
Q-RT-PCR	quantitative real-time PCR
RB400	HOOC-CH2-CO-Trp-NMe(Nle)-Asp-Phe-NH2
RP73870	({[(N-methyoxy-3-phenyl)-N-(N-methyl-N-phenyl-carbamoyl-methyl)-carbamoylmethyl]-3-ureido}-3-phenyl)-2-ethylsulfonate-(RS)
SR27897	1-([2-{4-(2-chlorophenyl)thiazole-2-yl}aminocarbonyl] indolyl)acetic acid
T0632	sodium (S)-3-(1-[2-fluorophenyl]-2,3-dihydro-3-[{3-isoquinolinyl}-carbonyl]amino-6-methoxy-2-oxo-1H-indole)propanoate
YM022	(R)-1-(2,3-dihydro-1-[2'-methylphenacyl]-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-3-(3-methylphenyl)urea

INTRODUCTION

Cholecystokinin (CCK) is a peptide molecule discovered in the small intestine (for review, see Rehfeld, 2004). The presence of CCK in the mammalian central nervous system (CNS) was suggested by the discovery of gastrin-like immunoreactivity in the rat brain subsequently identified as CCK (Vanderhaegen *et al.,* 1975; Dockray and Taylor, 1976). Further studies showed that the majority of neuronal CCK is expressed in the form of sulphated octapeptide (CCK-8s); larger forms (CCK-58, CCK-33) and smaller fragments (CCK-5, CCK-4) have also been detected (Rehfeld and Nielsen, 1995). CCK peptides are abundant throughout the brain, with the highest levels in the cerebral cortex, limbic structures and basal ganglia (Beinfeld *et al.,* 1981; Vanderhaegen and Schiffmann, 1992; Lindefors *et al.,* 1993). CCK acts as a neurotransmitter and exerts a neuromodulatory influence on several classical neurotransmitters, including dopamine, serotonin (5-hydroxytryptamine, 5-HT), noradrenaline, γ-aminobutyric acid (GABA), glutamate and opioid peptides (Crawley, 1995; Daugé and Roques, 1995). By now two CCK receptor subtypes and their distribution have been characterised (Moran *et al.,* 1986; Noble *et al.,* 1999). CCK_2 (formerly brain/gastrin subtype, CCK_B) receptors predominate in the brain (Wank, 1995; Noble *et al.,* 1999), while CCK_1 (formerly alimentary subtype, CCK_A) receptors are present in the pancreas, gallbladder and in some discrete brain areas (Hill *et al.,* 1990; Noble *et al.,* 1999). CCK peptides vary in their affinity to the subtypes of CCK receptors. CCK-8s and its amphibian analogue, caerulein, are nonselective agonists of CCK receptors, whereas desulphated CCK-8, pentagastrin (CCK-5) and CCK-4 are selective CCK_2 receptor agonists.

CCK is involved in the regulation of various physiological functions such as feeding, nociception, memory, learning, motivations and stress-related behaviours (Singh *et al.,* 1991; Costall *et al.,* 1991; Harro *et al.,* 1993; Crawley and Corwin, 1994; Shlik *et al.,* 1997). CCK_2 receptor knockout mice generated by Nagata et al. (1996) represent an excellent tool for exploring the role of CCK and CCK_2 receptors in the regulation of behaviour. Since 1996 behavioural profile of CCK_2 receptor-deficient mice has been characterised. Despite the atrophy of gastric mucosa, the oldest animals of this line have reached to the age of 24 months (Nagata *et al.,* 1996; Kopin *et al.,* 1999). According to the study of Kopin et al. (1999), the genetic invalidation of CCK_2 receptors does not affect weight gain and feeding behaviour in mice. However, CCK_2 receptor deficient mice display impairment of memory function and increased activity of opioid system (Sebret *et al.,* 1999; Pommier *et al.,* 2002). Animal and human research suggests that CCK is implicated in the regulation of anxiety (Harro *et al.,* 1993). However, very little is known about the emotional behaviour of CCK_2 receptor deficient mice. Therefore, a major goal of the present study was to characterise the emotional behaviour in mice lacking CCK_2 receptors. It has been shown that CCK is localised in the GABAergic neurons in the cerebral

cortex and hippocampus (Hendry *et al.*, 1984; Kosaka *et al.*, 1985; Cope *et al.*, 2002). CCK has been shown to increase the release of GABA in the cerebral cortex and hippocampus, and this effect is mediated via CCK_2 receptors (Perez de la Mora *et al.*, 1993; Miller *et al.*, 1997; Ferraro *et al.*, 1999). Moreover, the administration of CCK_2 receptor antagonists reverses the signs of diazepam withdrawal in rodents (Hughes *et al.*, 1990; Singh *et al.*, 1992; Rasmussen *et al.*, 1993). By taking into account the probable antagonistic interaction between GABA and CCK, we studied the role of GABAergic system in the behavioural changes induced by the genetic invalidation of CCK_2 receptors.

REVIEW OF LITERATURE

1. Cholecystokinin (CCK): distribution in the brain and receptor subtypes

Vanderhaeghen and colleagues first described neuropeptide CCK in the rat brain in 1975. Initially it was discovered as a gastrin-like immunoreactivity and only later it was identified as CCK (Dockray and Taylor, 1976; Rehfeld, 1985). CCK belongs to the group of peptides found in the digestive tract and central nervous system (CNS). Biochemical studies have shown that the majority of neuronal CCK is expressed in the form of sulphated (in position 7 from the -COOH terminal) octapeptide CCK-8s. The desulphated form (des-CCK-8) of this peptide also exists. Besides that, larger (CCK-58, CCK-33) and smaller fragments (CCK-5, CCK-4) have been detected (Rehfeld and Nielsen, 1995). These isoforms are cleaved from N-terminus of pre-pro-CCK, a 115-amino-acid precursor molecule (Figure 1) (Dockray, 1992; Noble and Roques, 2002).

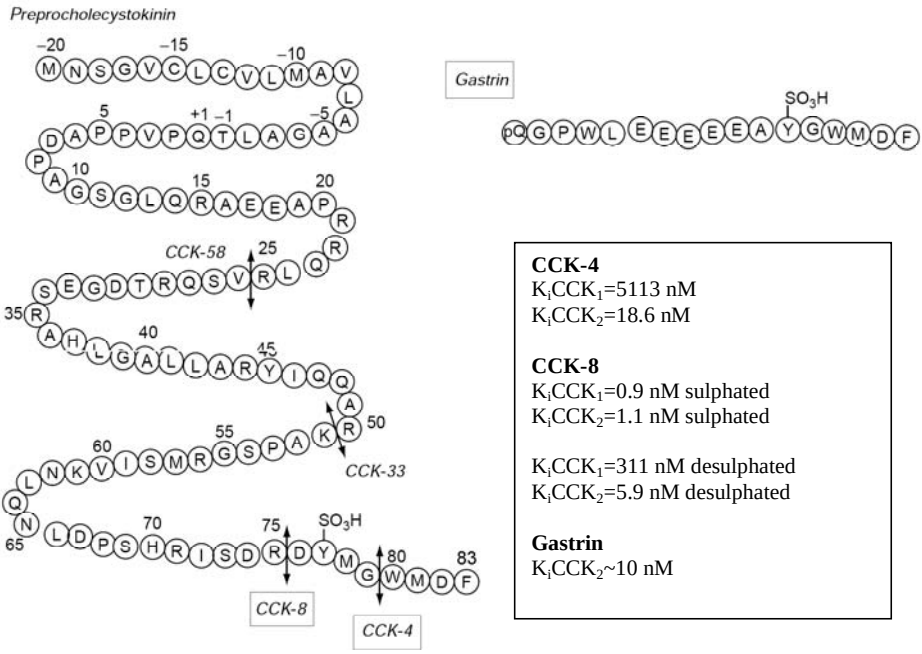

Figure 1. Predicted structure of human pre-pro-cholecystokinin (pre-pro-CCK)
The signal peptide consists of residues -20 to -1. The amino terminal flanking peptide consists of residues 1 to 25. The largest characterised form from brain and intestine, CCK-58, consists of residues 26 to 83. Other active molecular forms are derived from this precursor, such as CCK-39, CCK-33, CCK-22, CCK-7, and CCK-5 (Noble and Roques, 2002).

There are four other peptides, which show narrow homology with sequence of CCK. Gastrin, has an identical -COOH terminal pentapeptide sequence with CCK (Pisegna *et al.,* 1992). Caerulein and phyllocaerulein, isolated from the skin of the amphibians *Hyla caerulea, Xenopus laevis* and *Phyllomedusa sauvagei*, have a similar structure with CCK-8s (Anastasi *et al.,* 1967, 1969). Finally, cionin, from the protochordate *Ciona intestinalis*, might represent the common ancestor of gastrin and CCK (Johnsen and Rehfeld, 1990). CCK peptides are abundant throughout the brain, with the highest levels in the cerebral cortex, hippocampus, basal ganglia, hypothalamus, and periaqueductal grey matter (Beinfeld *et al.,* 1981; Vanderhaeghen and Schiffmann, 1992; Lindefors *et al.,* 1993). It has been proposed that the brain contains at least three sub-populations of CCK neurones with different post-translational processing pathways (Rehfeld and Hansen, 1986; Rehfeld *et al.,* 1992). According to this view, it is possible that different forms of CCK function independently in distinct neuronal settings. Pre-pro-CCK has been cloned in the rat, human and pig (Deschenes *et al.,* 1984; Takahashi *et al.,* 1985; Gubler *et al.,* 1984), and mapping studies of pre-pro-CCK mRNA using *in situ* hybridisation histochemistry have been conducted (Savasta *et al.,* 1988, 1990; Ingram *et al.,* 1989; Schalling *et al.,* 1989; Vanderhaeghen and Schiffmann, 1992). Certain discrepancies between the density of CCK-like immunoreactivity and CCK mRNA-containing neurones occur in some brain areas. This difference might come from the greater sensitivity of *in situ* hybridisation histochemistry compared to immunohistochemistry, because it allows to detect the putative neurones with long projections that synthesise CCK but transport it rapidly to the nerve terminals (Vanderhaeghen and Schiffmann, 1992). High levels of CCK-like immunoreactivity are present in synaptosomal preparations (Pinget *et al.,* 1978; Emson *et al.,* 1980), and CCK is synthesised *de novo* in the brain (Golterman *et al.,* 1980). CCK is released from the brain slices or synaptosomes exposed to depolarising stimuli in a calcium-dependent manner (Pinget *et al.,* 1979; Dodd and Kelly, 1981; Emson *et al.,* 1980; Verhage *et al.,* 1991). Furthermore, specific high-affinity binding sites for CCK are widely distributed throughout the CNS (Innis and Snyder, 1980; Saito *et al.,* 1980). CCK has been shown to induce excitation of central neurones (Dodd and Kelly, 1979, 1981; Ishibashi *et al.,* 1979; Boden and Hill, 1988 a, b). However, inhibitory postsynaptic effects have also been recorded (Ishibashi *et al.,* 1979; MacVicar *et al.,* 1987; Lopes da Silva *et al.,* 1990). This is in accordance with morphological studies suggesting that CCK is present in both excitatory and inhibitory neurones (Peters *et al.,* 1983). Mechanisms terminating the action of CCK are less clear, but the selective uptake into synaptosomal fraction *in vitro* has been demonstrated (Migaud *et al.,* 1993).

Two types of CCK receptors have been characterised (Hays *et al.,* 1980; Innis and Snyder, 1980; Saito *et al.,* 1980; Sankaran *et al.,* 1980; Moran *et al.,* 1986). Initially, CCK receptors were named according to their preferential localisation: the peripheral or alimentary type (CCK_A) and the central or brain

type (CCK_B). CCK_B receptors predominate in the brain (Wank, 1995) while CCK_A receptors are present in the visceral organs and in some discrete brain nuclei (Hill *et al.,* 1990). CCK_A and CCK_B receptors were cloned and the localisation of their mRNA in the brain was established (Kopin *et al.,* 1992; Wank *et al.,* 1992; Pisegna *et al.,* 1992; Lee *et al.,* 1993; Ulrich *et al.,* 1993). According to the nomenclature created by the authorised Committee on Receptor Nomenclature and Drug Classification of the International Union of Pharmacology, the receptors were renamed (Table 1): CCK_1 (formerly CCK_A) and CCK_2 (formerly CCK_B) receptors according to their affinity to different CCK analogues. Namely, CCK_1 receptors bind CCK-8 that is sulphated with high affinity, but CCK_2 receptors are less selective than CCK_1 receptors as they also bind desulphated CCK-8, gastrin and CCK-4 with high affinity (Woodruff and Hughes, 1991).

Table 1. Characterisation of subtypes of CCK receptors

Receptor	**CCK_1** CCK_A /Alimentary / Peripheral	**CCK_2** CCK_B / Brain / Central
Structure – human	428 – amino acid sequence (P32238 7TM)	447 – amino acid sequence (P32239 7TM)
Gene and location **Human:** **Mouse:**	*CCK_1* Chromosome 4 Chromosome 5	*CCK_2* Chromosome 11 Chromosome 7
Splice variants	No	Yes (long form, short form, Δ form)
Genetically induced disruption of gene in mice	Kopin *et al.*, 1999	Nagata *et al.,* 1996
Distribution	Gall bladder, pancreas, pylorus, intestine, spinal cord, vagus nerve, limited brain areas (nucleus tractus solitarius, area postrema, nucleus interpeduncularis, posteromedial part of nucleus accumbens)	Throughout the brain (with the highest densities in the cerebral cortex, nucleus caudatus, anterolateral part of nucleus accumbens), vagus nerve, stomach, pancreas
Endogenous ligands according to their affinity to specific receptor	CCK-8s >> gastrin, des-CCK-8 > CCK-4	CCK-8s ≥ gastrin, des-CCK-8, CCK-4
Agonists	Caerulein (amphibian CCK analogue); A71623; GW5823; JMV-180;	Caerulein; CCK-4; Boc-CCK-4; BC 197; BC 264; des-CCK-8; gastrin; RB400
Antagonists	Proglumide; Lorglumide; Devazepide; Lintitript (SR 27897) ; T0632 ; IQM95333 ; PD140548	Proglumide; L-365260; L-740093; LY 288513; CI-988; YM022; GV150013; RP73870; LY262691

Intracellular activation	$G_{q/11}/G_s$	$G_{q/11}/G_s$
Functional role	Mediates CCK actions on gall bladder contraction, secretion of pancreatic enzymes, gastric emptying, inhibits feeding and respiration, potentiates dopamine-mediated behaviours and dopamine release in shell of nucleus accumbens	Mediates CCK actions on increases in neuronal firing rates, nociception, anxiety, respiration, inhibits dopamine-mediated behaviours and dopamine release, regulates insulin release in pancreas

CCK_1 and CCK_2 receptors possess seven trans-membrane domains and belong to the G-protein linked receptor superfamily with considerable amino acid sequence similarities to the other members of this family. However, site directed mutagenesis experiments have shown that some amino acids present in trans-membrane or within extracellular domains often play a key role in binding various agonists and/or antagonists to G protein-coupled receptors. Jagerschmidt et al. (1996, 1998) have demonstrated the importance of amino acids Hist381, Phe227 and Phe347 residues of the rat CCK_2 receptor for CCK_2 vs CCK_1 antagonist selectivity. Amino acid Trp351 of the agonist binding site of the receptor is involved in CCK-4 binding (Jagerschmidt *et al.*, 1998). In addition, a segment of five amino acids in the second extracellular loop of the CCK_2 receptor was shown to be essential for the high affinity of the natural peptide agonist, gastrin (Silvente-Poirot *et al.*, 1998).

CCK_1 receptors (Table 1) are located mainly in peripheral tissues. However, CCK_1 receptors occur in certain brain regions, including area postrema, nucleus of the solitary tract, and interpeduncular nucleus (Moran *et al.*, 1986). Radioligand and electrophysiological studies have revealed that the distribution of CCK_1 receptors is even more widespread in the brain. These receptors have been identified in dorsal raphe, nucleus accumbens, substantia nigra, and ventral tegmental area (Barrett *et al.*, 1989; Gerhardt *et al.*, 1989; Vickroy and Bianchi, 1989; Hill *et al.*, 1990). CCK_1 receptors, at least in the gastrointestinal tract, are coupled to a guanine nucleotide binding regulatory protein (G-protein), which activates phospholipase C, breaks down inositol phospholipids, mobilises intracellular calcium and activates protein kinase C (Jensen *et al.*, 1989). CCK_1 receptors also exhibit selective stimulation of cAMP through G_s coupling (Yule *et al.*, 1993; Wu *et al.*, 1997).

In 1993, Song and colleagues described the gene for CCK_2 receptors in humans (Table 1). It consists of 5 exons and 4 introns and is conformed similarly in humans, mice (Nagata *et al.*, 1996), and rabbits (Blandizzi *et al.*, 1994). Three splice variants of CCK_2 gene [long form, short form (Song *et al.*, 1993) and Δ form (Miyake, 1995)] have been identified. The mRNA for CCK_2 receptor was also found to express a wide range of splice diversity and is

located also in the regions of CNS without the presence of functional receptors (Pélaprat *et al.*, 1987; Jagerschmidt *et al.*, 1994). Wank (1995) has shown that, in general, shorter and longer isoforms are pharmacologically indistinguishable, although the CCK_2 receptor antagonist L-365260 interacts with an increased affinity at the shorter form. Recently, Morton et al. (2003) have confirmed these results. Moreover, his team detected some small, but significant differences in the affinity for certain CCK_2 receptor ligands (YM022, PD140376, sulphated CCK-8, R-L-365260) between isoforms. However, it seems that the selectivity of these compounds is not large enough to assume the existence of two subtypes of the CCK_2 receptor previously described by Harper et al. (1999). Several binding studies (Durieux *et al.*, 1986; Knapp *et al.*, 1990; Talkad *et al.*, 1994; Harper *et al.*, 1996, 1999) and behavioural studies (Derrien *et al.*, 1994; Léna *et al.*, 1999; Ladurelle *et al.*, 1998) with various highly selective CCK_2 receptor ligands, have suggested the occurrence of two or three affinity states of CCK_2 receptors. Thus, the heterogeneity of pharmacological properties of CCK_2 receptor agonists reported in various studies (Derrien *et al.*, 1994; Daugé and Lena, 1998; Bellier *et al.*, 2004) could be related to the different coupling modes to G-proteins (triggering the activation of distinct second messengers) rather than to the existence of different subtypes of the CCK_2 receptor (Pommier *et al.*, 1999). CCK_2 receptors are widely distributed in the brain, with the highest concentration in the striatum, cerebral cortex, and limbic system (Beinfeld, 1983), but these receptors are also present in the gastrointestinal tract. For a long time the CCK_2 receptor has caused confusion for its similarity to the gastrin receptor. Recently it was revealed, that the canine parietal cell gastrin receptor and the brain CCK_2 receptor are highly homologous if not identical (Kopin *et al.*, 1992). Indeed, the reported Southern-blot hybridisation analysis of human genomic DNA indicates that a single gene encodes both CCK_2 and gastrin receptors (Lee *et al.*, 1993).

CCK_2 receptor is linked to two independent signal transduction pathways (arachidonic acid and inositol phosphate) via different G proteins. CCK_2 receptor is capable of coupling to phospholipase C (PLC) through pertussis toxin-insensitive G proteins, leading to inositol phosphates production (Roche *et al.*, 1990; Zhang *et al.*, 1992; Jagerschmidt *et al.*, 1994; Pommier *et al.*, 1999, 2003). Data from directed mutagenesis experiments have revealed a critical role of amino acid Phe347 residue in the sixth transmembrane domain of the CCK_2 receptor in the phosphatidylinositol pathway. Another residue, Asp100 has also been shown to be involved in signal transduction by means of phosphatidylinositols (Jagerschmidt *et al.*, 1994). It has been hypothesised that Asp100 points in the direction of a cluster of basic amino acid (Lys333/Lys334/Arg335) located in the third intracellular loop of the receptor at the bottom of the sixth transmembrane domain. This suggestion was confirmed by results reported by Wang (1997), showing that these three basic amino acids are involved in the CCK_2 receptor mediated activation of G_q proteins. Therefore, the amino acid residues, implicated in transduction processes, can play a key role in agonist-

induced changes in the receptor conformation triggering $G_{q/11}$ protein stimulation.

Arachidonic acid can be generated via two major pathways, one is phospholipase A_2 (PLA_2) (via pertussis toxin-sensitive G-proteins) and the other results from a subsequent activation of PLC and diacylglycerol (DAG) lipase (Pommier *et al.,* 2003). MAP kinase pathway has also been shown to be activated via the stimulation of CCK_2 receptors (Taniguchi *et al.,* 1994).

2. Functional role of CCK and interaction with other neurotransmitter systems

2.1. CCK and locomotor activity

Several studies have demonstrated that CCK agonists inhibit locomotor activity in mice, decreasing motility and the frequency of rearings, and blocking amphetamine-induced hyperlocomotion (Zetler, 1985; Moroji *et al.,* 1987; Hagino *et al.,* 1989; Vasar *et al.,* 1991; Hirosue *et al.,* 1992). Involvement of CCK_1 receptors in the regulation of motor activity has been shown with a selective CCK_1 receptor antagonist devazepide what can antagonise motor depressant action of systemically and intracerebroventricularly administered CCK agonists, CCK-8s and caerulein (Khosla and Crawley, 1988; O'Neill *et al.,* 1991; Vasar *et al.,* 1991). It is thought that the motor inhibition and suppression of dopaminergic activity induced by CCK agonists are of peripheral origin since their effects could be abolished by abdominal vagotomy in rats (Crawley and Kiss, 1985; Hamamura *et al.,* 1989; Vasar *et al.,* 1994a). However, not all authors have been able to reproduce the evidence that vagotomy can reverse the behavioural effects of CCK agonists in rodents. Moroji and Hagino (1987) have demonstrated that bilateral subdiaphragmatic vagotomy does not prevent the behavioural effects of subcutaneously injected caerulein in mice. The suppression of electrical self-stimulation by caerulein is also completely insensitive to vagotomy in rats (De Witte *et al.,* 1986). Moreover, L-365,260, an antagonist of CCK_2 receptors, caused an effect opposite to that of devazepide because the motor inhibition elicited by caerulein and CCK-8 became stronger under the influence of that CCK antagonist (Vasar *et al.,* 1991, 1994b). These data demonstrate the opposite effect of CCK in the regulation of locomotor activity, depending on the CCK receptor subtype involved.

It is well known that CCK is localised in the mesolimbic dopamine neurons (Hökfelt *et al.,* 1980a). Co-localisation of dopamine and CCK in the ventral tegmental area and in the ascending mesolimbic pathway suggests that CCK could act as a neuromodulator of dopaminergic transmission (Hökfelt *et al.,* 1980b; Vanderhaeghen *et al.,* 1980). Locomotor activity is obviously dependent on the functional activity of the mesolimbic dopaminergic system (Bradbury *et*

al., 1983; Costall *et al.*, 1985). Indeed, low doses of apomorphine, an agonist of dopamine D_1 and D_2 receptors, and L-DOPA, an amino acid, which is decarboxylated to dopamine in the brain, cause hypomotility due the stimulation of dopamine autoreceptors (Di Chiara *et al.*, 1976) belonging to the dopamine D_2 receptor family (Meltzer, 1980; Sokoloff *et al.*, 1992), but co-administration of caerulein with low doses of apomorphine induces an almost complete suppression of locomotor activity in mice (Vasar *et al.*, 1986, 1991). On the other hand, pretreatment of mice with devazepide significantly antagonises the motor suppression caused by apomorphine, whereas the CCK_2 receptor antagonist L-365,260 apparently potentiates the motor depressant effect of apomorphine (Vasar *et al.*, 1991). It shows that similar neurochemical mechanisms are responsible for the motor suppressant action of caerulein and apomorphine in mice. CCK-8s also significantly potentiates dopamine-induced hypolocomotion if simultaneously injected into the ventral tegmental area of rat brain, suggesting that CCK-8s acts as a facilitatory modulator of dopamine at CCK receptors on the dopamine A10 cell bodies (Crawley, 1989). High doses of apomorphine, L-DOPA, as well as some dopamine D_2 receptor agonists (bromocriptine, quinpirole) and an indirectly acting dopaminergic drug amphetamine significantly increase the locomotor activity of rodents (Jackson *et al.*, 1988; Koller and Herbster, 1988; Vaccarino and Rankin, 1989). Dopamine D_1 agonists seem to enhance the hypermotility elicited by dopamine D_2 agonists (Jackson *et al.*, 1988; Koller and Herbster, 1988). Higher doses of caerulein not only inhibit locomotor activity but also amphetamine-induced hyperlocomotion, showing that central mechanisms are probably involved in the action of peripherally administered CCK agonist. This effect of caerulein was also antagonised by devazepide, demonstrating the involvement of CCK_1 receptors (Vasar *et al.*, 1991). However, some investigators report that CCK_1 antagonists have no effect on acute amphetamine-induced locomotion (Higgins *et al.*, 1994; DeSousa *et al.*, 1999). The failure of CCK antagonists to alter spontaneous locomotion and acute amphetamine locomotion can be explained by the requirement for the experimental manipulation to induce a significant release of CCK. CCK antagonists have activity only in experimental protocols that induce higher release of CCK, like repeated psychostimulant exposure. Recently, Wunderlich et al. (2004) confirmed this theory, showing that the systemic administration of PD-140548, a CCK_1 receptor antagonist, into the caudal nucleus accumbens attenuates amphetamine-induced locomotion only in animals previously treated chronically with amphetamine and not in control animals, not exposed to chronic amphetamine treatment, suggesting that chronic stimulant pretreatment may sensitise CCK systems without changing the overall basal level of CCK or the number of CCK-positive cells within the mesoaccumbens system. In rats, the effect of centrally administered CCK-8s on amphetamine-induced hyperlocomotion is dependent on the brain site of administration. In the anterolateral part of nucleus accumbens CCK-8s suppresses via CCK_2 receptors the amphetamine-induced hyperlocomotion,

whereas in the posteromedial part CCK-8s potentiates via CCK_1 receptors the action of amphetamine (Crawley and Corwin, 1994).

Brain dopamine also plays a role in reward-related and motivated behaviours (Wise and Rompre, 1989). Therefore, by influencing the dopamine system, CCK affects the regulation of motivated behaviours. As it can be expected, considering the existence of multiple CCKergic subsystems in the mesolimbic area and the complex nature of CCK-dopamine interactions (Crawley, 1991), the action of CCK receptor agonists and antagonists on motivated behaviours is diverse. Peripherally injected CCK-8s can produce a conditioned place aversion in food-deprived rats (Swerdlow *et al.,* 1983). However, microinjection of CCK-8s into the ventral tegmental area potentiates amphetamine-conditioned place preference (Pettit and Mueller, 1989). Intra-accumbal injection of CCK-8s can either enhance or reduce the behavioural effects of amphetamine, dependent on the injection site and receptor subtype (Vaccarino and Rankin, 1989). A part of the anxiogenic-like effect of CCK-8s is also mediated via the dopaminergic mechanisms in the nucleus accumbens. For example, CCK-8s injected into the posterior part of nucleus accumbens reduces novelty-related exploratory activity through CCK_1 receptors, and this effect is probably related to the reduction of dopamine metabolism and mediated by the modulation of dopamine D_2 receptors (Derrien *et al.,* 1993).

2.2. CCK and pain

The suggestion that CCK plays a critical role in the regulation of pain comes from the study of Faris and colleagues, demonstrating that the administration of CCK reduces opioid-dependent analgesia (Faris, 1985). The ability of CCK-8s to antagonise opioid-induced analgesia was confirmed by Itoh et al. (1985). These findings are the cornerstones for the hypothesis that CCK-8s acts as an endogenous antagonist of the opioidergic system (Faris, 1985). The distribution of CCK peptides in the CNS matches with opioid peptides — enkephalin, β-endorphin, and dynorphin (Gall *et al.,* 1987; Baber *et al.,* 1989; Skinner *et al.,* 1997). CCK_2 antagonists are able to prevent the development of opioid tolerance and dependence (Idanpään-Heikkila *et al.,* 1997; Kayser *et al.,* 1998). Literature offers some evidence that CCK_1 receptors are responsible for the rewarding properties of morphine, whereas CCK_2 receptors modulate the antinociceptive activity of morphine (Singh *et al.,* 1996). Interestingly, an increased number of CCK receptors in the supraoptic nucleus after chronic morphine treatment suggests that CCK could have a role in the development of tolerance to the anti-nociceptive effect of morphine (Munro *et al.,* 1998). Recently Xie et al. (2005) reported that CCK_2 receptors in the rostral ventro-medial medulla are responsible for the development of tolerance to the antinociceptive effect of morphine. Moreover, they suggest that the tone of the CCK system in the rostral ventromedial medulla, induced by long-term

administration of morphine, diminishes the antinociceptive potency of morphine at the spinal level by activating the descending pain facilitatory mechanisms (Xie *et al.,* 2005). Electrophysiological studies demonstrate that CCK-8s diminishes morphine-induced inhibition of dorsal horn neurons in response to painful stimuli, whereas CCK antibodies and antagonists enhance this inhibition (Suberg and Watkins, 1987). This effect seems to involve intracellular calcium content, because CCK-8s has been shown to increase the level of intracellular calcium in presynaptic terminals by mobilisation from the intracellular stores and, therefore, antagonises the suppression of cytosolic calcium levels induced by opioid agonists (Wang *et al.,* 1992).

2.3. CCK and anxiety

In the last fifteen years, particular attention has been directed towards understanding the part of CCK in the neurobiology of fear and anxiety (Harro *et al.,* 1993; Bradwejn and Vasar, 1995; Crawley, 1995). Interestingly, a specific association between CCK and anxiety or panic disorder was discovered in a rather serendipitous way. In 1979 Rehfeld and one of his co-workers injected each other with CCK-4, a CCK_2 receptor agonist, to see whether CCK-4 might stimulate growth hormone secretion. In addition to stimulation of growth hormone secretion, CCK-4 produced entirely unexpected anxiety, dyspnea and depersonalisation (Rehfeld, 2000). These symptoms were very similar seen in patients, suffering from panic disorder, during their panic attacks. Further investigations of the effects of CCK-4 in psychiatric patients and healthy volunteers subsequently confirmed the panic-like action of CCK-4 (De Montigny, 1989; Koszycki *et al.,* 1991; Shlik *et al.,* 1997). Evidence for the anxiogenic-like properties of CCK arises also from electrophysiological experiments where the anxiolytic drugs belonging to benzodiazepines block the excitatory effect of CCK on the rat hippocampal pyramidal neurons (Bradwejn and De Montigny, 1984, 1985). The anxiogenic-like potency of CCK-related peptides was established in various animals, including mice, rats, cats, and monkeys (Fekete *et al.,* 1984; Csonka *et al.,* 1988; Harro *et al.,* 1990; Harro and Vasar, 1991; Singh *et al.,* 1991; Palmour *et al.,* 1992). CCK receptor agonists CCK-8s and CCK-4 inhibit the exploratory activity in mice and rats, reducing the time spent on open arms and the number of entries into the open arms in the elevated plus-maze, time spent and exploratory activity in the light compartment of the light/dark exploration test and retention in fear-motivated tests (Harro *et al.,* 1990; Rex *et al.,* 1994; Shlik *et al.,* 1997). The anxiogenic-like action of CCK agonists is mediated via CCK_2 receptors (Shlik *et al.,* 1997; Noble *et al.,* 1999; Hernandez-Gomez *et al.,* 2002). Anxiogenic manipulations, like social isolation, increase the density of CCK_2 receptors in the frontal cortex of rodents (Vasar *et al.,* 1993; Shlik *et al.,* 1997). It is obvious that dose efficacy and behavioural responses after CCK peptide challenge depend on the baseline

anxiety of animals. For example, rats with low exploratory behaviour in the elevated plus-maze (i.e. anxious rats) display an increased density of CCK_2 receptors in the forebrain structures compared to rats with high exploratory behaviour (i.e. non-anxious rats) (Kõks *et al.,* 1997). African green monkeys, "uptight animals", who are typically restless, submissive to threat and excessively reactive to the environment, become anxious after low doses of CCK-4 while the behaviour of basically calm conspecifics seems to be rather different after CCK-4 injection (Palmour *et al.,* 1992). The administration of caerulein, a non-selective CCK agonist, induces anxiogenic-like effect only in stressed rats, whereas it is ineffective in habituated and non-stressed animals (Kõks *et al.,* 2000).

3. Phenotype of CCK_2 receptor deficient mice

Nagata et al. (1996) generated CCK_2 receptor deficient mice by replacing a part of exon 2, exons 3, 4 and 5. Mice with targeted disruption of CCK_2 receptor gene are fertile and without obvious behavioural abnormalities up to the age of 24 months (Nagata *et al.,* 1996). Kopin et al. (1999) were unable to establish differences between CCK_2 receptor deficient mice and their wild-type (+/+) littermates in the food intake, weight gain and pancreatic function. On the other hand, Miyasaka et al. (2002) and Weiland et al. (2004) have shown that the lack of CCK_2 receptors results in an increased energy expenditure, higher basal metabolic rate, increased body weight, water consumption, elevated body temperature and decreased scotophase locomotor activity. Miyasaka et al. (2004) have also proposed that the enhanced gastric empting in mice, lacking CCK_2 receptors, may partly be responsible for the increased food intake, although the real mechanism is unknown. CCK_2 receptor deficient mice display markedly impaired gastric acid secretion, atrophy of the oxyntic mucosa and hypergastrinaemia. The impaired acid secretion may be the result of a reduced parietal cell mass, a reduced proportion of actively secreting parietal cells (with secretory canaliculi), and a replacement of ECL cells by histamine-free ECL-like cells (Nagata *et al.,* 1996; Chen *et al.,* 2002).

3.1. Impairment of memory functions in CCK_2 receptor deficient mice

Sebret et al. (1999) revealed that mice with targeted disruption of CCK_2 receptor gene displayed impaired memory. In a two-trial memory task, which is based upon the spontaneous tendency of animals to explore novelty, CCK_2 receptor deficient mice spent significantly less time in the novel arm compared to wild-type mice (Sebret *et al.,* 1999). However, other models like passive avoidance and Morris water maze tests did not support this finding in mice

without CCK_2 receptors (our unpublished data). On the contrary, in the Morris water maze, CCK_2 receptor deficient mice showed a normal learning curve and a normal performance in the probe trial. In a long-term memory test, which consisted of a probe trial conducted 14 days after training, male CCK_2 receptor deficient mice performed even better than their wild-type littermates. Passive avoidance test with an electric shock (2 s, 0.6 mA) gave a similar result, demonstrating that nine days after the shock, a decline of memory had occurred in male wild-type mice, but not in their littermates lacking CCK_2 receptors. In view of contradictory evidence, further studies are needed to clarify the precise role of CCK_2 receptors in memory mechanisms.

3.2. Dopamine-dependent hyperactivity in CCK_2 receptor deficient mice

CCK_2 receptor deficient mice display increased locomotor activity in the open-field test. Daugé et al. (2001a) demonstrated that the behavioural activation in CCK_2 receptor deficient mice was suppressed by treatment with the selective dopamine D_2 antagonist sulpiride, suggesting the increased sensitivity of dopamine D_2 receptors in CCK_2 receptor deficient mice. Further behavioural studies with the unselective dopamine agonist apomorphine, which at low doses inhibits the locomotor activity via dopamine D_2 autoreceptors, confirmed the increased sensitivity of presynaptic dopamine receptors in mice, lacking CCK_2 receptors (Kõks *et al.*, 2001, 2003). Kõks et al. (2001, 2003) demonstrated that the indirectly acting dopaminergic drug amphetamine caused a stronger hyperlocomotion in genetically modified mice compared to their wild-type littermates, also demonstrating the enhanced sensitivity of postsynaptic dopamine receptors in mice, lacking CCK_2 receptors. This finding was confirmed by radioligand studies where the increased density of dopamine D_2 receptors was established in the striatum of male mice, lacking CCK_2 receptors (Kõks *et al.*, 2001). There is also evidence that the hyperactivity of mutant mice could be partly due to an increased function of the opioidergic system. Pommier et al. (2002) demonstrated that administration of morphine or inhibition of enkephalin metabolism induces a significantly stronger hyperlocomotion in homozygous (-/-) mice compared to wild-type (+/+) littermates.

3.3. Reduced pain sensitivity in CCK_2 receptor deficient mice

Recent evidence suggests that the function of the opioidergic system is significantly altered in mice, lacking CCK_2 receptors. Pommier et al. (2002) have found that these mice display hyperalgesia in the hotplate test. Reduced jumping latency of homozygous (-/-) mice in this test was confirmed by our group (Veraksitš *et al.*, 2003). However, if licking/shaking of a hindpaw was

used as the endpoint of nociceptive behaviour the significantly delayed response to the noxious stimuli was established in the CCK_2 receptor deficient mice. Interestingly, in the other widely used pain test, plantar analgesia test, pain sensitivity of CCK_2 receptor deficient mice was again significantly reduced compared to wild-type littermates, leading authors to the statement that CCK_2 receptor deficient mice have a decreased pain sensitivity but a reduced pain tolerance (Veraksitš *et al.,* 2003). According to our unpublished data, we established differences in the gene expression between genetically modified and wild-type (+/+) mice. In the striatum of CCK_2 receptor deficient mice, the expression of μ-opioid receptor gene was increased, whereas the expression of pre-pro-enkephalin and nociceptin genes was reduced. Recently Kurrikoff et al. (2004) reported that CCK_2 receptor deficient mice display mechanical hyposensitivity, which can be reversed to the level of wild-type (+/+) animals by the administration of naloxone (Kurrikoff *et al.,* 2004). The finding that mice, lacking CCK_2 receptors, express higher expression levels of lumbar CCK_1, opioid delta and kappa receptor genes could be the possible explanation for the reduced mechanical sensitivity established in genetically modified animals. Moreover, our group demonstrated that CCK_2 receptor deficient mice do not develop mechanical hyperalgesia in the Bennett's neuropathic pain model. Induction of neuropathy resulted in a decrease of lumbar pro-opiomelanocortin (POMC) gene expression in wild-type (+/+) mice, whereas the opposite change was found in CCK_2 receptor deficient mice. These findings confirm the evidence that the genetic invalidation CCK_2 receptors results in an upregulation of the opioidergic system in mice (Pommier *et al.,* 2002; Kurrikoff *et al.,* 2004).

OBJECTIVES

The general aim of the present study was to further characterise the behavioural and biochemical phenotype of CCK_2 receptor deficient mice. The evidence suggests that CCK is implicated in the regulation of anxiety (Harro *et al.,* 1993). Therefore, the first major goal was to establish possible changes in the emotional behaviour of mice, lacking CCK_2 receptors. It has been shown that CCK is a co-mediator of GABA, a major inhibitory neurotransmitter, in the cerebral cortex, hippocampus and basolateral amygdala (Hendry *et al.*, 1984; Kosaka *et al.*, 1985; Cope *et al.,* 2002). By taking into account the probable antagonistic interaction between GABA and CCK, the second major goal was to reveal possible changes in the GABAergic system in mice, lacking CCK_2 receptors. The specific objectives of the present study were as follows:

1. To evaluate the behaviour of genetically modified mice in models reflecting their emotional behaviour (exploratory models of anxiety, fear conditioning test).
2. To reveal the effect of social isolation on the behaviour of male and female CCK_2 receptor deficient mice.
3. To determine the behavioural effects of diazepam, a benzodiazepine agonist, and β-carboline DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), an inverse agonist of benzodiazepine receptors, in mice, lacking CCK_2 receptors.
4. To measure the parameters of benzodiazepine receptors in the cerebral cortex, hippocampus and cerebellum of mice with targeted disruption of CCK_2 receptor gene.
5. To evaluate the expression levels of α1, α2 and γ2 subunits of $GABA_A$ receptors, responsible for the action of anxiolytic and anxiogenic drugs, in the frontal cortex, hippocampus and cerebellum of genetically modified mice.

MATERIALS AND METHODS

1. Animals

CCK_2 receptor-deficient mice were provided from the original background 129Sv/C57Bl/6 mice (Nagata *et al.*, 1996). CCK_2 receptor-deficient mice were generated by homologous recombination by replacing a part of exon 2 and exons 3, 4, and 5 (Nagata *et al.*, 1996). Breeding and genotype analysis were performed in the Department of Physiology, University of Tartu. Genotyping was carried out by means of polymerase chain reaction (PCR) using two pairs of primers. HE2F (TGG AGT TGA CCA TTC GAA TCA C) and LacZrev (GTG CTG CAA GGC GAT TAA GTT G) were designed to detect the mutant allele, and HE3F (TAT CAG TGA GTG TGT CCA CTC T) and HE3R (ACA TTT GTT GGA CAC GTT CAC) were designed for the wild-type allele. For PCR we used the following protocol: 96°C for 10 min (initial denaturation); 96°C for 50 s, 60°C for 50 s and 72°C for 2 min (25 cycles); and 72°C for 10 min (final amplification). PCR products were stored at 4°C until electrophoresis. During the studies, mutant mice were crossed back six times to the C57Bl/6 background to minimise possible genetic effects from the 129Sv strain. The mice were kept in the animal house at 20±2°C under a 12-h/12-h light/dark cycle (lights on at 0700). Tap water and food pellets were available *ad libitum*. Female mice were used throughout the studies, except in social isolation studies where male mice were also studied. All animal procedures were approved by Animal Ethics Committee of the University of Tartu in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

2. Drugs

All injections were performed intraperitoneally (i.p.) in a volume of 10 ml/kg. The behavioural effects of diazepam, a benzodiazepine agonist, and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), an anxiogenic β-carboline, were studied in separate groups of mice. For the study of anxiolytic action of diazepam in the elevated plus-maze and light/dark exploration tests, different doses of diazepam were used (0.5–3 mg/kg, Sigma). The action of diazepam (0.5 and 3 mg/kg) was also studied in the rotarod test. Diazepam was suspended in physiological saline (0.9% sodium chloride solution) with the help of a few drops of Tween-80 (Sigma) and was administered i.p. 30 min prior to the study. Anxiogenic β-carboline, DMCM (0.25–1 mg/kg) was injected i.p. 15 min before the study. DMCM was dissolved in 0.25 ml of 0.2 M HCl, neutralised with 0.05 ml of 1 M NaOH and then diluted with physiological saline.

3. Behavioural experiments

The animals were brought into the experimental room one hour before the experiment. All behavioural experiments were performed between 1100 and 1900. Ethological models of anxiety, rotarod and conditioned place preference tests were performed on separate groups of animals. Since some behavioural experiments lasted 6–8 h, precautions were taken to control the possible daily fluctuations in the exploratory behaviour of animals. Therefore, the experiments were always performed in randomised order, that is, wild-type (+/+) mice were used in parallel with their genetically modified littermates. In one part of a study, the effect of social isolation was studied on the exploratory behaviour of male and female genetically modified mice. Social isolation was used in order to induce a strong emotional stress in mice. Animals were kept isolated in cages (33cm×12cm×13cm) for 21 days, while their age- and genotype-related littermates were kept in groups of 8–11. On day 21, all subjects were exposed to the plus-maze test, which was immediately followed by the locomotor activity test in the motility boxes.

3.1. Exploratory behaviour

3.1.1. Elevated plus-maze

The elevated plus-maze is one of most popular tasks for measuring anxiety-like behaviour. This test is nonshock, naturalistic conflict between the tendency of mice to explore a novel environment and aversive properties of a brightly lit, open area and height. Elevated plus-maze allows to control for locomotor activity and thus a separate test for general locomotion in the motility box is not necessary to verify a positive finding. The major advantage of this task is that it permits a quick screening of anxiety modulating drugs, mouse genotypes without training or food/water deprivation or use of noxious stimulation (Rodgers *et al.*, 1997).

In the pilot study, we compared the exploratory behaviour of male and female mice from the 129 Sv/C57BL/6 background in the elevated plus-maze. This pilot experiment confirmed the findings of a previous study (Johnston and File, 1991) in that female animals showed reduced aversion to the open arms compared to male mice. Therefore, for further studies, female mice were chosen. According to our previous experiments, the anxiogenic-like effect of CCK agonist (caerulein, CCK-4) depends on pre-experimental manipulations (Kõks *et al.*, 2000). The chance to find an anxiolytic effect of a drug is higher in stressed animals. Thus, the experiment was performed in a brightly lit room. Animals were not handled before studies, and they were placed singly in the cages for 30 min prior to the plus-maze exposure. The plus-maze consisted of two opposite open (17.5x5 cm) arms without sidewalls and two enclosed arms of the same size with 14-cm-high sidewalls and an end wall. The arms extended

from a common central square (5x5 cm) and were angled at 90° to each other, making the shape of a plus sign. To determine locomotor activity, the open arms were divided by lines into three equal parts. The entire plus-maze apparatus was elevated to the height of 30 cm and placed in a brightly lit room (illumination level ~750 lux). In order to encourage open arm exploration, a slightly raised edge (0.25 cm) was put around the perimeter of the open arm, providing a grip for animals. Testing began by placing an animal on the central platform of the maze facing a closed arm. The mice clearly preferred the enclosed arms. An arm entry was counted only when all four limbs were within a given arm. Standard 5-min test duration was employed (Pellow *et al.*, 1985; Lister, 1987), and the maze was wiped with damp and dry towels between the subjects. Test sessions were video-recorded and the videotapes were subsequently blind-scored by a trained observer. The following measures were taken by the observer: 1) time spent on the central square and open arms of the plus-maze; 2) number of closed and open arm entries; 3) number of line crossings; 4) ratio between open and total arm entries. Time spent on open arms, number of open arm entries, and ratio between open and total arm entries are conventional measures of anxiety in the elevated plus-maze (Pellow *et al.*, 1985; Lister, 1987). The frequencies of closed and total arm entries and the number of line crossings were used as measures of locomotor activity (Johnson and Rodgers, 1996). Each animal was used only in one experiment.

3.1.2. Light/dark exploration test

The test is nonshock, naturalistic conflict between the tendency of mice to explore a novel environment vs. the aversive properties of a brightly lit open field. This task is very simple and therefore widely used.

Light/dark exploration test was performed with animals subjected to the gentle handling. The handling habituation of mice was performed once daily for three consecutive days in the room where the experiment was conducted on the fourth day. Light/dark exploration test is an unconditioned test of anxiety-like behaviour designed for mice (Crawley and Goodwin, 1980). The Plexiglas box (45x20x20 cm) was divided into two parts: 2/3 was brightly illuminated (~500 lux) by a 60 W light bulb fixed 30 cm above the floor, 1/3 was painted black, covered by a lid and separated from the white compartment with a partition containing an opening (13x5 cm) at the floor level. The animal was placed in the centre of the light compartment facing away from the opening, and the latency to move to the dark, the time spent in the light compartment and the number of transitions between the two compartments were measured. Additionally, we measured the number of rearings in the light part. The duration of the test was 5 min beginning from the first entry to the dark (the test was terminated if this time exceeded 300 s).

3.2. Fear conditioning test

This is a form of classical conditioning where a simple association of a conditioned stimulus (tone, CS) with an electric foot-shock is analysed. The procedures were based on published methods (Paylor *et al.*, 1998) with some modifications. Experiments were carried out with a computer-controlled fear conditioning system (TSE, Bad Homburg, Germany). Context and tone-dependent experiments took place in a lit room. The animals were not handled before the experiment. During the training period, mice were kept in their home-cages, on the day of the experiment the animals were placed singly in cages for 30 min before the test. Training was conducted in a transparent acrylic chamber (110x160x160 mm/110x135x155 mm) containing 3 mm stainless steel rod floor, spaced 0.5 cm, through which a foot shock could be administered. The test chamber was placed inside a sound-attenuated chamber and was constantly illuminated (~500 lux). Mice were observed through a window in the front wall of the sound-attenuated chamber. Animals were placed in the conditioning context for 120 s and were then exposed to a 10 kHz tone (CS) for 30 s. The tone was terminated by a foot-shock (2 s, 0.5 mA), which served as an unconditioned stimulus (US). 120 s later another CS-UC pairing was presented. The mouse was removed from the chamber 15–30 s later and returned to its home cage.

Twenty-four hours later contextual memory was tested. The animal was placed back into the test chamber for 5 min. The CS was not applied during the test. Total time of freezing (defined as absence of any movements for more than 3 sec) was measured using the standard interval sampling procedure every 10 sec. Four hours later, the mouse was tested for its freezing behaviour to the auditory CS. Testing was performed in a different acrylic chamber (220x160x160 mm/220x135x155 mm) the floor of which was covered with white cardboard. The background colour was black. Duration of the test was 6 minutes: 3 minutes without the tone (pre-CS phase) and 3 minutes with the tone (CS phase). During this time freezing intervals were measured. The number of freezing intervals was converted to a % of freezing. For the auditory CS test, the % of freezing was obtained by subtracting the % of freezing in the pre-CS period from the % of freezing when the auditory CS was present.

3.3. Locomotor activity

For the study of locomotor activity, each animal was placed singly in a photoelectric motility box (448×448×450 mm) connected to a computer (TSE Technical and Scientific Equipment GMBH, Germany). The illumination level of the transparent test boxes was ~500 lux. After removing the mouse from the box, the floor was cleaned with a 5% ethanol solution. Time in locomotion (s), distance of locomotion (m), the number of rearings and corner entries was registered during the 30 min observation period.

3.4. Rotarod test

A 1 min training session was given to mice on the rotarod (diameter 8 cm, 9 rpm) 5 min before the first measurement. Time until the first fall was registered during a 2 min session on three consecutive days to evaluate the motor performance of mice.

4. Radioligand binding studies

In the radioligand binding studies we used animals that had not been exposed to behavioural testing. After decapitation, the brains were rapidly dissected on ice, and cooled down in liquid nitrogen. The cerebral cortex (including frontal and parietal cortices), hippocampus, and cerebellum were dissected (Franklin and Paxinos, 1997) and stored in liquid nitrogen at –80°C until sample preparation. The brain structures from six mice were pooled. The radioligand binding studies were performed according to the method of Kõks et al. (1997). [^{3}H]-flunitrazepam (specific activity 96 Ci/mmol, Amersham Radiochemicals) was used for the labelling of benzodiazepine receptors. The parameters of benzodiazepine receptors were determined in the presence of 0.5–16 nM [^{3}H]-flunitrazepam at 4°C for 60 min. Diazepam (Sigma, 10 μM) was added to determine the non-specific binding at benzodiazepine receptors. The brain tissue was homogenised in 20 volumes of ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) using a Potter-S glass-teflon homogeniser (1,000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48,000 x g for 20 min) and resuspension. After the last centrifugation, crude brain membranes were suspended in the incubation buffer: 50 mM Tris-HCl (pH 7.4 at 4°C). The protein content was measured according to the method of Bradford (1976). The saturation curves of [^{3}H]-flunitrazepam binding were analysed using GraphPad Prism (Version 3.00) for Windows software. The experiments were repeated four times.

5. Gene expression studies

In gene expression studies, wild-type (+/+) and homozygous (-/-) mice (12 animals in both groups) not exposed to any behavioural testing were used. We did not include heterozygous (+/-) mice in gene expression studies as their behaviour did not significantly differ from that of wild-type (+/+) littermates. After decapitation, brains were quickly dissected into four parts (cerebellum, brainstem, hippocampus, frontal cortex) and frozen in liquid nitrogen. mRNA was extracted, using Rneasy Midi Kit (QIAGEN) according to the manufacturer's protocol. First strand cDNA was synthesised, using First Strand cDNA Synthesis Kit (Fermentas).

Brain samples from animals in both groups were pooled as follows: six pools (two mice per pool) were formed per tissue per group (n=6). Pooling was performed to minimise the fluctuations resulting from individual differences. As a rule, the experiments with wild-type (+/+) and homozygous (-/-) animals were conducted in parallel. We studied the expression levels of the following subunits of $GABA_A$ receptors: α1, α2, and γ2. For establishing differences in gene expression, quantitative real-time PCR (qRT-PCR) was applied. This method has several advantages compared to other PCR methods. qRT-PCR offers enhanced sensitivity, high throughput, use of closed-tube system, reduced variation, and lack of post-PCR manipulations. For establishing differences in gene expression, ABI PRISM 7700 Sequence Detection System equipment (PE Applied Biosystems, USA) and ABI PRISM 7000 SDS Software were used. In all quantification experiments hypoxanthineguanine phosphoribosyl transferase (HPRT) was used as the endogenous reference gene. Primers were designed with the Primer ExpressTM software (PE Applied Biosystems, USA). The primer sequences for $GABA_A$ receptor α1, α2, and γ2 subunits are presented in Table 2. All reactions were performed by using SYBR® Green I Master Mix (Roche, USA). Instructions of the equipment and reagent manufacturers were always followed. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. All samples to be compared were run in the same experiment. The amount of the target gene was compared to the housekeeper gene in the homozygous (-/-) and wild-type (+/+) groups by means of the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Every reaction was made in four parallel samples to minimise possible errors. The mRNA level in wild-type (+/+) mice was always defined as 1 and the increase of mRNA amounts was shown as the fold increase.

6. Data analysis

Age- and weight-matched mice were used. The results are expressed as mean values ± S.E.M. The results were analysed by using one- or/and two-way analysis of variance (ANOVA/MANOVA). *Post hoc* comparisons between the individual groups were performed by means of the Tukey HSD test and Newman-Keuls test, using Statistica for Windows software. The Student's t-test was applied for radioligand binding and gene expression studies.

Table 2. Sequences of primers used for qRT-PCR studies

Gene	**Forward primer**	**Reverse primer**
$GABA_A$ receptor, α1 subunit	5’-CACCAGTTTCGGACCAGTTT-3’	5’-ACAGCAGAGTGCCATCCTCT-3’
$GABA_A$ receptor, α2 subunit	5’-CACAGAGGATGGCACTCTGCT-3’	5’-TTCAGCTCTCACGGTCAACCT-3’
$GABA_A$ receptor, γ2 subunit	5’-TGACAACAAACTTCGACCTGACA-3’	5’CTGTATGAATTAATGTTGGTTTCACTC-3’
HPRT	5’-GCAGTACAGCCCCAAAATGG-3’	5’-AACAAAGTCTGGCCTGTATCCAA-3’

RESULTS

1. Behavioural differences between wild-type (+/+) and CCK_2 receptor deficient mice (Papers I, III)

1.1. Exploratory activity in the elevated plus-maze test (Paper I)

Comparison of exploratory activity of female CCK_2 receptor deficient mice relative to the wild-type (+/+) littermates established several differences. Homozygous (-/-) mice visited open arms more frequently than wild-type (+/+) mice (Figure 2). Also, homozygous (-/-) mice spent significantly more time on the open arms and the central square of the plus-maze. However, changes in the other parameters of plus-maze exploration (the number of line crossings, ratio between open and total arm entries) did not reach a statistically significant level. The frequency of closed arm entries in homozygous (-/-) mice did not differ from that of the wild-type (+/+) littermates, but was different from the number of closed arm entries of heterozygous (+/-) animals.

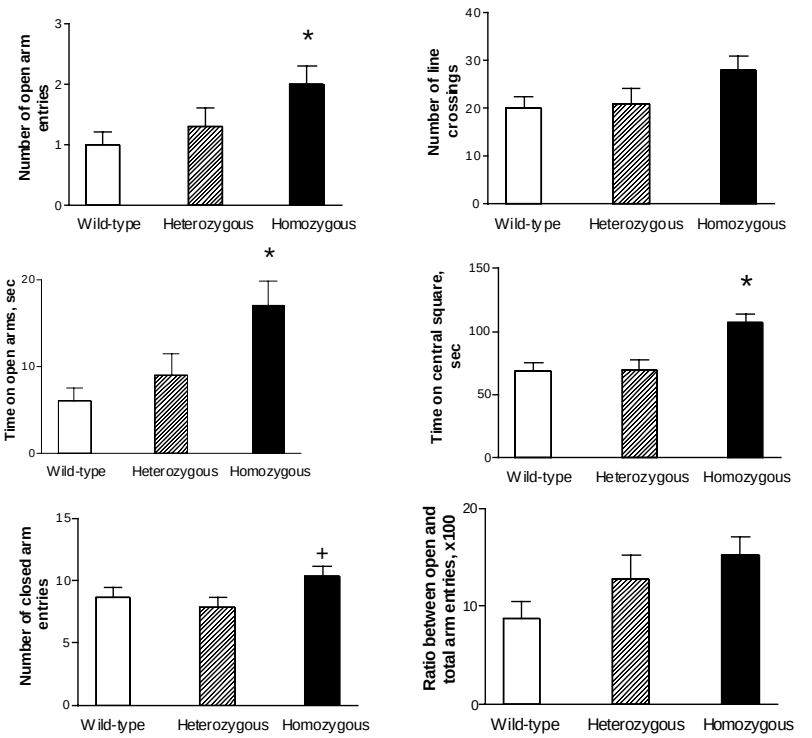

Figure 2. The exploratory activity of CCK_2 receptor deficient mice in the elevated plus-maze. The number of animals in each group was between 28 and 30. White bars – wild-type (+/+) mice; striped bars – heterozygous (+/-) animals; black bars – homozygous (-/-) animals. $^{*}p<0.05$ (compared to wild-type (+/+) mice, Tukey HSD test after significant one-way ANOVA); $^{+}p<0.05$ (compared to heterozygous (+/-) mice).

1.2. Exploratory activity in the light/dark exploration test (Paper III)

The exploratory behaviour of CCK_2 receptor deficient mice in the light/dark test was studied in two different experiments. In the first experiment, the exploratory activity of wild-type (+/+) mice was higher compared to the second one. Nevertheless, in both cases the exploratory activity of CCK_2 receptor deficient mice was significantly increased compared to their wild-type (+/+) littermates (Figure 3). In the first study, the frequency of transitions of homozygous (-/-) mice was significantly higher than that of heterozygous (+/-) and wild-type (+/+) mice, whereas the other parameters did not differ between the genotypes. In the second experiment, all the parameters of exploratory behaviour of homozygous (-/-) mice were different from that of wild-type (+/+) and heterozygous (+/-) littermates (Figure 3). The frequency of transitions and time spent in the light compartment was significantly higher in homozygous (-/-) mice compared to their wild-type (+/+) and heterozygous (+/-) littermates. The number of rearings was also higher in homozygous (-/-) mice, but this difference was significant only if homozygous (-/-) and heterozygous mice (+/-) were compared.

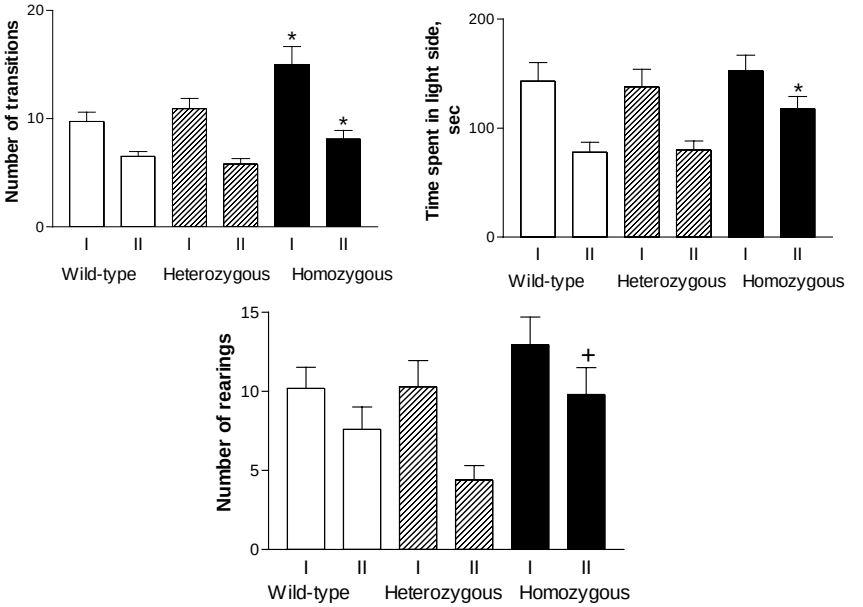

Figure 3. The exploratory behaviour of CCK_2 receptor deficient mice in the light-dark exploration test.
The results of two different experiments (I and II) are presented. Number of mice in the first experiment was as follows: wild-type (+/+) – 32, heterozygous (+/-) – 33 and homozygous (-/-) – 26. In the second study, the number of animals was as follows: wild-type (+/+) – 25, heterozygous (+/-) – 25 and homozygous (-/-) – 21. White bars – wild-type (+/+) mice; striped bars – heterozygous (+/-) animals; black bars – homozygous (-/-) animals. *$p<0.05$ (compared to wild-type (+/+) mice, Newman-Keuls test after significant one-way ANOVA); $^{+}p<0.05$ (compared to heterozygous (+/-) mice).

1.3. Fear conditioning test (Paper III)

Differently from the light/dark exploration test, the behaviour of CCK_2 receptor deficient mice in the fear conditioning test did not differ significantly from that of wild-type (+/+) littermates. In the pre-conditioning test, wild-type (+/+) and homozygous (-/-) mice showed comparable freezing behaviour (data not shown). In the contextual memory test, all groups demonstrated a significant increase in freezing, but no differences between the genotypes were found. In the new context, the freezing of mice was comparable to that established in the pre-conditioning test. The testing of cued fear in the altered context induced a significant increase in freezing in all groups, but, again, we did not find any differences between the genotypes.

1.4. Motor coordination in rotarod test (Paper I)

The performance of wild-type (+/+), heterozygous (+/-), homozygous (-/-) mice in the rotarod test did not differ on days 1, 2 and 3 (Figure 4). This is different from previous studies performed with male CCK_2 receptor deficient mice showing a significant impairment of motor coordination in homozygous (-/-) mice (Daugé *et al.,* 2001b; Kõks *et al.,* 2001). These differences might be related to gender, since the present study was performed on female mice. It is also noteworthy that in the rotarod test C57Bl/6 mice show much longer latencies to fall than 129Sv mice (Homanics *et al.,* 1999). Thus, it is possible that the targeted mutation of CCK_2 receptors induces motor disturbances in mice predominately having the genes of a 129Sv strain. This suggestion is supported by our studies on male mice showing that backcrossing of mice to C57Bl/6 genetic background reverses the suppression of motor activity evident in our first studies with homozygous (-/-) mice (Kõks *et al.*, 2001, 2003).

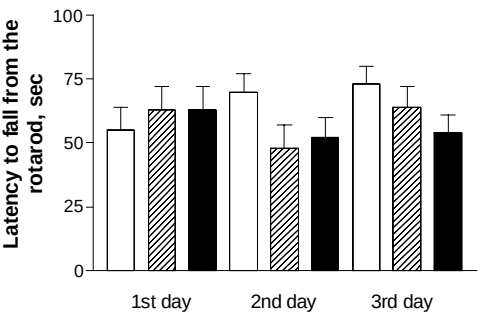

Figure 4. The performance of CCK_2 receptor deficient mice in the rotarod test.
The number of animals in each group was as follows: 30 wild-type, 28 heterozygous and 28 homozygous mice. The study was repeated on 3 consecutive days. White bars – wild-type (+/+) mice; striped bars – heterozygous (+/-) animals; black bars – homozygous (-/-) animals.

1.5. Locomotor activity in motility boxes (Paper III)

There were no differences in locomotor activity between genotypes (Figure 10). As for time in locomotion, distance travelled, number of rearings and corner entries, there were no differences between female wild-type (+/+) and homozygous (-/-) mice.

2. Social isolation-induced behavioural changes in wild-type (+/+) and CCK_2 receptor deficient mice (Paper II)

Social isolation induced genotype-dependent as well as gender-dependent changes in exploratory behaviour. The number of open arm entries tended to be higher in group-housed female mice compared to male group-housed animals. Social isolation remarkably increased the number of open arm entries in male wild-type (+/+) mice. In female wild-type (+/+) mice, social isolation induced the opposite effect: a significant reduction of open arm entries (Figure 5). In female and male homozygous (-/-) mice, individual housing tended to increase the frequency of open arm entries, but this increase was not statistically significant. In contrast to open arm entries, social isolation did not induce significant changes in time spent on open arms. Statistical analysis revealed that social isolation induced a significant increase in closed arm visits in male mice compared to female animals, regardless of genotype. A remarkable reduction in the ratio between open and total arm entries was revealed in isolated female wild-type (+/+) animals compared to other groups (Figure 5). This behavioural change was not established in male mice and female homozygous (-/-) animals. Moreover, there was a significant difference between isolated female wild-type (+/+) and CCK_2 receptor deficient mice. Social isolation affected differently the number of attempts in male and female, but also in wild-type (+/+) and homozygous (-/-) mice. Group-housed male wild-type (+/+) mice made significantly more attempts to enter the central square than group-housed male homozygous (-/-) and group-housed female wild-type (+/+) rodents. This behaviour indicates a higher level of anxiety in group-housed male wild-type (+/+) mice. However, a significant decrease in the number of attempts was seen in isolated male wild-type (+/+) animals compared to respective group of normally housed mice. As for locomotor activity, social isolation induced a different effect in homozygous (-/-) and wild-type (+/+) mice. Number of line crossings in isolated male wild-type (+/+) animals was more than two-fold higher compared to group-housed male wild-type (+/+) littermates. This effect was not evident in any other groups. There were significant differences between isolated male and female wild-type (+/+) mice.

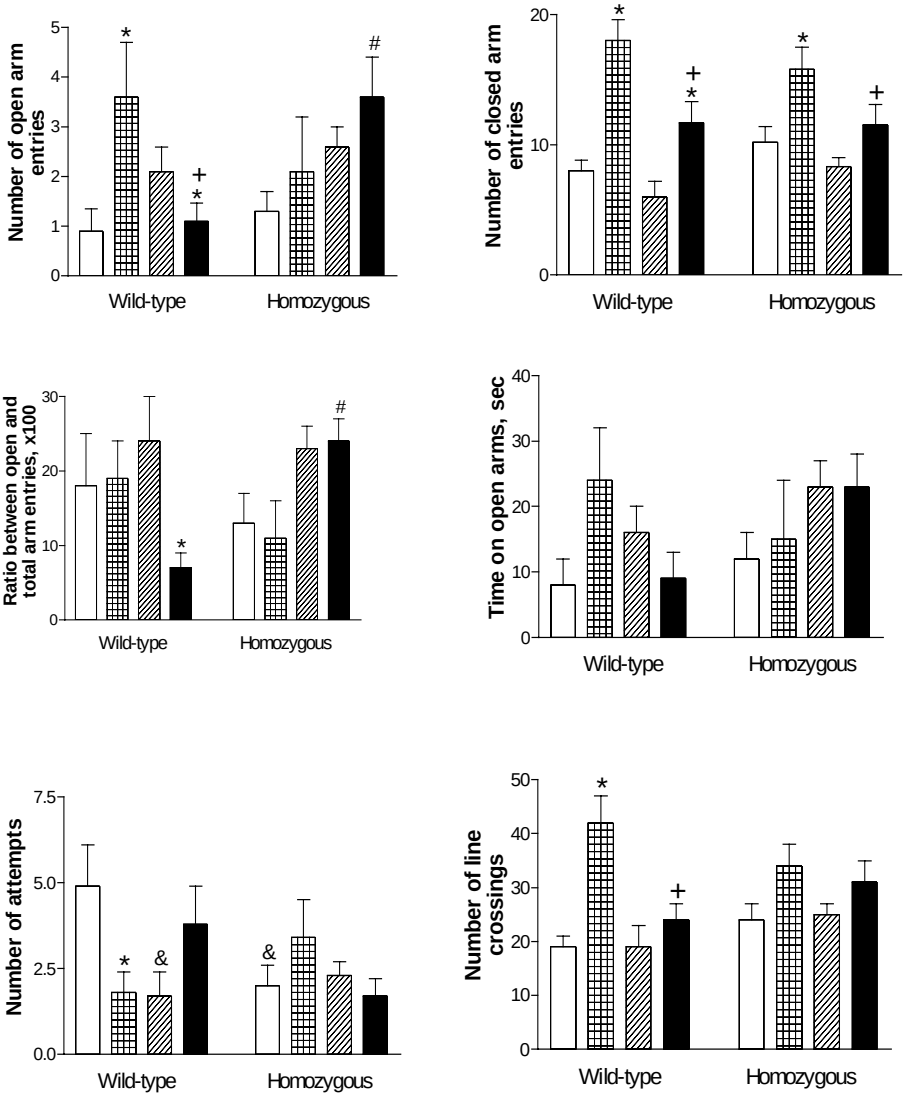

Figure 5. The effect of social isolation on the exploratory activity of male and female CCK_2 receptor deficient mice in the elevated plus-maze.
White bars – male, group-housed mice; hatched bars – male, isolated mice; striped bars – female, group-housed mice; black bars – female, isolated mice. *$p<0.05$ Newman-Keuls test after significant MANOVA, compared to respective group of normally housed mice; $^{+}p<0.05$ a statistically significant difference between isolated female and male mice, $^{\#}p<0.05$ a statistically significant difference between isolated homozygous (-/-) and wild-type (+/+) female mice $^{\&}p<0.05$ a statistically significant difference compared to group-housed wild-type (+/+) male mice.

3. Drug-induced behavioural changes in wild-type (+/+) and CCK_2 receptor deficient mice (Papers I, III)

3.1. The effect of diazepam in the elevated plus-maze test (Paper I)

The effect of diazepam in the plus-maze was studied in a separate group of mice. In this experiment, the baseline exploratory activity of heterozygous (+/-) mice was higher than in the above described plus-maze experiment. However, differences in exploratory activity between wild-type (+/+) and homozygous (-/-) mice remained at the same level in these two separated studies (Figures 2 and 6). The administration of diazepam (0.5–3 mg/kg) caused a dose-dependent anxiolytic-like effect in wild-type (+/+) mice. Diazepam increased the number of open arm entries, time spent on the open arms, and the ratio between open and total arm entries (Figure 6). The number of open arm entries was differently affected by diazepam in wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice. The lowest dose of diazepam (0.5 mg/kg) induced a statistically significant increase in open arm entries in wild-type (+/+) mice. After treatment with diazepam at a dose of 1 mg/kg, a significant change in the exploratory behaviour was found in the homozygous (-/-) mice. However, in heterozygous (+/-) animals this effect of the benzodiazepine agonist was not significant. The highest dose (3mg/kg) caused an increase in open arm entries in wild-type (+/+) mice, but not in genetically modified animals. Also, diazepam affected differently the number of line crossings in these groups of mice. At lower doses (0.5 and 1 mg/kg), diazepam tended to increase the number of line crossings in wild-type (+/+) mice, but this effect was not statistically significant (Figure 6). The highest dose of diazepam (3mg/kg) reduced this behavioural measure in heterozygous (+/-) and homozygous (-/-) mice, but only in homozygous (-/-) animals was this change significant. Diazepam (3 mg/kg) did not change time spent on the central square and the number of closed arm entries in wild-type (+/+) mice as compared to vehicle-treated animals. However, diazepam significantly reduced these parameters of exploratory behaviour in genetically modified mice. Diazepam (3 mg/kg) induced a statistically significant increase in the ratio between open and total arm entries only in wild-type (+/+) mice, but not in heterozygous (+/-) or homozygous (-/-) CCK_2 mutant mice.

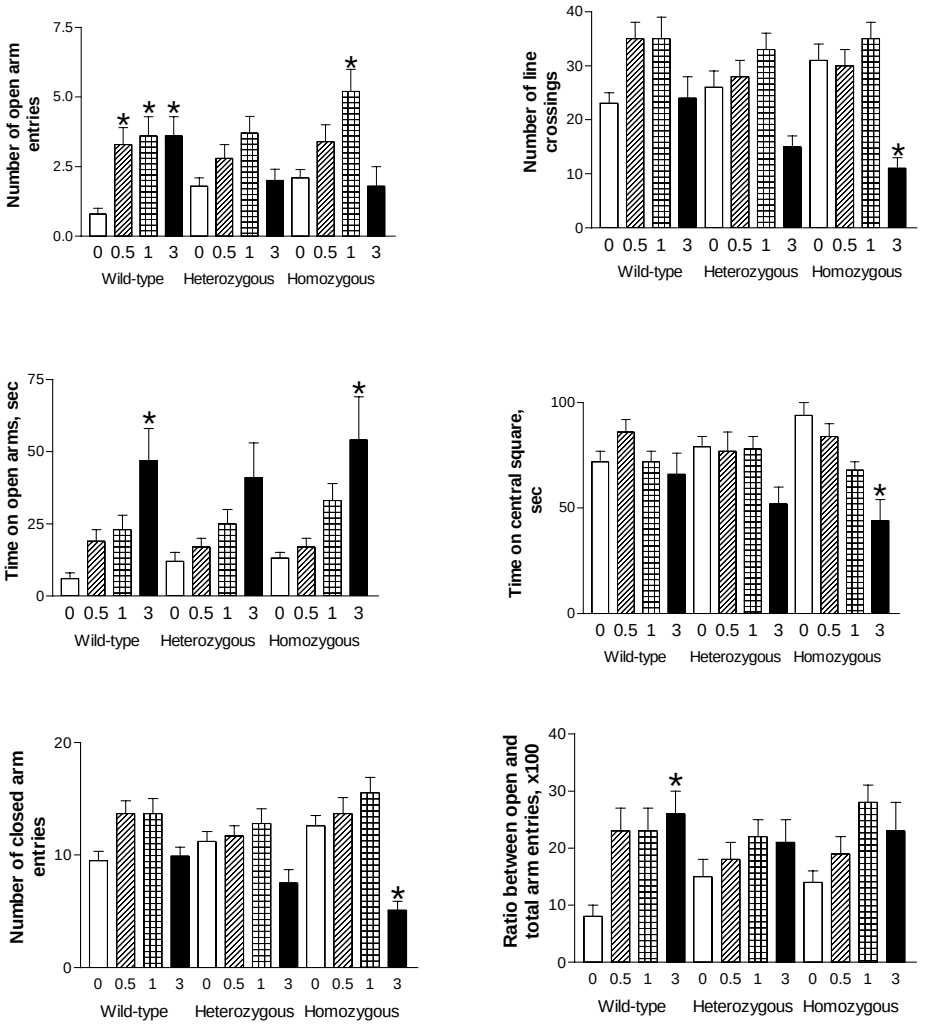

Figure 6. The effect of diazepam (0.5-3 mg/kg) on the exploratory activity of CCK_2 receptor deficient mice in the elevated plus-maze.

The number of animals in each group was between 30 and 37. White bars – saline; striped bars – diazepam 0.5 mg/kg; hatched bars – diazepam 1 mg/kg; black bars – diazepam 3 mg/kg. *p<0.05 (compared to respective saline-treated group, Tukey HSD test after significant two-way ANOVA)

3.2. The effect of diazepam and DMCM on the exploratory behaviour in the light/dark test (Paper III)

Administration of diazepam (0.5–2 mg/kg) had a different effect on the exploratory activity of wild-type (+/+) and CCK_2 receptor deficient mice. In wild-type (+/+) mice, the lowest dose of diazepam (0.5 mg/kg) increased the number of transitions between the two compartments, whereas in genetically modified mice the highest dose of diazepam (2 mg/kg) caused a significant suppression of transitions (Figure 7). The highest dose of diazepam also decreased time spent in light compartment by heterozygous (+/-) and homozygous (-/-) mice. The number of rearings was inhibited by the highest dose of diazepam in all groups (Figure 7).

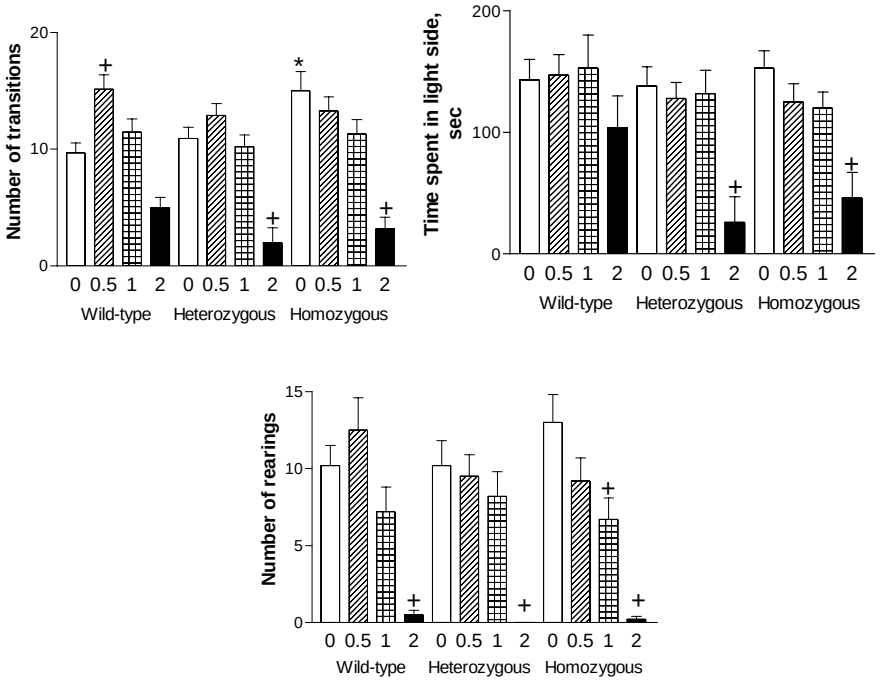

Figure 7. The effect of diazepam (0.5–2 mg/kg) on the exploratory behaviour of CCK_2 receptor deficient mice in the light-dark exploration test.
The number of animals in each group was between 23 and 27. White bars – vehicle treated mice; striped bars – diazepam 0.5 mg/kg; hatched bars – diazepam 1 mg/kg; black bars – diazepam 2 mg/kg. $^{*}p<0.05$ (compared to vehicle-treated wild-type (+/+) mice, Newman-Keuls test after the significant two-way ANOVA); $^{+}p<0.05$ (compared to vehicle-treated group of respective genotype).

Treatment with DMCM (0.25–1 mg/kg) induced an anxiogenic-like action in genetically modified mice (Figure 8). In heterozygous (+/-) mice, DMCM did not affect exploratory behaviour and in wild-type (+/+) animals it tended to increase exploratory activity. The administration of DMCM induced a dose-dependent reduction in transitions between the two compartments in homozygous (-/-) mice. As for time spent in the light compartment, treatment with DMCM also had different effects on different genotypes. In wild-type (+/+) mice, DMCM caused an unexpected increase in time spent in the light part (Figure 8). In homozygous (-/-) mice, the administration of DMCM caused an anxiogenic-like response by reducing the time spent in the light compartment in a dose-dependent manner. As for rearings, the action of DMCM was again different in various genotypes. The administration of DMCM tended to increase the frequency of rearings in wild-type (+/+) mice, but the effect was not significant. However, in homozygous (-/-) mice DMCM induced a dose-dependent inhibition of rearings.

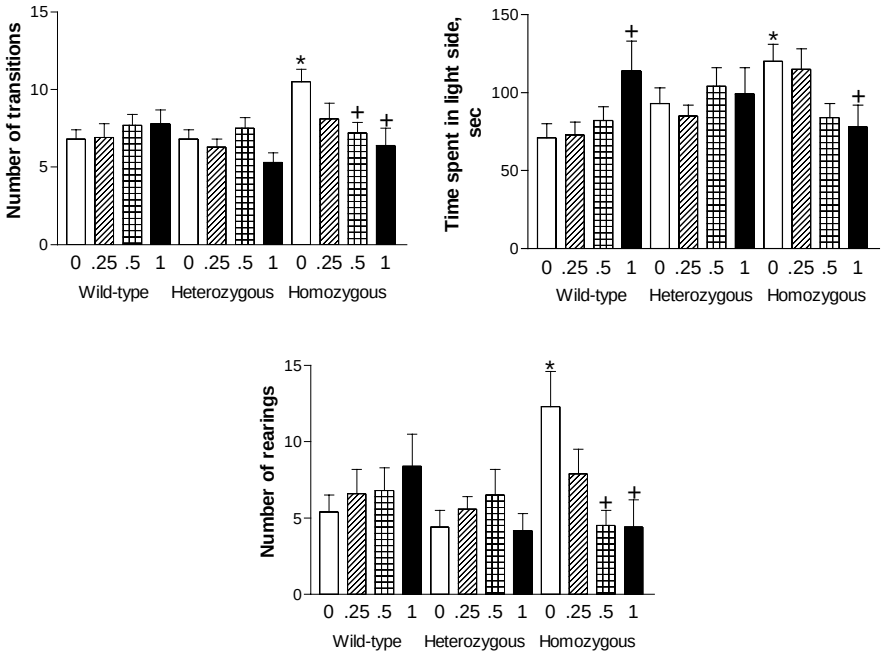

Figure 8. The effect of DMCM (0.25–1 mg/kg) on the exploratory behaviour of CCK_2 receptor deficient mice in the light-dark exploration test.
The number of animals in each group was between 14 and 16. White bars – vehicle treated mice; striped bars – DMCM 0.25 mg/kg; hatched bars – DMCM 0.5 mg/kg; black bars – DMCM 1 mg/kg. $^{*}p<0.05$ (compared to vehicle-treated wild-type (+/+) mice, Newman-Keuls test after significant two-way ANOVA); $^{+}p<0.05$ (compared to vehicle-treated group of respective genotype).

3.3. The effect of diazepam on motor coordination in the rotarod test (Paper I)

Treatment with diazepam (0.5 and 3 mg/kg) on day 4 caused a dose-dependent impairment of motor coordination in all genotypes (Figure 9). However, a significantly stronger impairment of motor coordination induced by diazepam (0.5 and 3 mg/kg) was seen in homozygous (-/-) mice compared to wild-type (+/+) littermates.

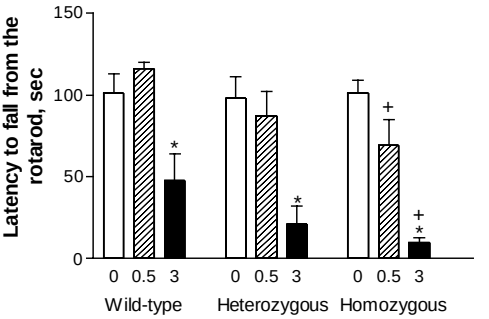

Figure 9. The effect of diazepam (0.5 and 3 mg/kg) on the performance of CCK_2 receptor deficient mice in the rotarod test.
The number of animals in each group was between 8 and 11. White bars–saline; striped bars – diazepam 0.5 mg/kg; black bars – diazepam 3 mg/kg. *$p<0.05$ (compared with respective saline-treated group, Tukey HSD test after significant one-way ANOVA). $^{+}p<0.05$ (compared with diazepam-treated wild-type mice)

3.4. The effect of DMCM on locomotor activity in motility boxes (Paper III)

The administration of DMCM (0.25–1 mg/kg) did not change the locomotor activity of wild-type (+/+) and homozygous (-/-) mice (Figure 10). Time in locomotion was not influenced if the effect of DMCM was compared in wild-type (+/+) and homozygous (-/-) mice. Distance travelled was not affected either by the treatment with DMCM. Number of rearings and corner entries also remained unchanged.

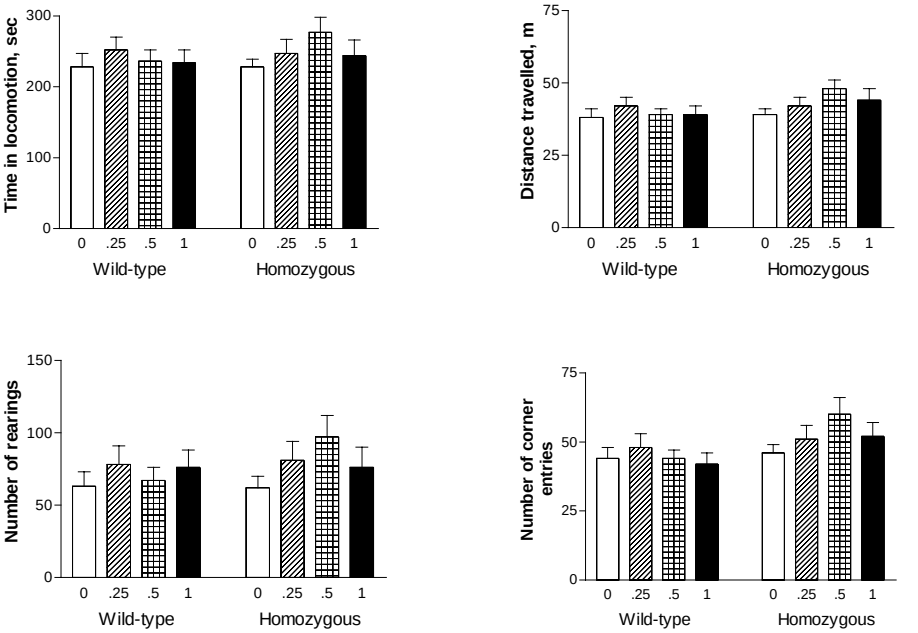

Figure 10. The effect of DMCM (0.25–1 mg/kg) on the locomotor activity of CCK_2 receptor deficient mice in the motility boxes.
The number of animals in each group was 14 or 15. White bars – vehicle-treated mice; striped bars – DMCM 0.25 mg/kg; hatched bars – DMCM 0.5 mg/kg; black bars – DMCM 1 mg/kg.

4. Radioligand binding studies (Paper I)

The density of [^{3}H]-flunitrazepam binding sites (B_{max}) in the cerebellum was increased in homozygous (-/-) mice compared to their wild-type (+/+) littermates (Figure 11). No such difference between wild-type (+/+) and homozygous (-/-) mice was found in the cerebral cortex and hippocampus. The density of benzodiazepine binding sites tended to be higher in the cerebral cortex and hippocampus of heterozygous (+/-) animals relative to wild-type (+/+) mice. However, these differences were not statistically significant. The affinity of benzodiazepine binding sites (K_d) in the cerebral cortex, hippocampus and cerebellum did not differ in wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice (Figure 11).

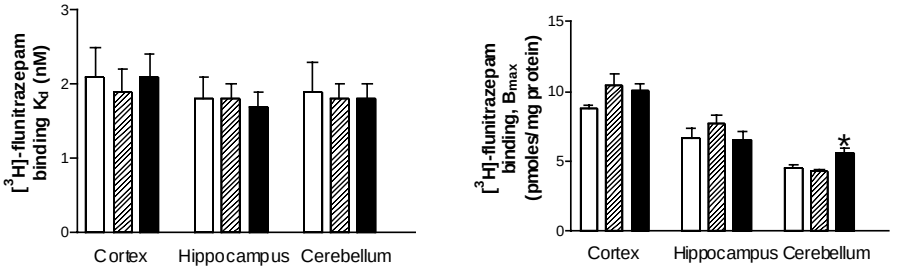

Figure 11. The parameters of [^{3}H]–flunitrazepam binding in the brain structures of CCK_2 receptor deficient mice.
The number of animals in each group was 24, the brains of six mice were pooled and the mean is a result of four experiments. White bars – wild-type; striped bars – heterozygous; black bars – homozygous. *p<0.05 (compared to wild-type mice, Student's t-test)

5. Gene expression studies (Paper III)

In most cases the expression levels of $GABA_A$ receptor (α1, α2 and γ2 subunits) related genes did not differ in wild-type (+/+) and homozygous (-/-) mice (Figure 12). The only difference was established for the α2 subunit in the frontal cortex. In this particular case, the expression level in homozygous (-/-) mice was 1.6-fold higher compared to their wild-type (+/+) littermates.

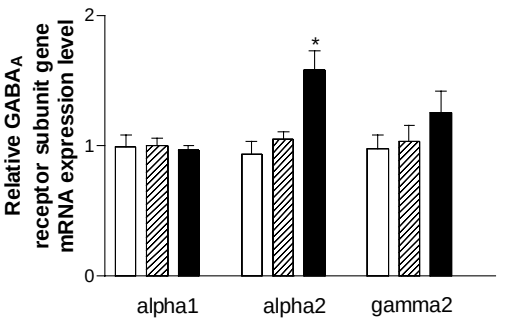

Figure 12. Relative $GABA_A$ receptor subunit (α1, α2, and γ2) genes mRNA expression levels in the frontal cortex, hippocampus and cerebellum of the CCK_2 receptor deficient mice.
Number of samples in each group was 6. White bars – cerebellum; striped bars – hippocampus; black bars – frontal cortex. *p<0.05 (compared to wild-type (+/+) mice, Student's t-test).

DISCUSSION

1. Changes in exploratory behaviour: evidence for altered function of the GABAergic system (Papers I–III)

1.1. Exploratory activity and changes in anxiety-related behaviour induced by social isolation

Anxiety-like behaviour in mice was measured by conditioned fear paradigm and classical ethological models of anxiety (elevated plus-maze and light/dark tests). In the elevated plus-maze, female wild-type (+/+) mice displayed significantly higher exploratory activity compared to male mice. This finding is in a good agreement with the study of Johnston and File, who also demonstrated sex differences in exploratory activity (Johnston and File, 1991). The exploratory behaviour established in female wild-type (+/+) mice can be increased as well as decreased by genetic and pharmacological manipulations, whereas in male mice only anxiolytic-like effect can be studied. This was the reason why female mice were chosen for the other tests of anxiety. The results obtained from both ethological models demonstrate that female mice, lacking CCK_2 receptors, display increased exploratory activity. In the elevated plus-maze, homozygous (-/-) animals visited open arms more often and spent longer time on the open arms and central square compared to wild-type (+/+) mice. Daugé et al. (2001b) also observed an increased number of open arm entries in CCK_2 receptor deficient mice, but this effect was not statistically significant. Our study showed that the ratio between open and total arm entries was also increased in homozygous (-/-) mice, but this increase did not reach statistical significance. Locomotor activity, measured as the number of closed arm entries, did not differ in wild-type (+/+) and genetically modified mice.

The exploratory behaviour of CCK_2 receptor deficient mice in the light/dark test was different in two separate experiments. This diversity of behaviour seems to be depending on the baseline exploratory activity of wild-type (+/+) mice. When the exploratory activity of wild-type (+/+) mice was lower, both the number of transitions between compartments and time spent in the light side were significantly increased in homozygous (-/-) mice. However, when the activity of wild-type (+/+) animals was higher, only the increase in number of transitions was statistically significant in CCK_2 receptor deficient mice. Chesler et al. (2002) have proposed that, among other factors, the experimenter seems to be of crucial importance in producing differences. However, we can exclude this influence, because the same person was responsible for handling of mice and performing of experiments in both studies. We have seen in our previous studies that the exploratory behaviour of male Wistar rats can be different in various studies (Kõks *et al.*, 2000). For example, the exploratory activity of rats was higher in winter compared to the study performed in summer. We found

that the variations in exploratory activity were related to the density of CCK_2 receptors in the frontal cortex and hippocampus. Namely, the density of CCK_2 receptors was higher in rats displaying reduced exploratory activity. Therefore, it is possible that the differences in the exploratory activity of wild-type (+/+) mice can also be attributed to the differences in the density of CCK_2 receptors in the forebrain. This assumption seems to be supported by the fact the effect of the genetic invalidation of CCK_2 receptors was stronger in mice displaying reduced exploratory activity. Nevertheless, this suggestion needs further studies to demonstrate that not only in rats, but also in mice the density of CCK_2 receptors in the brain follows seasonal changes and that these changes are related to differences in exploratory activity. Horinouchi et al. (2004) also showed an increased number of transitions between the compartments in mice, lacking CCK_2 receptors, in the light/dark exploration test. It has to be noted that they performed their studies in different conditions compared to our study. Namely, they used a reversed light/dark cycle in the animal house. The exploratory behaviour was measured during the dark phase and in a dark room under red light. Obviously such conditions increase exploratory activity in wild-type (+/+) mice and this is probably the reason why the effect of genetic manipulation on the behaviour was rather small.

The study of behaviour of transgenic mice in fear conditioning test did not reveal any differences between the genotypes. The intensity of freezing of transgenic mice in context- and cue-test did not differ from that of wild-type (+/+) littermates. Studies performed with CCK_2 receptor antagonists in models of anxiety provide controversial evidence. Dawson et al. (1995) did not find any effect if CCK_2 receptor antagonists were studied in exploratory and conditioned models of anxiety. By contrast, other studies have shown an increased exploratory activity in mice after the treatment with CCK_2 receptor antagonists in the light/dark exploration test (Hughes *et al.,* 1990; Costall *et al.,* 1991). Tsutsumi et al. (1999) demonstrated that PD135158, an antagonist of CCK_2 receptors, reversed freezing behaviour in the conditioned fear model. Farook et al. (2004) proposed that various CCK_2 antagonists may not affect the baseline anxiety state, but instead they modulate heightened states of anxiety via CCK_1/CCK_2 receptors. Nevertheless, it seems likely that the role of CCK is more obvious in models where innate mechanisms that regulate exploratory behaviour dominate, whereas in conditioned models of anxiety its significance is rather marginal.

Social isolation has been reported to produce CCK-related anxiety-like behaviour in male rats in the elevated plus-maze (Vasar *et al.,* 1993; Molina-Hernandez *et al.,* 2001). Recent studies with mice show that male and female animals are differently affected by individual housing (Palanza, 2001). Isolated female mice display remarkably lower exploratory activity compared to group-housed females, whereas male mice show the opposite behaviour (Palanza, 2001). Our study confirmed these results showing that social isolation causes a different effect on the exploratory behaviour of female and male mice. Isolated female wild-type (+/+) mice demonstrated remarkably lower exploratory

activity compared to group-housed females. Suppressive effect of social isolation on exploratory behaviour was especially evident if classical measures of anxiety were taken such as the number of open arm entries, and the ratio between open and total arm entries. Isolation induced in male wild-type (+/+) mice a significant increase in the frequency of open arm and closed arm entries, but also decreased significantly the number of attempts to enter the open part. This finding most likely reflects an increased exploratory drive and reduced anxiety in male wild-type (+/+) mice after social isolation, confirming the statements of Palanza (2001). Present study also indicates that female wild-type (+/+) mice display increased anxiety compared to female mice, lacking CCK_2 receptors. Several studies have described differences in the baseline level of anxiety between 129Sv and C57Bl/6 strains. The basal level of anxiety of mice belonging to the 129Sv strain is significantly higher compared to that of C57Bl/6 mice (Contet *et al.,* 2001; Võikar *et al.,* 2001; Rodgers *et al.,* 2002). It has been shown that animals with the dominating 129Sv background develop place preference under the influence of morphine when drugs suppressing anxiety are administered together with morphine (Dockstader and van der Kooy, 2001). Despite backcrossings into the C57Bl/6 background, the influence of genes from the 129Sv background is still strong in our population. Therefore, the high basal anxiety of wild-type (+/+) mice significantly affects their exploratory behaviour. The results of the present study demonstrate that CCK_2 receptor invalidation antagonises the behavioural effects of isolation in female, but not in male mice. Accordingly, CCK_2 receptors play a critical role in the isolation-induced behaviour of female animals.

1.2. The effects of diazepam and DMCM on the behaviour of CCK_2 receptor deficient mice

The action of diazepam, an anxiolytic agonist of benzodiazepine receptors, was studied in the elevated plus-maze and light/dark tests. Both tests showed that the effect of diazepam on exploratory activity of mice differed significantly between the genotypes. It is obvious that the effect of an anxiolytic drug depends on the baseline exploratory activity of animals. In the elevated plus-maze, the baseline activity of homozygous (-/-) mice was higher compared to wild-type (+/+) mice. The same was true about the dark/light exploration test, where genetically modified mice displayed a significantly higher activity. The administration of diazepam (0.5 mg/kg) significantly increased open arm entries in wild-type (+/+) mice in the elevated plus-maze, whereas to get similar an increase in homozygous (-/-) animals we had to inject diazepam at the dose of 1 mg/kg. In the light/dark test, the same low dose of diazepam (0.5 mg/kg) also increased exploratory behaviour in wild-type (+/+), but not homozygous (-/-) mice. Interestingly, this dose of diazepam increased the number of transitions in wild-type (+/+) mice exactly to the level of homozygous (-/-) animals. A further

increase of the dose of diazepam leads to an inhibition of locomotor activity in mice and, therefore, the suppression of locomotor activity masks the anxiolytic action of a drug. This inhibitory effect was very clearly demonstrated by the highest dose of diazepam (3 mg/kg), inducing a strong suppression of exploratory activity in the elevated plus-maze in mice, lacking CCK_2 receptors. Moreover, the administration of diazepam (3 mg/kg) also caused a significantly greater impairment of motor coordination in the rota-rod test in homozygous (-/-) mice compared to their wild-type (+/+) littermates.

The administration of DMCM, an inverse agonist of benzodiazepine receptors, caused opposite changes in the exploratory behaviour of wild-type (+/+) and homozygous (-/-) mice. In wild-type (+/+) animals, an unexpected increase in exploratory activity was established. As mentioned above, the higher basal anxiety level of wild-type (+/+) mice was probably caused by dominating genes of 129Sv strain. This could explain why DMCM increased exploratory activity in wild-type (+/+) mice. Interestingly, Vasar et al. (1993) established a similar unexpected anxiolytic-like action of caerulein, an agonist of CCK, in the male Wistar rats kept in social isolation and displaying, therefore, increased anxiety. By contrast, in CCK_2 receptor deficient mice, the administration of DMCM caused a dose-dependent reduction in exploratory behaviour. Data from the motility boxes showed that the effect of DMCM on the exploratory activity of wild-type (+/+) and homozygous (-/-) mice was not associated with the action of DMCM on the locomotor activity, because DMCM in the studied doses did not influence the motor activity in mice. Altogether these results from pharmacological studies with diazepam and DMCM in the elevated plus-maze and light/dark tests again support the suggestion that wild-type (+/+) mice of 129Sv/C67Bl/6 background display increased anxiety compared to homozygous (-/-) animals.

2. Changes in binding of [^{3}H]-flunitrazepan and expression levels of $GABA_A$ receptor subunit genes (Papers I, III)

Radioligand binding studies revealed an increased binding of benzodiazepine receptors in the cerebellum, but not in the hippocampus and cerebral cortex of CCK_2 receptor deficient mice. The cerebellum has a key role in the regulation of motor coordination (Mason and Sotelo, 1997). Diazepam-induced ataxia in rodents is most probably related to the stimulation of $GABA_A$ receptors located in the cerebellum (Korpi *et al.*, 1999). Accordingly, increased density of benzodiazepine receptors in the cerebellum could be a reason for the increased impairment of motor co-ordination and suppression of locomotor activity established in homozygous (-/-) mice after the administration of diazepam (3 mg/kg). In further experiments, the expression levels of selected $GABA_A$ receptor subunit (α1, α2, and γ2) genes, playing a role in the action of anxiolytic

drugs, were studied in the frontal cortex, hippocampus and cerebellum. These studies revealed a 1.6-fold increase of α2 subunit of $GABA_A$ receptors in the frontal cortex of homozygous (-/-) mice. This subunit mediates the anxiolytic action of diazepam and the genetic invalidation of this gene abolishes this effect of diazepam (Löw *et al.,* 2000; Möhler *et al.,* 2002). We found some increase in the expression of γ2 subunit of $GABA_A$ receptors (1.24-fold) in the frontal cortex, but this was not statistically significant. Still, it is interesting to note that heterozygous (+/-) γ2 subunit deficient mice display increased anxiety in the elevated plus-maze (Crestani *et al.,* 1999). Davidson and Irwin (1999) suggest that the frontal cortex promotes adaptive goals in the face of strong competition from behavioural alternatives that are linked to immediate emotional consequences. Moreover, stressful manipulations with mice and rats increase the release of CCK and the number of CCK_2 receptors in the frontal cortex (Shlik *et al.,* 1997; Becker *et al.,* 2001). Also, an increase of CCK_2 receptor mRNA in the frontal cortex was established in response to the exposure of rats to a cat (Farook *et al.,* 2001). CCK is localised only within GABAergic neurons in the cerebral cortex (Hendry *et al.,* 1984) and, therefore, CCK strongly modulates the activity of these neurons (Ferraro *et al.,* 1999). The lack of CCK_2 receptors leads to a situation where the balancing influence from the side of CCK is lost for GABAergic neurons. This could be a reason why the expression of the α2 subunit of $GABA_A$ receptors is increased in the frontal cortex of mice, lacking CCK_2 receptors. Despite some discrepancies between gene expression and binding data, there is clear evidence about the increased function of GABAergic system in the brain of CCK_2 receptor deficient mice.

3. Concluding remarks and suggestions for further studies

The present study shows that the targeted mutation of the CCK_2 receptor gene induces alterations in the function of GABAergic system. The anatomical finding that CCK is co-localised with GABA in the neurons of the cerebral cortex and hippocampus (Hendry *et al.,* 1984; Kosaka *et al.,* 1985; Cope *et al.,* 2002) and the neurochemical evidence that CCK can induce the release of GABA in the cerebral cortex and hippocampus (Perez de la Mora *et al.,* 1993; Miller *et al.,* 1997; Ferraro *et al.,* 1999) have indicated that these two neurotransmitter systems are in close interaction in certain structures of the brain. In this work, we established that the genetic invalidation of CCK_2 receptors increased the exploratory activity of mice, but did not affect their behaviour in the fear conditioning test. The changes in the action of diazepam and DMCM, compounds having an opposite influence on $GABA_A$ receptors, on the animal behaviour suggest that the activity of the GABAergic system is affected by the genetic invalidation of CCK_2 receptors. Alterations in the activity of the GABAergic system, established in the pharmacological studies, are confirmed by the data from radioligand binding

studies with [^{3}H]-flunitrazepam and expression studies using the genes of $GABA_A$ receptor subunits. An increased density of benzodiazepine receptors in the cerebellum and an increased expression of $GABA_A$ receptor subunit α2 gene are clear indications of an increased function of the GABAergic system in the brain. Altogether, the data of behavioural, pharmacological and neurochemical studies reflect an increased tone of GABAergic system in mice, lacking CCK_2 receptors.

This study also demonstrates that the genetic background of animals has a crucial importance for the anxiety-like behaviour. It has been demonstrated that the baseline anxiety level of the 129Sv strain is significantly higher compared to the C57Bl/6 strain (Võikar *et al.*, 2001, 2004; Holmes *et al.*, 2003). Holmes et al. (2003) have shown that the genetic invalidation of 5-hydroxytryptamine transporter (5-HTT) gene induces different changes in these two backgrounds. The increased anxiety was evident in mice belonging to the C57Bl/6 strain, whereas in animals of 129Sv genetic background this genetic manipulation did not change the behaviour of mice. Therefore, it is clear that the anxiolytic-like effect of genetic invalidation of the CCK_2 receptor gene can be established in animals with dominating genes from the 129Sv background. This explains the discrepancy between our study and the experiments performed by Miyasaka et al. (2002). This diversity of data probably results from the fact that the basal exploratory activity of wild-type (+/+) mice (they performed more than 7 open arm entries per session) in the mentioned study was too high to see any further increase in exploratory activity due to the invalidation of CCK_2 receptors. The anxiolytic-like action of CCK_2 receptor gene invalidation is difficult to establish if the baseline anxiety is low like in the case of C57Bl/6 genetic background.

Our study also indicates that female mice of 129Sv/C57Bl/6 background are more suitable for the study of anxiety compared to their male littermates. This is in good agreement with the experiments performed by Võikar and colleagues (2001). The exploratory activity of female wild-type (+/+) mice in the elevated plus-maze is significantly higher compared to male animals. This behaviour of female mice can be increased and reduced by genetic and pharmacological manipulations, whereas in male mice the effect of anxiolytic-like manipulations can be established. Moreover, social isolation induces opposite effects on the behaviour of male and female mice. In male wild-type (+/+) mice, individual housing increases exploratory activity, whereas in female mice a significant suppression of behaviour is evident. Therefore, stress-induced anxiety-like states can be more easily studied in female animals. The model of social isolation-induced behavioural alterations is a feasible target for our further studies, because the genetic invalidation of CCK_2 receptors antagonised the effect of individual housing. The comparison of wild-type (+/+) and homozygous (-/-) animals helps us to determine the neurochemical networks playing a crucial role in the development of an anxiety-like state due to social isolation.

CONCLUSIONS

1. Female mice, lacking CCK_2 receptors, display increased exploratory activity in the exploratory models of anxiety compared to female wild-type (+/+) mice. The genetic invalidation of CCK_2 receptors does not affect their behaviour in the fear conditioning paradigm.
2. The effect of social isolation on exploratory behaviour differs significantly between male and female wild-type (+/+) mice. Individual housing increases the exploratory activity in male mice, whereas in female mice an anxiety-like state is evident. Targeted mutation of CCK_2 receptors antagonises the effect of social isolation in female, but not in male mice.
3. The anxiolytic-like action of diazepam, an agonist of benzodiazepine receptors, is significantly stronger in female wild-type (+/+) mice compared to their homozygous (-/-) littermates in exploratory models of anxiety. By contrast, DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), an inverse agonist of benzodiazepine receptors, induces an anxiogenic-like action in homozygous (-/-) mice, whereas in wild-type (+/+) mice an unexpected anxiolytic-like effect is evident. These data can be explained in the light of evidence that mice, with dominating genes from 129Sv background, display increased basal anxiety. This is a reason for the increased effectiveness of diazepam and the paradoxical effect of DMCM in wild-type (+/+) mice.
4. The genetic invalidation of CCK_2 receptors elevates the density of benzodiazepine binding sites in the cerebellum. This neurochemical change can be attributed to the increased effect of diazepam on motor coordination in mice, lacking CCK_2 receptors.
5. The targeted mutation of CCK_2 receptors increases the expression of α2 subunit gene of $GABA_A$ receptors, responsible for the anxiolytic action of diazepam, in the frontal cortex. Altogether, the results of behavioural, pharmacological and neurochemical studies reflect the increased tone of GABAergic system in mice, lacking CCK_2 receptors.

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SUMMARY IN ESTONIAN

Muutused GABAergilise süsteemi talitluses koletsüstokiniini teist tüüpi retseptori puudulikkusega hiirtel

Sissejuhatus

Koletsüstokiniin (CCK) on neuropeptiid, mis esineb nii seedetraktis kui ka kesknärvisüsteemis (KNS). Ehkki nimetatud neuropeptiid võib organismis esineda mitme alavormina, domineerib KNS-s kaheksast aminohappest koosnev CCK sulfateeritud vorm -CCK-8s. CCK olulisusest organismis annab tunnistust osalemine mitmesuguste funktsioonide regulatsioonis (toitumine, mälu, valu, õppimine, motivatsioonid, stress). Mitmed loomade ja inimestega tehtud uuringud on näidanud, et CCK mängib tähtsat rolli ärevuse mehhanismides. Nagata ja kaasautorite (1996) poolt loodud CCK_2 retseptori puudulikkusega hiir annab meile uued võimalused selle neuropeptiidi funktsionaalse rolli uurimiseks ajus.

Uuringute eesmärk

Käesoleva töö esimeseks eesmärgiks oli iseloomustada CCK_2 retseptori puudulikkusega hiirte emotsionaalset käitumist. Anatoomilised uuringud on näidanud, et ajukoores ja hipokampuses paikneb CCK ainult γ-aminovõihapet (GABA) sisaldavates neuronites. Elektrofüsioloogilistes uuringutes on leitud, et bensodiasepiinid, mis suurendavad närviülekannet $GABA_A$ retseptorite tasemel, antagoniseerivad CCK erutavat toimet hipokampuse närvirakkudele (Bradwejn ja De Montigny, 1984). Arvestades CCK- ja GABA-ergilise süsteemi võimalikku antagonistlikku interaktsiooni, seati teiseks olulisemaks eesmärgiks selgitada muutusi GABAergilises süsteemis CCK_2 retseptorite geneetilise väljalülitamise mõjul.

Konkreetsemad ülesanded käesoleva töö jaoks olid alljärgnevad:

1. selgitada võimalikke muutusi CCK_2 retseptori puudulikkusega emaste hiirte emotsionaalses käitumises, kasutades selleks uudistamiskäitumise ja tingitud hirmu mudeleid;
2. uurida sotsiaalse isolatsiooni mõju emaste ja isaste homosügootsete (-/-) hiirte uudistamisaktiivsusele;
3. selgitada muutusi bensodiasepiini retseptorite agonisti diasepaami ning bensodiasepiini retseptorite pöördagonisti DMCM-i (metüül-6,7-dimetoksü-4-etüül-β-karboliin-3-karboksülaadi) toimes geneetiliselt modifitseeritud katseloomade uudistamiskäitumisele;
4. määrata bensodiasepiini retseptorite afiinsust ja tihedust CCK_2 retseptori puudulikkusega emaste hiirte suurajukoores, hipokampuses ja väikeajus;

5. määrata $GABA_A$ retseptori α_1, α_2, γ_2 alaühikute geenide ekspressiooni geneetiliselt modifitseeritud katseloomade otsmikukoores, hipokampuses ja väikeajus.

Meetodid

Katseloomadeks olid CCK_2 retseptori puudulikkusega hiired. Algne hiirte liin on saadud Toshimitsu Matsui laboratooriumist (Nagata *et al.*, 1996), kuid katsete tarvis paljundati hiiri kohapeal TÜ Biomeedikumi vivaariumis. Ärevuskäitumise hindamiseks teostati tõstetud pluss-puuri, hele-tumeda puuri ja hirmu tingimise test. Samuti hinnati pluss-puuris sotsiaalse isolatsiooni ja anksiolüütilise toimega diasepaami mõju uudistamiskäitumisele. Hele-tumeda puuri testis uuriti diasepaami ja DMCM-i mõju uudistamiskäitumisele. Loomade motoorset aktiivsust hinnati fotosensoritega varustatud katseseadmes. Hinnati DMCM-i mõju motoorsele aktiivsusele. Motoorse koordinatsiooni hindamiseks kasutati rotarodi testi, kus uuriti katseloomade võimet püsida pöörleval silindril. Selle testiga selgitati diasepaami mõju motoorsele koordinatsioonile.

Radioligandi sidumiskatsetes mõõdeti bensodiasepiini retseptorite afiinsust ja nende tihedust suurajukoores, hipokampuses ja väikeajus. Bensodiasepiini retseptorite märgistamiseks kasutati [^{3}H]-flunitrasepaami ja mittespetsiifilise sidumise määramiseks bensodiasepiini retseptorite agonisti diasepaami.

Geenikspressiooni uuringu puhul määrati $GABA_A$ retseptori α_1, α_2 ja γ_2 alaühikute ekspressiooni taset otsmikukoores, hipokampuses ja väikeajus, kasutades selleks kvantitatiivset reaalaja polümeraasi ahelreaktsiooni (Q-RT-PCR). Kõik reaktsioonid tehti SYBR® Green I Master Mixiga. $\Delta\Delta C_T$ meetodi abil võrreldi uuritava geeni hulka endogeense võrdlusgeeni HPRT kogusega ja arvutati uuritava geeni ekspressioonitase.

Tulemused

Rakendades erinevaid etoloogilisi ärevusmudeleid, pluss-puuri ja hele-tumedat puuri, selgus, et emastel CCK_2 retseptori puudulikkusega hiirtel on suurenenud uudistamisaktiivsus. Katsetest ilmnes, et sotsiaalse isolatsiooni mõju uudistamiskäitumisele on soost sõltuv. Nimelt “metsikut tüüpi” isastel loomadel suurendab 21-päevane isolatsioon uudistamiskäitumist, “metsikut tüüpi” emaste hiirte puhul kutsub üksikpuuri paigutamine esile suurenenud ärevuse. CCK_2 retseptori geneetiline väljalülitamine antagoniseerib sotsiaalsest isolatsioonist tingitud käitumuslikke muutusi emastel, aga mitte isastel hiirtel. Tingitud hirmu mudelis ei õnnestunud ”metsikut tüüpi” ja geneetiliselt modifitseeritud emaste hiirte käitumise võrdlemisel erinevusi leida. Farmakoloogilised uuringud näitasid, et bensodiasepiini retseptorite agonisti diasepaami anksiolüütiline toime avaldub “metsikut tüüpi” hiirtel tugevamini kui geneetiliselt modifitseeritud

pesakaaslastel. Samas avaldab diasepaam CCK_2 retseptori puudulikkusega hiirte puhul oluliselt tugevamat motoorikat pärssivat toimet kui "metsikut tüüpi" liigikaaslaste puhul. Bensodiasepiini retseptorite pöördagonist DMCM põhjustab geneetiliselt modifitseeritud hiirtel doosist sõltuva uudistamisaktiivsuse languse, kuid "metsikut tüüpi" katseloomadel paradoksaalsel viisil suurendab uudistamisaktiivsust. Diasepaami tugevamat anksiolüütilist toimet ning DMCM-i paradoksaalset efekti "metsikut tüüpi" hiirtel võib põhjendada sellega, et neil hiirtel domineerivad ühe vanemliku liini (129Sv liini) geenid, mis tingivad suurema basaalärevuse.

Radioligandi sidumiskatsed ja geenieksпressiooni uuringud kinnitasid farmakoloogiliste uuringute andmeid, mille järgi CCK_2 retseptori geneetiline väljalülitamine põhjustab olulisi muutusi GABAergilises süsteemis. Sidumiskatsetest selgus, et homosügootsetel hiirtel on väikeajus bensodiasepiini retseptorite arv suurenenud. See tulemus aitab meil põhjendada, miks diasepaam pärssis CCK_2 retseptori puudulikkusega hiirtel motoorset aktiivsust tunduvalt enam kui "metsikut tüüpi" pesakaaslastel. Geenieksпressiooni uuringud näitasid, et diasepaami anksiolüütilise toimega seotud $GABA_A$ retseptori $\alpha 2$ alaühiku ekspressioonitase on mutanthiirtel otsmikukoores märkimisväärselt tõusnud. Seega, antud töös tehtud käitumiskatsete ning farmakoloogiliste ja neurokeemiliste uuringute tulemuste põhjal võib väita, et CCK_2 retseptori geeni väljalülitamine põhjustab GABAergilise süsteemi toonuse olulist tõusu KNS-is.

Järeldused

1. CCK_2 retseptori puudulikkusega hiirtel on suurenenud uudistamisaktiivsus tõstetud pluss-puuris ja hele-tume puuris. CCK_2 retseptori geneetiline väljalülitamine ei mõjuta homosügootsete hiirte käitumist tingitud hirmu mudelis.
2. Sotsiaalne isolatsioon avaldab erinevat mõju emaste ja isaste "metsikut tüüpi" hiirte uudistamiskäitumisele. Isoleeritud isastel suureneb uudistamisaktiivsus, kuid emastel hiirtel põhjustab isolatsioon ärevuse suurenemist. CCK_2 retseptori geneetiline väljalülitamine antagoniseerib sotsiaalsest isolatsioonist tingitud käitumuslikke muutusi emastel, aga mitte isastel hiirtel.
3. Bensodiasepiini retseptorite agonisti diasepaami anksiolüütiline toime on "metsikut tüüpi" hiirtel tugevam kui CCK_2 retseptori puudulikkusega hiirtel. Bensodiasepiini retseptorite pöördagonist DMCM kutsub geneetiliselt modifitseeritud hiirtel esile doosist sõltuva uudistamisaktiivsuse languse. "Metsikut tüüpi" katseloomadel avaldab DMCM vastupidist toimet, suurendades uudistamisaktiivsust. Diasepaami ja DMCM-i mõju "metsikut tüüpi" hiirtele saab seletada suuremat basaalärevust põhjustavate 129Sv geenide domineerimisega nende genotüübis.
4. Homosügootsetel hiirtel on suurenenud bensodiasepiini retseptorite arv väikeajus. See neurokeemiline muutus on seostatav diasepaami oluliselt tuge-

vama motoorset koordinatsiooni häiriva toimega CCK_2 retseptori puudulikkusega hiirtel.

5. CCK_2 retseptori geneetiline väljalülitamine suurendab märkimisväärselt $GABA_A$ retseptori α2 alaühiku, mis on seotud diasepaami anksiolüütilise toimega, ekspressiooni otsmikukoores. Käitumiskatsete ning farmakoloogiliste ja neurokeemiliste uuringute tulemuste alusel võib väita, et CCK_2 retseptori geeni väljalülitamine kutsub esile GABAergilise süsteemi toonuse olulise tõusu KNS-is.

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Teadustöö

Teadustöö: CCK_2 retseptori puudulikkusega hiirte emotsionaalse käitumise ja neurokeemiliste muutuste iseloomustamine.