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ORIGINAL INVESTIGATION

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Targeted invalidation of CCK_2 receptor gene induces anxiolytic-like action in light–dark exploration, but not in fear conditioning test

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Abstract *Rationale:* Evidence suggests that γ-aminobutyric acid (GABA) and cholecystokinin (CCK) have opposite roles in the regulation of anxiety. *Objectives:* The aim of our work was to study the behaviour of CCK_2 receptor deficient mice in light–dark exploration and fear conditioning tests. Moreover, the action of diazepam and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), having the opposite effect on $GABA_A$ receptors, was evaluated on the exploratory behaviour in these mice. Expression levels of $GABA_A$ receptor subunit genes were also measured. *Methods:* Light–dark exploration and fear conditioning tests were used to determine changes in anxiety of mice. The action of diazepam (0.5–2 mg/kg i.p.) and DMCM (0.25–1 mg/kg i.p.) was studied in the light–dark box. The effect of DMCM was also evaluated in the motor activity test to demonstrate that its anti-exploratory action was not related to motor suppression. Expression levels of $GABA_A$ receptor subunit genes were determined by means of real-time polymerase chain reaction (qRT-PCR). *Results:* Female mice lacking CCK_2 receptors displayed increased exploratory activity in the light–dark box compared to their wild-type (+/+) littermates. Locomotor activity in the motility boxes and the intensity of freezing did not differ in wild-type (+/+) and homozygous (−/−) mice. Treatment with diazepam (0.5 mg/kg) increased the number of transitions in wild-type (+/+) animals, whereas in homozygous (−/−) mice diazepam (0.5–2 mg/kg) reduced exploratory activity. Administration of DMCM (0.25–1 mg/kg) induced an anxiogenic-like effect in homozygous (−/−) mice, but did not change their locomotor activity. Gene expression analysis established a 1.6-fold increase in the expression of the α2 subunit of $GABA_A$ receptors in the frontal cortex of homozygous (−/−) mice. *Conclusion:* Genetic invalidation of CCK_2 receptors induced an anxiolytic-like action in exploratory, but not in conditioned models of anxiety. The observed reduction in anxiety in homozygous (−/−) mice is probably related to an increased function of GABAergic system in the brain.

Keywords CCK_2 receptors · Transgenic animals · $GABA_A$ receptors · Diazepam · DMCM · Exploratory activity · Light–dark exploration test · Freezing · Fear conditioning test · Anxiety · Gene expression · qRT-PCR

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Introduction

Cholecystokinin (CCK) is a neuropeptide widely distributed in the brain. Several studies suggest that CCK is implicated in the regulation of anxiety. Fekete et al. (1984) were the first to demonstrate the anxiogenic potential of neuronal CCK on the basis of animal experiments. CCK receptor agonists inhibit exploratory behaviour of mice and rats in the elevated plus-maze test, decrease the time spent and exploratory activity in the light compartment of the light–dark compartment test, and support acquisition and retention in fear-motivated tests (Harro et al. 1993; Shlik et al. 1997). The anxiogenic-like action of CCK agonists is mediated via CCK_2 receptors (Shlik et al. 1997; Nobel et al. 1999; Hernandez-Gomez et al. 2002). Also, rats selected according to their anxiety-like behaviour in the elevated plus-maze display an increased density of CCK_2

receptors in the forebrain structures compared to animals having reduced anxiety (Kõks et al. 1997). Anxiogenic manipulations with rodents increase the density of CCK_2 receptors in the frontal cortex (Vasar et al. 1993; Shlik et al. 1997).

Recently, mice with targeted disruption of CCK_2 receptor gene have been generated (Nagata et al. 1996). Animals without CCK_2 receptors display disturbances in the development of gastric mucosa (Nagata et al. 1996) and in learning abilities (Sebret et al. 1999). The activity of dopaminergic system is also affected in mice with corrupted function of CCK_2 receptors. An increased sensitivity of dopamine D_2 receptors was established in these mice (Daugé et al. 2001; Kõks et al. 2001). Recent evidence suggests altered function of the endogenous opioid system in mice without CCK_2 receptors. Pommier et al. (2002) demonstrated that mice without CCK_2 receptors display hyperalgesia and a reduced response to morphine-induced analgesia in the hot-plate test. Our previous study established that female mice lacking CCK_2 receptors had reduced anxiety in the elevated plus-maze and the anxiolytic-like action of diazepam is weaker in mutant mice compared to their wild-type (+/+) littermates (Raud et al. 2003). The present study was designed to analyse further the exploratory behaviour of female mice without CCK_2 receptors in the dark–light box exploration paradigm. CCK_2 receptor antagonists are shown to be ineffective in conditioned fear tests (Dawson et al. 1995). Therefore, the behaviour of homozygous (−/−) and wild-type (+/+) animals was also studied in the fear conditioning test.

The initial suggestion that CCK interacts with γ-aminobutyric acid (GABA) in the regulation of anxiety came from the experiments performed by Bradwejn and de Montigny (1984). They demonstrated that benzodiazepine receptor agonists could attenuate CCK-induced excitation of rat hippocampal neurons. Several studies have shown that CCK is localized in 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). Administration of CCK_2 receptor antagonists reverses the signs of diazepam withdrawal in rodents (Singh et al. 1992; Rasmussen et al. 1993). Taking into account the probable antagonistic interaction between GABA and CCK, we studied the effect of diazepam, a benzodiazepine agonist, interacting with the different subunits of $GABA_A$ receptors (Möhler et al. 2002), on the exploratory activity of CCK_2 receptor deficient mice in the light–dark exploration test. Also the action of anxiogenic beta-carboline DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), an inverse agonist of benzodiazepine receptors, was studied on the exploratory behaviour of CCK_2 receptor deficient mice. The behavioural effects of diazepam are mediated via α1 and α2 subunits of $GABA_A$ receptors (Möhler et al. 2002), whereas γ2 subunit is responsible for the action of zolpidem and heterozygous (+/−) γ2 subunit deficient mice display increased anxiety (Crestani et al. 1999; Cope et al. 2004). Therefore, the expression levels of α1, α2 and γ2 subunits of $GABA_A$ receptors were studied in the frontal cortex, hippocampus and cerebellum of wild-type (+/+) and homozygous (−/−) mice.

Materials and methods

Animals

Nagata et al. (1996) generated CCK_2 receptor deficient mice by replacing a part of exon 2, and exons 3, 4 and 5. Breeding and genotype analysis were performed at the Department of Physiology of the 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 performing 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. In our previous experiments we found that female mice from the 129Sv/C57Bl/6 background showed reduced aversion to the open arms compared to male mice (Raud et al. 2003; Abramov et al. 2004). This was a main reason why female mice were used throughout the present study. Wild-type (+/+) and CCK_2 receptor deficient mice were used in behavioural and gene expression studies. Genetically modified mice were crossed back six times to the C57BL/6 background to minimize possible genetic influence 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 h). Tap water and food pellets were available ad libitum. All animal procedures were approved by the Animal Care Committee of the University of Tartu in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

Behavioural experiments

The animals were brought into the experimental room 1 h before the experiment. All behavioural experiments were performed between 1100 and 1900 h. The light–dark box exploration, fear conditioning and locomotor activity were studied in separate groups of animals. The animals were used in the behavioural experiments only once. Since behavioural experiments lasted 6–8 h, precautions were taken to control for possible daily fluctuations in the exploratory behaviour of animals. Therefore, the experiments were always performed in randomized order, that is, wild-type (+/+) mice were used in parallel with their genetically modified littermates.

Light–dark exploration test

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. The experiments were carried out in a lit room (illumination ~500 lux). As mentioned above CCK and GABA systems have antagonistic interaction in the regulation of anxiety. In order to reveal the functional changes in GABA system, due to the genetic invalidation of CCK_2 receptors, two drugs having opposite effects on $GABA_A$ receptors were used. These drugs are diazepam, stimulating via the subtypes of $GABA_A$ receptors the activity of GABA system, and DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), an anxiogenic β-carboline, inducing the behavioural effects opposite to diazepam. Diazepam (Sigma, 0.5 mg/kg, 1 mg/kg, 2 mg/kg) was administered intraperitoneally (i.p.) 30 min before the study and the animals were placed singly into the cages. Diazepam was suspended in physiological saline (0.9% of sodium chloride solution) with the help of a few drops of Tween 80 (Sigma). DMCM (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) was injected i.p. 15 min before the study and after the injection mice were placed separately into the cages. DMCM was dissolved in 0.25 ml of 0.2 M HCl, neutralized with 0.05 ml of 1 M NaOH and then diluted with physiological saline. Light–dark exploration test is an unconditioned test of anxiety-like behaviour designed for mice (Crawley and Goodwin 1980). The Plexiglas box (45×20× 20 cm) was divided into two parts: 2/3 was brightly illuminated (~500 lx) 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 (13×5 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 was 300 s).

Fear conditioning (FC)

This is a form of classical conditioning where a simple association of a conditioned stimulus (tone, CS) with an electric foot shock was analysed. The study was performed according to the method described by 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 experiment the animals were placed into single cages 30 min before the test. Training was conducted in a transparent acrylic chamber (110×160×160 mm/110×135×155 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 lx). 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 [conditioned stimulus (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 s) was measured using the standard interval sampling procedure every 10 s. Four hours later, the mouse was tested for its freezing behaviour to the auditory CS. Testing was performed in a different acrylic chamber (220×160×160 mm/220×135×155 mm) the floor of which was covered with white cardboard. The background colour was black. Duration of the test was 6 min: 3 min without the tone (pre-CS phase) and 3 min 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.

Locomotor activity

The purpose of locomotor activity test is to prove that the changes in the exploratory activity of CCK_2 receptor deficient mice and the anti-exploratory action of DMCM are not related to the changes in the locomotor activity. For the measurement of locomotor activity animals, not exposed previously to motility boxes, were used. In the beginning of the study the animals were placed individually into photoelectric motility boxes (448×448×450 mm) connected to a computer [Technical and Scientific Equipment (TSE), GmBH, Germany]. The illumination level of the transparent test boxes was ~500 lx. After removing the mouse from the box the floor was cleaned with a 5% alcohol solution. Time in movement (s), total distance travelled (m), and the number of rearings and corner entries were registered during the 30-min observation period. The locomotor effects of DMCM (0.25, 0.5 and 1 mg/kg) were studied in wild-type (+/+) and homozygous (−/−) mice. Similarly to the dark/light exploration test, DMCM was injected i.p. 15 min before the experiment. In this experiment, no prior handling or adaptation of animals to the experimental environment were used.

Table 1 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′

Dissection of brain structures, RNA isolation and cDNA synthesis

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 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 synthesized, using First Strand cDNA Synthesis Kit (Fermentas).

Gene expression studies with quantitative real-time PCR (qRT-PCR)

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 coming 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 used. For that purpose ABI PRISM 7700 Sequence Detection System equipment (PE Applied Biosystems, USA) and ABI PRISM 7000 SDS Software were used. In all quantification experiments hypoxanthineguanine phos-

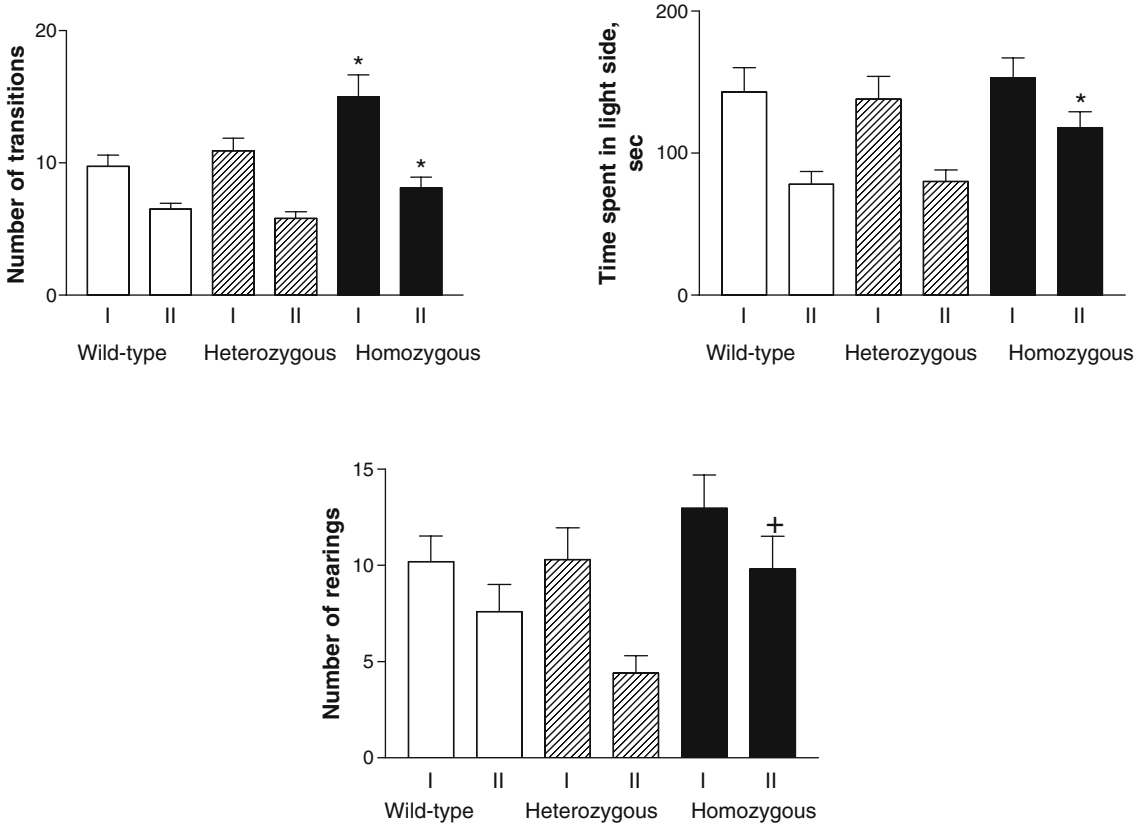

Fig. 1 The exploratory behaviour of CCK_2receptor deficient mice in the light–dark exploration test. The results of two different experiments (I and II) are presented. Number of mice in the first experiment was as follows: wild-type (+/+) 32, heterozygous (+/−) 33 and homozygous (−/−) 26. In the second 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 (+/05 (compared to heterozygous−) mice)

Table 2 The freezing behaviour of CCK_2 receptor deficient mice in the fear conditioning test

Genotype	Freezing, %		
	Context test	Novelty test	Cue test
Wild type (+/+)	37±4	9±3	48±3
4gous (+/−)	52±5	5±3	54±5
Homozygous (−/−)	48±7	1±1	55±6

The number of mice in each group was following: wild type (+/+) 27, heterozygous (+/−) 29 and homozygous (−/−) 26

phoribosyl 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 $\alpha1$, $\alpha2$, and $\gamma2$ subunits are presented in Table 1. All reactions were performed by using SYBR Green I Master Mix (Roche, USA). Instructions of the equipment and reagent manufactures 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 is shown as the fold increase.

Statistics

The results are expressed as mean values ± SEM. The behavioural studies were analysed using one- or two-way analysis of variance. Post hoc comparisons between the individual groups were performed by means of the Newman–Keuls test, using Statistica for Windows software. Student's t-test was applied for the analysis of gene expression data.

Results

Light–dark exploration test

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. The application of two-way ANOVA estab-

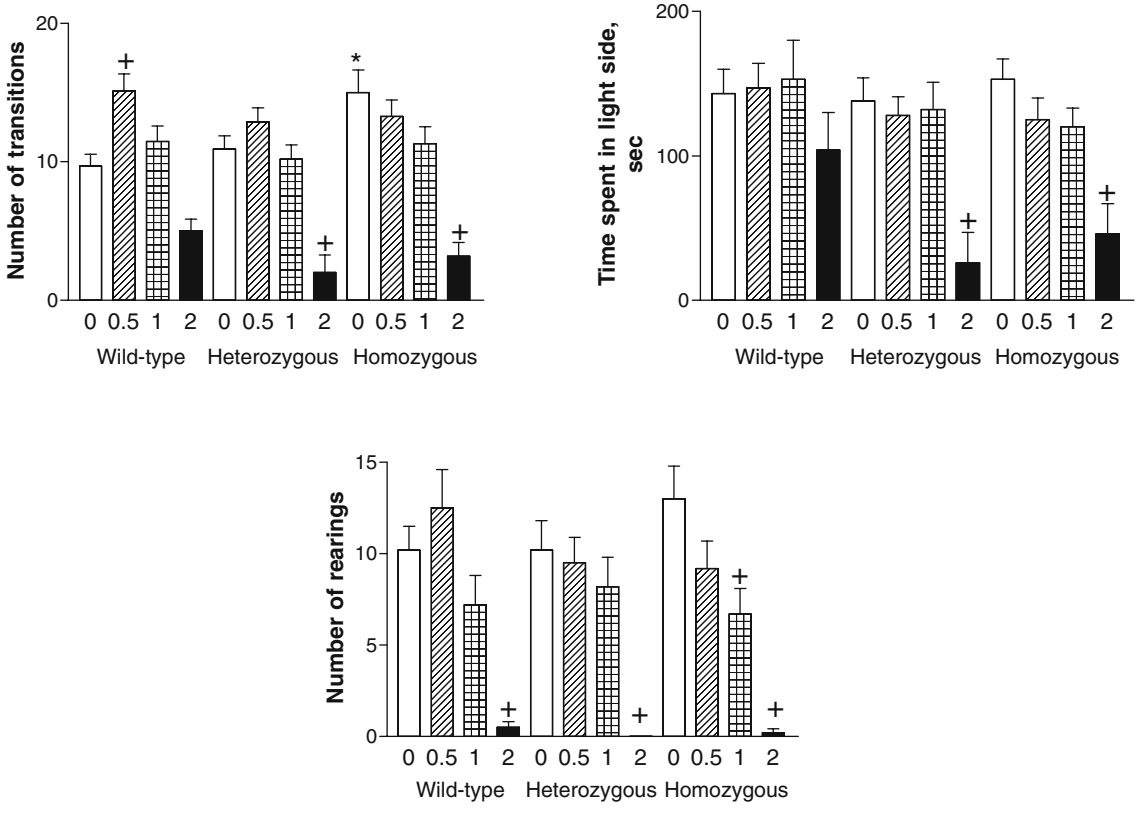

Fig. 2 The effect of diazepam (0.5–2 mg/kg) on the exploratory behaviour of CCK_2 receptor deficient mice in the light–dark exploration test. The number of animals in each group was between 23 and 27. White bars-vehicle treated mice; striped bars-diazepam 0.5 mg/kg; hatched bars-diazepam 1 mg/kg; black 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)

lished the significant differences between two studies. For the number of transitions both genotype- ($F_{2,156}$=7.1, p< 0.01) and experiment-dependent ($F_{1,156}$=39.9, p<0.01) differences were evident, whereas the effect of genotype was not different in these two experiments ($F_{1,156}$=1.67, p= 0.19). For the time spent in light compartment only the experiment-dependent change was established (genotype $F_{2,156}$=2.1, p=0.12; experiment $F_{1,156}$=20.9, p<0.01; genotype x experiment $F_{1,156}$=0.64, p=0.53). For the number of rearings the genotype- and experiment-dependent effects were significant (genotype $F_{2,156}$=3.49, p<0.05; experiment $F_{1,156}$=10.2, p<0.01; genotype x experiment $F_{1,156}$=0.68, p= 0.51). The post hoc analysis revealed that two groups of wild-type (+/+) mice differed significantly by the number of transitions (Newman–Keuls test, p<0.05) and time spent in light compartment (p<0.01) in these experiments, whereas difference in the number of rearings was not statistically significant. Despite the significant differences between two experiments, in both cases the exploratory activity of CCK_2 receptor deficient mice was significantly increased compared to wild-type (+/+) littermates (Fig. 1). In the first study the frequency of transitions performed by homozygous (−/−) mice was significantly elevated compared to heterozygous (+/−) and wild-type (+/+) mice ($F_{2,88}$=5.48; p<0.01), whereas the other parameters did not differ between the genotypes. The difference in transitions was significant, as revealed by post hoc analysis, between wild-type (+/+) and homozygous (−/−) mice (Newman–Keuls test, p<0.05). In the second experiment, all the parameters of exploratory behaviour of homozygous (−/−) mice were different from that of wild-type (+/+) and heterozygous (+/−) littermates ($F_{2,68}$=3.67, p<0.05 for number of transitions; $F_{2,68}$=5.48, p<0.01 for time spent in the light compartment; $F_{2,68}$=4.19, p<0.05 for number of rearing). The application of post hoc analysis (Newman–Keuls test) established that the frequency of transitions was statistically higher in homozygous (−/−) mice compared with heterozygous (+/−) and wild-type (+/+) animals (Fig. 1). Time spent in the light compartment was significantly higher in homozygous (−/−) mice compared to their wild-type (+/+) and heterozygous (+/−) littermates (p<0.01). The number of rearings was also higher in homozygous (−/−) mice, but this difference was significant only if homozygous (−/−) and heterozygous (+/−) were compared (p<0.05).

Fear conditioning test

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

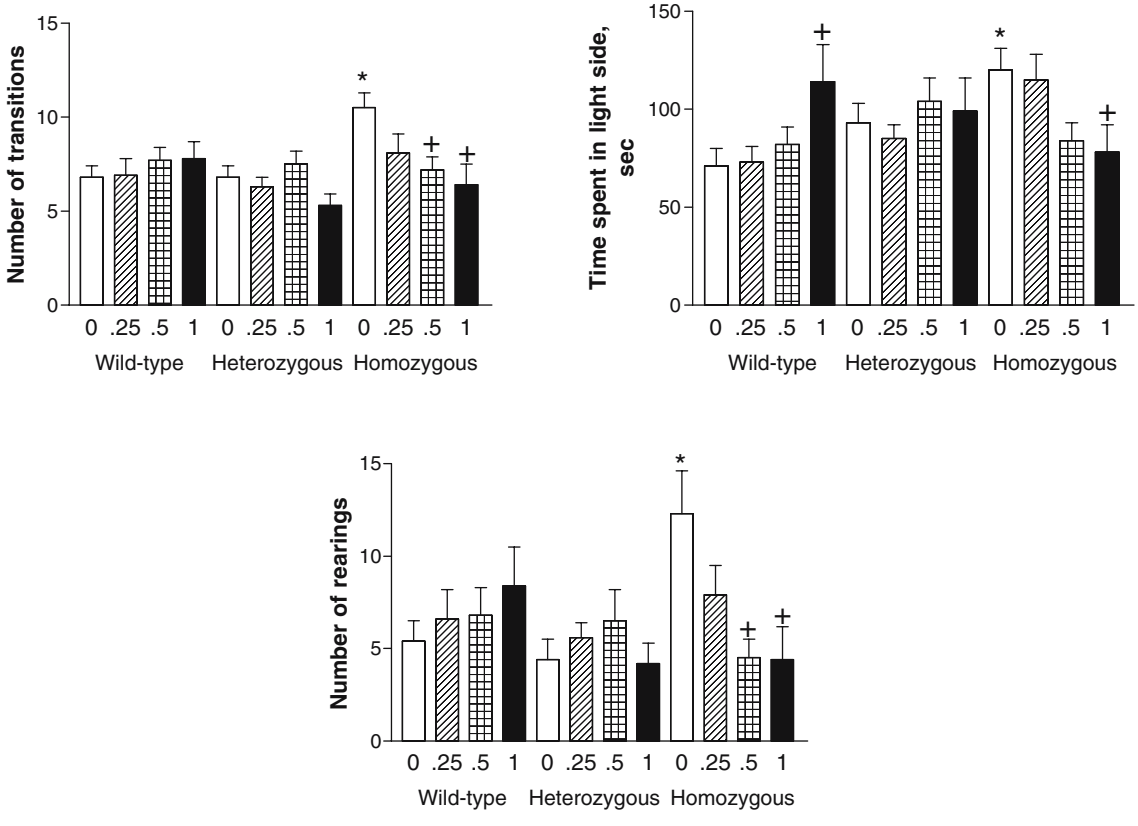

Fig. 3 The effect of DMCM (0.25–1 mg/kg) on the exploratory behaviour of CCK_2 receptor deficient mice in the light–dark exploration test. The number of animals in each group was between 14 and 16. White bars-vehicle treated mice; striped bars-DMCM 0.25 mg/kg; hatched bars-DMCM 0.5 mg/kg; black bars-DMCM 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)

(+/+) 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 ($F_{2,79}$=1.3, p=0.28) (Table 2). 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 ($F_{2,79}$=0.6, p=0.58).

Effect of diazepam and DMCM on the exploration in the light–dark test

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 (Fig. 2). In wild-type (+/+) mice the lowest dose of diazepam (0.5 mg/kg) increased the number of transitions between two compartments, whereas in genetically modified mice the highest dose of diazepam (2 mg/kg) caused a significant suppression of transitions (two-way ANOVA; genotype: $F_{2,292}$=1.81, p=0.16; treatment: $F_{3,292}$=23.2, p<0.01; genotype vs treatment: $F_{6,292}$=2.01, p=0.064). The highest dose of diazepam also decreased time spent in light compartment by heterozygous (+/−) and homozygous (−/−) mice (two-way ANOVA; genotype: $F_{2,292}$=2.76, p=0.065; treatment: $F_{3,292}$=7.78, p<0.01; genotype vs treatment: $F_{6,292}$=0.72, p=0.63). The number of rearings was inhibited by the highest dose of diazepam in all groups (two-way ANOVA: $F_{2,292}$=0.14, p=0.87; treatment: $F_{3,292}$=16.4, p<0.01; genotype vs treatment: $F_{6,292}$=0.79, p=0.58) (Fig. 2).

Treatment with DMCM (0.25–1 mg/kg) induced an anxiogenic-like action in homozygous (−/−) mice (Fig. 3). 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 (two-way ANOVA, $F_{2,164}$=4.02, p<0.05; treatment: $F_{3,164}$=1.36, p=0.11; genotype vs treatment: $F_{6,164}$=2.47, p<0.05). As for time spent in the light compartment, treatment with DMCM also had different effects on different genotypes (two-way ANOVA, genotype: $F_{2,164}$=0.26, p=0.26; treatment: $F_{3,164}$=0.17, p=0.92; genotype vs treatment: $F_{6,164}$=3.09, p<0.01). In wild-type (+/+) mice an unexpected increase of time spent in light part occurred after the treatment with DMCM (Fig. 3). In homozygous (−/−) mice the administration of DMCM caused an anxiogenic-like response by reducing time spent in the light compartment in a dose-dependent manner. As for rearings, the action of DMCM

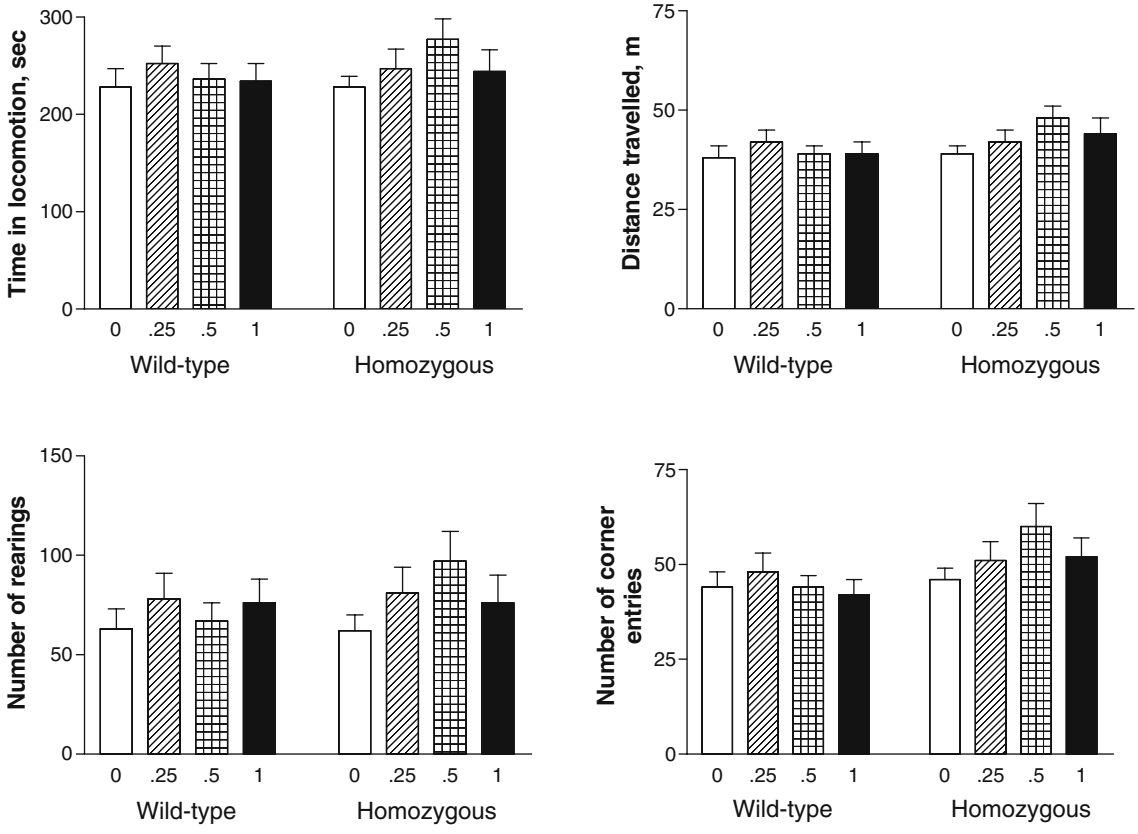

Fig. 4 The effect of DMCM (0.25–1 mg/kg) on the locomotor activity of CCK_2 receptor deficient mice in the motility boxes. The number of animals in each group was 14 or 15. White bars-vehicle-treated mice; striped bars-DMCM 0.25 mg/kg; hatched bars-DMCM 0.5 mg/kg; black bars-DMCM 1 mg/kg

was again different in various genotypes (two-way ANOVA, genotype: $F_{2,164}$=2.05, p=0.13; treatment: $F_{3,164}$=0.77, p= 0.51; genotype vs treatment: $F_{6,164}$=3.10, p<0.01). The administration of DMCM tended to increase the frequency of rearing in wild-type (+/+) mice, but the effect was not significant. However, in homozygous (−/−) mice DMCM induced a dose-dependent inhibition of rearing (Fig. 3).

Effect of DMCM on locomotor activity

The administration of DMCM (0.25–1 mg/kg) did not change the locomotor activity of wild-type (+/+) and homozygous (−/−) mice (Fig. 4). Time in locomotion was not influenced if the effect of DMCM was compared in wild-type (+/+) and homozygous (−/−) mice (two-way ANOVA, genotype: $F_{1,108}$=0.78, p=0.38; treatment: $F_{3,108}$= 0.96, p= 0.41; treatment vs genotype: $F_{3,108}$=0.61, p= 0.063). Distance travelled was not affected either by the treatment with DMCM (two-way ANOVA, genotype: $F_{1,108}$=3.51, p= 0.063; treatment: $F_{3,108}$=1.20, p=0.31; treatment vs genotype: $F_{3,108}$=1.04, p=0.38). Number of rearings (two-way ANOVA, genotype: $F_{1,108}$=0.92, p=0.34; treatment: $F_{3,108}$= 1.10, p=0.35; treatment vs genotype: $F_{3,108}$=0.75, p=0.52) and corner entries (two-way ANOVA, genotype: $F_{1,108}$= 6.16, p<0.05; treatment: $F_{3,108}$=0.91, p=0.44; treatment vs genotype: $F_{3,108}$=1.22, p=0.31) also remained unchanged (Fig. 4).

Expression levels of GABA-related genes in the brain structures

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 (Fig. 5). 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. The expression of γ2 subunit was increased 1.24-fold in the frontal cortex, but this change was not statistically significant.

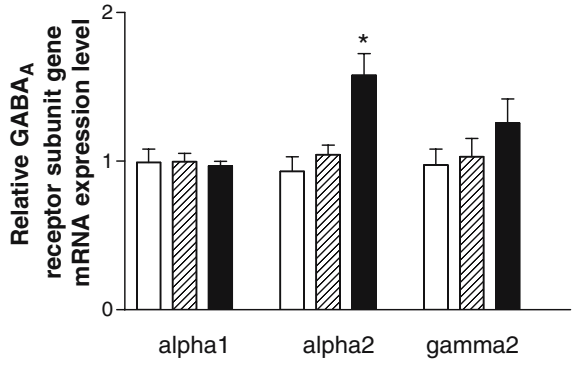

Fig. 5 Relative $GABA_A$ receptor subunit (α1, α2, and γ2) genes mRNA expression levels in the frontal cortex, hippocampus and cerebellum of the CCK_2 receptor deficient mice. Number of samples in each group was 6. White bars-cerebellum; striped bars-hippocampus; black bars-frontal cortex. * -p<0.05 (compared to wild-type (+/+) mice, Student's t-test)

Discussion

The results of the present study demonstrate that female mice lacking CCK_2 receptors display increased exploratory activity in the dark–light test. It is worthy to stress that the locomotor activity of wild-type (+/+) and homozygous (−/−) mice did not differ in the motility boxes. Therefore, the established increase in exploratory activity is probably due to reduced anxiety in mice without CCK_2 receptors. The anxiolytic action of genetic invalidation of CCK_2 receptors is not robust, but it is comparable to the action of diazepam (0.5 mg/kg) in wild-type (+/+) mice. The established effects are in accordance with our previous studies where female CCK_2 receptor deficient mice demonstrated reduced anxiety in the elevated plus-maze (Raud et al. 2003; Abramov et al. 2004). However, it is clear that the effect of genetic invalidation of CCK_2 receptors also depends on the baseline exploratory activity of wild-type (+/+) mice. Indeed, we found that the exploratory behaviour of wild-type (+/+) mice apparently differs in various studies. 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 increased in homozygous (−/−) mice. However, when the activity of wild-type (+/+) animals was higher, only the number of transitions was elevated in CCK_2 receptor deficient mice. Horinouchi et al. (2004) also showed an increased number of transitions between the compartments in mice lacking CCK_2 receptors in the dark–light exploration test. It has to be noted that they performed their studies in different conditions compared to the present study. Namely, they used a reversed dark–light cycle in the animal house. The exploratory behaviour was measured during the dark phase and in a dark room under the red light. Obviously such conditions increase exploratory activity of wild-type (+/+) mice and this is a reason why the effect of genetic manipulation on the behaviour was rather small. Moreover, we established rather big difference between two different studies. Indeed, these differences established in wild-type (+/+) mice were statistically significant if the number of transitions and time spent in light compartment were measured. 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 (Kõks et al. 2000). For example, the exploratory activity of rats was different in summer and winter. The rats were significantly more active in winter compared to the study performed in summer. We found that the established differences in the exploratory activity of rats were related to the density of CCK_2 receptors in the frontal cortex and hippocampus. Namely, the density of CCK_2 re-

ceptors was higher in rats displaying the reduced exploratory activity. Therefore, it is possible that differences in the exploratory activity of wild-type (+/+) mice can be also attributed to the differences in the density of CCK_2 receptors in the brain. This assumption seems to be supported by the fact that the effect of genetic invalidation of CCK_2 receptors was stronger if wild-type (+/+) mice displayed the reduced exploratory activity. Nevertheless, this statement needs the further studies to demonstrate that not only in rats, but also in mice the density of CCK_2 receptors in the brain follows the seasonal changes and these changes are related to the differences in the exploratory activity.

The study of behaviour of transgenic mice in fear conditioning test does 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 the models of anxiety provide controversial evidence. Dawson et al. (1995) did not find any effect if CCK_2 receptor antagonists were studied in the exploratory and conditioned models of anxiety. By contrast, other studies have shown an increased exploratory activity of mice after the treatment with CCK_2 receptor antagonists in the dark–light 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. Nevertheless, it seems likely that the role of CCK in various models of anxiety is uneven. The role of CCK is more obvious in the exploratory models, whereas its significance is limited in the conditioned models of anxiety.

The administration of diazepam, an anxiolytic agonist of benzodiazepine receptors, increased at a low dose (0.5 mg/kg) the exploratory activity of wild-type (+/+) mice, whereas the highest dose induced an opposite effect. A low dose of diazepam increased the exploratory activity of wild-type (+/+) mice exactly to the level of homozygous (−/−) mice. It is important to stress that the same dose of diazepam (0.5 mg/kg) also induced a significant increase in the exploratory activity of wild-type (+/+) mice in the elevated plus-maze (Raud et al. 2003). Chaouloff et al. (1999) suggested that the light–dark exploration test is a less sensitive model, compared to the elevated plus-maze, to reveal the anxiolytic properties of diazepam. The performance of mice in the dark–light test depends not only on the level of anxiety, but also on the locomotor activity of mice. A further increase of the dose of diazepam leads to an impairment of locomotor activity in mice and, therefore, the inhibition of locomotor activity also masks the anxiolytic action of the drug. As a matter of fact, the highest dose of diazepam (2 mg/kg) induced a significant reduction of rearings in wild-type (+/+) mice. In mice lacking CCK_2 receptors the lowest dose of diazepam (0.5 mg/kg) did not significantly affect the exploratory activity in the dark–light test, but starting from the dose of 1 mg/kg suppressed the frequency of rearings in mice. This finding can be explained in the light of our previous study demonstrating that this dose of diazepam caused a significant impairment in the rotarod test in homozygous (−/−), but not in wild-type (+/+) mice (Raud et al. 2003). This is probably a reason why we were not able to see the anxiolytic action of diazepam in genetically modified animals. The present study also supports the finding of our previous experiments (Raud et al. 2003) where wild-type (+/+) mice of 129Sv/C67Bl/6 background displayed increased sensitivity to the anxiolytic action of diazepam compared to homozygous (−/−) animals. This effect can be attributed to the increased anxiety established in wild-type (+/+) animals if their behaviour was compared to their genetically modified littermates. It is obvious that the anxiolytic action of drugs or genetic manipulations can be achieved more easily in animals with an increased anxiety. Use of animals with a reduced anxiety may therefore cause problems in this respect. It is obvious that mice of C57Bl/6 and 129Sv strains differ by their basal anxiety level. 129Sv mice are much more anxious compared to the C57Bl/6 strain (Võikar et al. 2001, 2004). Therefore, the backcrossing of mice into the C57Bl/6 background may mask the anxiolytic action of a drug or genetic manipulation, especially, if this effect is not robust. This may be a reason why Miyasaka et al. (2002) showed a reduction of exploratory behaviour in CCK_2 receptor deficient mice. The basal exploratory activity of wild-type (+/+) mice (they performed more than seven open arm entries per session) in this study was too high to see any further increase in exploratory activity due to the invalidation of CCK_2 receptors.

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. This can be explained in the light of evidence that the basal anxiety of mice of 129Sv strain is significantly higher compared to C57Bl/6 mice (Contet et al. 2001; Rodgers et al. 2002; Võikar et al. 2001). 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. We 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 an increased anxiety (Vasar et al. 1993). By contrast, in CCK_2 receptor deficient mice the administration of DMCM caused a dose-dependent reduction of exploratory behaviour. The effect of DMCM on the exploratory activity of wild-type (+/+) and homozygous (−/−) mice is not associated with the action of DMCM on the locomotor activity, because DMCM in the studied doses does not influence the locomotor activity in mice. Accordingly, the administration of DMCM selectively affects anxiety in these mice and the effect of inverse agonist of benzodiazepine receptors on the exploratory behaviour of mice depends on the basal anxiety of animals. As discussed above, genetic background is an important factor for achieving anxiogenic or anxiolytic effect due to genetic

manipulation. Holmes et al. (2003) demonstrated that the genetic invalidation of 5-HT transporter gene induces an anxiety-like state in the C57Bl/6, but not in the 129Sv strain.

In the previous study we established an increased binding of benzodiazepine receptors in the cerebellum, but not in the hippocampus and cerebral cortex of CCK_2 receptor deficient mice (Raud et al. 2003). In the present study we analysed the expression levels of selected $GABA_A$ receptor subunit ($\alpha1$, $\alpha2$, and $\gamma2$) genes, that play a role in the action of anxiolytic drugs, in the frontal cortex, hippocampus and cerebellum. Generally the gene expression levels in homozygous (−/−) mice did not differ from that of wild-type (+/+) mice. However, there was a 1.6-fold increase in the $\alpha2$ subunit of $GABA_A$ receptors in the frontal cortex of CCK_2 receptor deficient mice. It has been shown that this subunit mediates the anxiolytic action of diazepam and that 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 $\gamma2$ 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 (+/−) $\gamma2$ subunit deficient mice display an 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. It is interesting to note that 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 noted in response to the exposure of rats to a cat (Farook et al. 2001). CCK is localized 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 $\alpha2$ subunit of $GABA_A$ receptors is increased in the frontal cortex of mice lacking CCK_2 receptors. Despite some discrepancies between the 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.

In conclusion, the present study supports the data from our previous experiments (Raud et al. 2003) that genetic invalidation of CCK_2 receptors reduces anxiety in mice of 129Sv/C57Bl/6 background. The data obtained from the pharmacological and gene expression studies suggest an increased tone of GABAergic system in the brain of female mice lacking CCK_2 receptors. The increased activity of GABAergic system could be a reason, at least partly, for the reduced anxiety in mice with corrupted CCK_2 receptors.

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