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ORIGINAL INVESTIGATION

Sirli Raud · Kertu Rünkorg · Alar Veraksitš · Ain Reimets · Aleksei Nelovkov · Urho Abramov · Toshimitsu Matsui · Michel Bourin · Vallo Volke · Sulev Kõks · Eero Vasar

Targeted mutation of CCK_2 receptor gene modifies the behavioural effects of diazepam in female mice

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Abstract *Rationale:* Evidence suggests that GABA and CCK have opposite roles in the regulation of anxiety. *Objective:* The aim of the present work was to study diazepam-induced anxiolytic-like action and impairment of motor co-ordination, and the parameters of benzodiazepine receptors in mice lacking CCK_2 receptors. *Methods:* The action of diazepam (0.5–3 mg/kg IP) was studied in the elevated plus-maze model of anxiety and rotarod test using mice lacking CCK_2 receptors. The parameters of benzodiazepine receptors were analysed using [^{3}H]-flunitrazepam binding. *Results:* In the plus-maze test, the exploratory activity of the homozygous (–/–) mice was significantly higher compared to their wild-type (+/+) littermates. However, the wild-type (+/+) mice displayed higher sensitivity to the anxiolytic-like action of diazepam. Even the lowest dose of diazepam (0.5 mg/kg) induced a significant increase of open arm entries in the wild-type (+/+) mice. A similar effect in the homozygous (–/–) mice was established after the administration of diazepam 1 mg/kg. The highest dose of diazepam (3 mg/kg) caused a prominent anxiolytic-like effect in the wild-type (+/+) mice, whereas in the homozygous (–/–) animals suppression of locomotor activity was evident. The performance of the homozygous (–/–) mice in the rotarod test did not differ from that of the wild-type (+/+) littermates. However, a difference between the wild-type (+/+) and homozygous (–/–) animals became evident after treatment with diazepam. Diazepam (0.5 and 3 mg/kg) induced significantly stronger impairment of motor co-ordination in the homozygous (–/–) mice compared to their wild-type (+/+) littermates. The density of benzodiazepine binding sites was increased in the cerebellum, but not in the cerebral cortex and hippocampus, of the homozygous (–/–) mice. *Conclusions:* Female mice lacking CCK_2 receptors are less anxious than their wild-type (+/+) littermates. The reduced anxiety in homozygous (–/–) mice probably explains why the administration of a higher dose of diazepam is necessary to induce an anxiolytic-like action in these animals. The highest dose of diazepam (3 mg/kg) induced significantly stronger suppression of locomotor activity and impairment of motor co-ordination in the homozygous (–/–) mice compared to the wild-type (+/+) littermates. The increase in the action of diazepam is probably related to the elevated density of benzodiazepine receptors in the cerebellum of homozygous (–/–) mice. The present study seems to be in favour of increased tone of the GABAergic system in mice without CCK_2 receptors.

Keywords Targeted mutagenesis · Wild-type · Heterozygous · Homozygous · Benzodiazepine receptors · GABA · Diazepam · Cholecystokinin · Cholecystokinin$_2$ receptors · Rotarod test · Motor co-ordination · Elevated plus-maze · Exploratory behaviour · Anxiety

S. Raud · K. Rünkorg · A. Veraksitš · A. Reimets · A. Nelovkov · U. Abramov · V. Volke · S. Kõks (✉) · E. Vasar
Department of Physiology, Biomedicum,
University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia
e-mail: Sulev.Koks@ut.ee
Tel.: +372-7-374335
Fax: +372-7-374332

T. Matsui
Division of Hematology/Oncology, Department of Medicine,
Kobe University School of Medicine, 650-0017 Kobe, Japan

M. Bourin
Department of Pharmacology, University of Nantes,
44035 Nantes Cedex, France

Introduction

Cholecystokinin (CCK) is a neuropeptide 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). However, the anxiogenic-like effects of the administered CCK peptides in animal experiments have not been observed by all investigators, and this considerable body of negative findings should not be ignored (Shlik et al. 1997). The conflicting data reported in the animal literature are attributable in part to failure to address the various factors that potentially influence susceptibility to the anxiogenic effect of CCK (Bradwejn and Vasar 1995). For instance, rats with low exploratory behavior (i.e. anxious rats) have been reported to exhibit a higher density of CCK receptor-binding sites in the frontal cortex and hippocampus relative to that in rats with high exploratory behaviour (i.e. non-anxious rats) (Harro et al. 1990; Kõks et al. 1997). In African green monkeys, "uptight" animals, 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 an anxiogenic-like effect only in stressed rats, whereas it was ineffective in habituated and non-stressed animals (Kõks et al. 2000). It appears that the dose efficacy and behavioural patterns after CCK challenge depend on the baseline anxiety of the animal and on its hierarchial position in its social group. These findings could suggest that CCK_2 receptor stimulation induces anxiety only in animals already in distress (Griebel 1999).

Recently, mice with targeted disruption of CCK_2 receptor gene have been bred (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 the dopaminergic system is also affected in mice with non-functional CCK_2 receptors because of increased sensitivity of dopamine D_2 receptors (Daugé et al. 2001a; Kõks et al. 2001). Recent evidence suggests altered function of the endogenous opioid system in mice without CCK_2 receptors. Pommier et al. (2002) have demonstrated that mice without CCK_2 receptors display hyperalgesia and a reduced response to morphine-induced analgesia in the hotplate test. Our pilot study showed that female mice lacking CCK_2 receptors displayed reduced anxiety in the elevated plus-maze (Vasar et al. 2002). The present study was designed to analyse further the exploratory behaviour of female mice without CCK_2 receptors in the elevated plus-maze paradigm. The initial suggestion that CCK interacts with γ-aminobutyric acid (GABA) in the regulation of anxiety came from the experiments by Bradwejn and de Montigny (1984) showing 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 is 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). The 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 subtypes of $GABA_A$ receptors (Möhler et al. 2002), on the exploratory activity of mice without CCK_2 receptors. It is worth noting that male CCK_2 receptor deficient mice have an impaired performance in the rotarod test (Kõks et al. 2001; Daugé et al. 2001a). Diazepam not only induces an anxiolytic action, but also causes the impairment of motor co-ordination in laboratory animals (Möhler et al. 2002). For this reason, the effect of diazepam was also studied on the motor performance of CCK_2 receptor deficient mice in the rotarod test. The parameters of benzodiazepine receptors were analysed simultaneously with the behavioural studies in the cerebral cortex, hippocampus, and cerebellum of 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 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. Female mice were used throughout the studies. Altogether, 180 homozygous (–/–) CCK_2 receptor deficient, 197 heterozygous (+/–), and 200 wild-type (+/+) (3 months old) mice were used in the behavioural and radioligand binding studies. The genetically modified mice were crossed back six times to the C57BL/6 background to minimize possible genetic influence from the 129 Sv 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 hours). 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 testing

The animals were brought into the experimental room 1 h before the experiment. All behavioural experiments were performed between 1100 and 1900 hours. The rotarod and elevated plus-maze tests were performed using separate groups of animals. Since the 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 randomized order, that is, the wild-type (+/+) mice were always used in parallel with the genetically modified littermates. In the

radioligand binding study, we used animals that had not been exposed to behavioural testing.

Elevated plus-maze

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) 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 agonists (caerulein, CCK-4) depends on pre-experimental manipulations (Kõks et al. 2000). For example, caerulein, a non-selective CCK agonist, caused the strongest effect in rats if administered in a novel aversive environment. Also, the possibility of establishing the anxiolytic effect of drugs is higher in stressed animals. Therefore, the experiment was performed in a brightly lit room, the animals were not handled before the studies, and they were placed singly into the cages for 30 min prior to the plus-maze exposure.

The plus-maze consists of two opposite open (17.5×5 cm) arms without side walls and two enclosed arms of the same size with 14-cm-high side walls and an end wall. The arms extended from a common central square (5×5 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 a 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 the 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. A 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 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 the open and total arm entries. Time spent in the open arms, number of open arm entries, and ratio between the open and total arm entries are the 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 (Rodgers et al. 1997). Each animal was used in only one experiment. Diazepam (Sigma, 0.5 mg/kg, 1 mg/kg and 3 mg/kg) was administered intraperitoneally 30 min prior to the study. Diazepam was suspended in physiological saline (0.9% of sodium chloride solution) with the help of a few drops of Tween-80 (Sigma).

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. Motor performance (time until the first fall) was registered during a 2-min session on three consecutive days. The effect of diazepam (0.5 and 3 mg/kg) was studied on day 4. Diazepam and vehicle (a few drops of Tween 80 in saline) were administered intraperitoneally 30 min before the experiment.

Radioligand binding studies

After decapitation, the brains were quickly dissected on ice. The cerebral cortex (including the frontal and parietal cortices), hippocampus, and cerebellum were dissected (Franklin and Paxinos 1997). 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 homogenized in 20 vol ice-cold 50 mM TRIS-HCl (pH 7.4 at 4°C) using a Potter-S glass-teflon homogeniser (1000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48,000 *g* for 20 min) and re-suspension. After the last centrifugation, the 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 experiment was repeated four times.

Statistics

The results are expressed as mean values±SEM. The behavioural studies were analysed using one- and two-way analysis of variance. Post hoc comparisons between the individual groups were performed by means of the Tukey HSD test, using the Statistica for Windows software. Student's *t*-test was applied for the analysis of radioligand binding data.

Results

Elevated plus-maze

Comparison of exploratory activity of the CCK_2 receptor deficient mice relative to the wild-type (+/+) littermates established several differences. The homozygous (–/–) mice visited the open arms more frequently than wild-type (+/+) mice [Fig. 1; $F(2,85)=4.50$, $P<0.05$]. Also, the homozygous (–/–) mice spent a significantly longer time on the open arms and central square of a plus-maze [open arms: $F(2,85)=5.30$, $P<0.01$; central square: $F(2,85)=3.77$, $P<0.05$]. However, changes in the other parameters of plus-maze exploration did not reach a statistically significant level: the number of line crossings [$F(2,85)=2.24$, $P=0.11$], and ratio between the open and total entries [$F(2,85)=2.69$, $P=0.07$]. Frequency of the closed arm entries in the homozygous (–/–) mice did not differ from the respective value of the wild-type (+/+) littermates, but was different from the number of closed arm entries of heterozygous (+/–) animals [Fig. 1, $F(2,85)=4.66$, $P<0.05$].

The effect of diazepam in the plus-maze was studied in a separate group of mice. It should be noted that the baseline exploratory activity of heterozygous (+/–) mice was higher in this experiment compared to the above described experiment. However, the differences in the exploratory activity of wild-type (+/+) and homozygous (–/–) mice remained at the same level in these two separate studies (Fig. 1, Fig. 2). The administration of diazepam (0.5–3 mg/kg) caused a dose-dependent anxiolytic-like effect in the wild-type (+/+) mice. Diazepam increased the number of open arm entries, time spent on the open arms, and ratio between the open and total arm

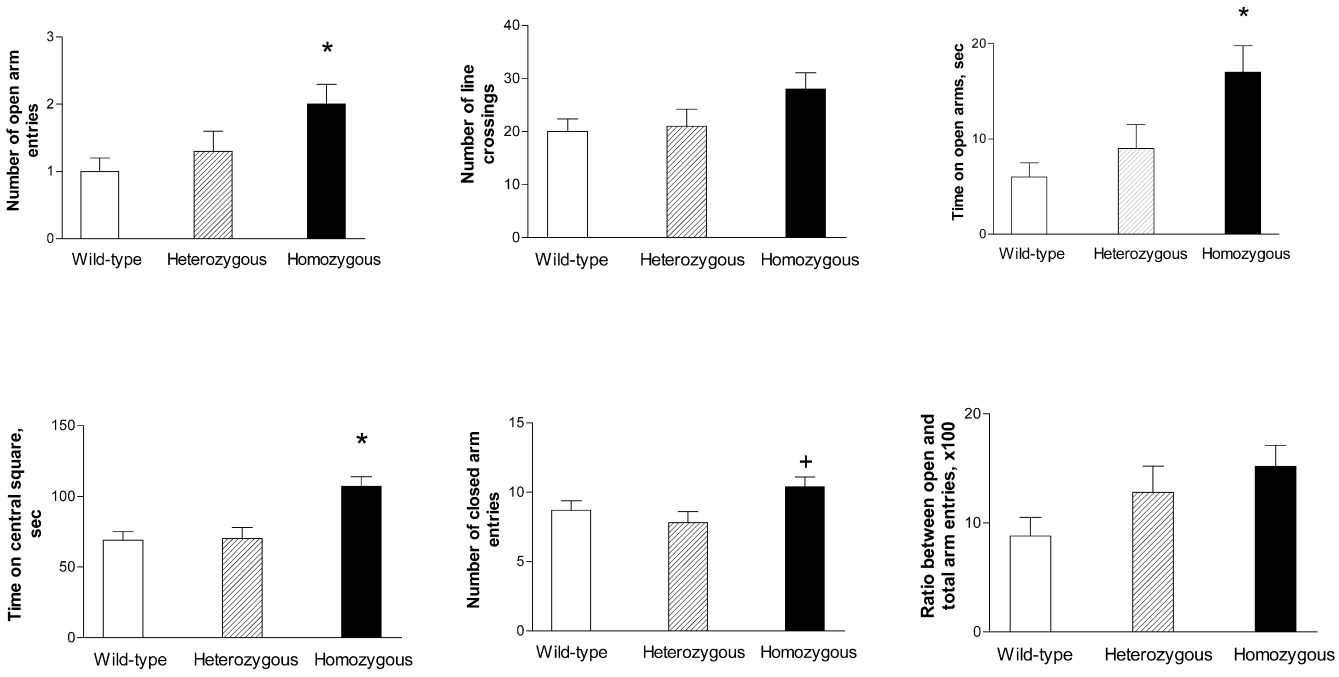

Fig. 1 The exploratory activity of CCK_2 receptor deficient mice in the elevated plus-maze. The number of animals in each group was between 28 and 30. *White bars* wild-type; *striped bars* heterozygous; *black bars* homozygous. **P*<0.05 (compared to wild-type mice, Tukey HSD test after significant one-way ANOVA); +*P*<0.05 (compared to heterozygous mice)

entries (Fig. 2). Two-way ANOVA was applied in order to compare the behavioural effects of diazepam in the wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice (Fig. 2). The number of open arm entries was differently affected by diazepam in wild-type (+/+), heterozygous (–/+) and homozygous (–/–) mice [genotype $F(2,391)=1.16$, $P=0.32$, treatment $F(3,391)=11.84$, $P<0.01$; genotype×treatment $F(6,391)=2.19$, $P<0.05$]. The following post hoc analysis (Tukey HSD test) established that the lowest dose of diazepam (0.5 mg/kg) induced a statistically significant increase ($P<0.05$) of open arm entries in the wild-type (+/+) mice. In the homozygous (–/–) mice a significant change was found after the administration of diazepam at a dose of 1 mg/kg ($P<0.01$), whereas in the heterozygous (+/–) animals the effect of a benzodiazepine agonist was not significant ($P=0.36$). The highest dose (3 mg/kg) caused an increase in open arm entries in the wild-type (+/+) mice ($P<0.05$), but not in the genetically modified animals. Frequency of open arm visits in the heterozygous (+/–) and homozygous (–/–) mice after the administration of diazepam (3 mg/kg) did not differ from that in the vehicle-treated animals (Fig. 2). Subsequent post hoc analysis established a statistically significant difference ($P<0.01$) between the action of two doses (1 and 3 mg/kg) of diazepam in the homozygous (–/–) mice. Two-way ANOVA also demonstrated that diazepam affected differently the number of line crossings in these groups of mice [genotype $F(2,391)=1.96$, $P=0.14$, treatment $F(3,391)=21.06$, $P<0.01$; genotype×treatment $F(6,391)=2.22$, $P<0.05$]. Diazepam at lower doses (0.5 and 1 mg/kg) tended to increase the number of line crossings in the wild-type (+/+) mice, but this effect was not statistically significant (Fig. 2). The highest dose (3 mg/kg) of diazepam reduced this behavioural measure in the heterozygous (+/–) and homozygous (–/–) mice, but only in the homozygous (–/–) animals was it significant (Tukey HSD test: $P=0.21$ for the heterozygous (+/–) and $P<0.01$ for homozygous (–/–) animals). The statistical analysis did not reveal any differences between the genotypes if time spent on the open arms was studied [genotype $F(2,391)=0.85$, $P=0.43$, treatment $F(3,391)=15.31$, $P<0.01$; genotype×treatment $F(6,391)=0.32$, $P=0.92$]. Nevertheless, the post hoc analysis demonstrated that the action of diazepam (3 mg/kg) was statistically significant in the wild-type (+/+) ($P<0.01$) and homozygous (–/–) ($P<0.01$) animals, but not in the heterozygous (+/–) ($P=0.14$) mice (Fig. 2). Diazepam (3 mg/kg) did not change time spent on the central square and the number of closed arm entries in the wild-type (+/+) mice if compared to the vehicle-treated animals. However, diazepam significantly reduced these parameters of exploratory behaviour in the genetically modified mice [time spent on the central square: genotype $F(2,391)=0.18$, $P=0.84$, treatment $F(3,391)=11.57$, $P<0.01$; genotype×treatment $F(6,391)=2.22$, $P<0.05$, number of closed arm entries: genotype $F(2,391)=0,64$, $P=0.53$, treatment $F(3,391)=26.24$, $P<0.01$; genotype×treatment $F(6,391)=2.62$, $P<0.05$]. Again, post hoc analysis established that these effects of diazepam were significant in the homozygous (–/–) mice ($P<0.01$ for time spent on the central square and $P<0.01$ for the number of closed arm entries), but not in the heterozygous (+/–) animals

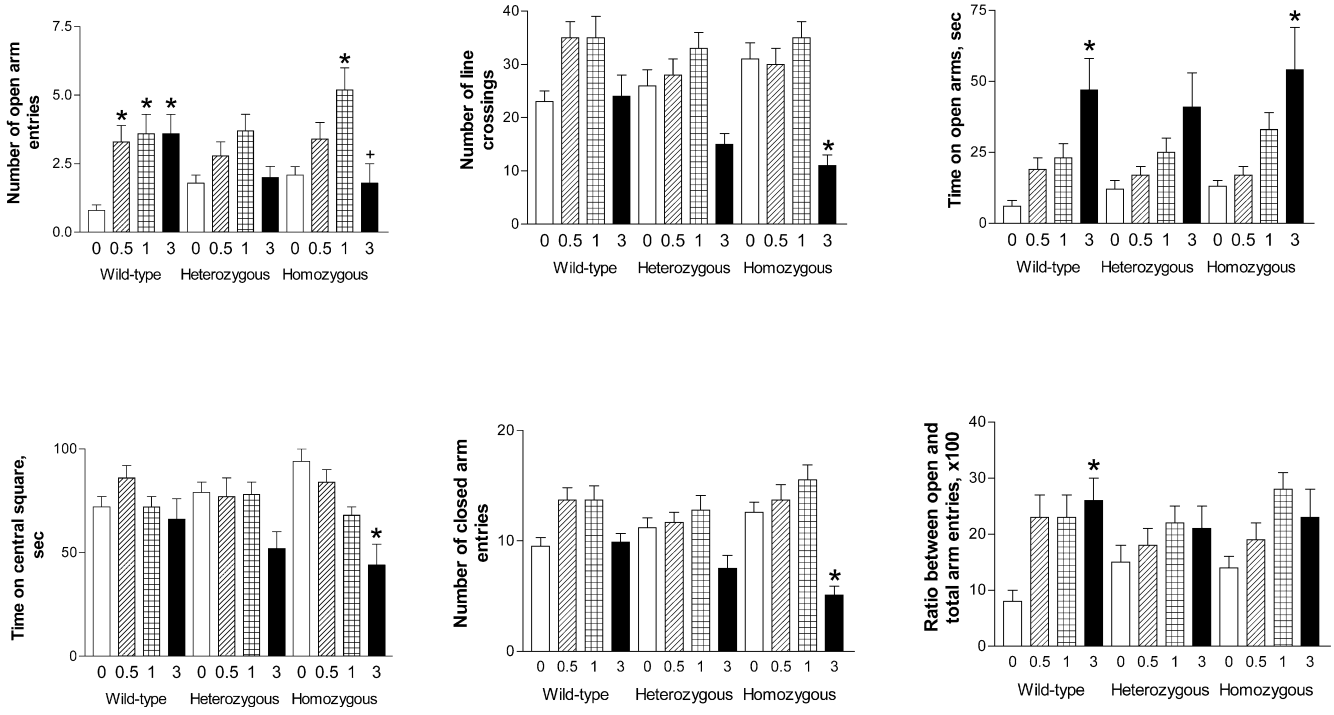

Fig. 2 The effect of diazepam (0.5–3 mg/kg) on the exploratory activity of CCK_2 receptor deficient mice in the elevated plus-maze. The number of animals in each group was between 30 and 37. *White bars* vehicle; *striped bars* diazepam 0.5 mg/kg; *hatched bars* diazepam 1 mg/kg; *black bars* diazepam 3 mg/kg. *P<0.05 (compared to the respective vehicle-treated group, Tukey HSD test after significant two-way ANOVA); +P<0.01 [compared to the effect of diazepam (1 mg/kg) in homozygous mice]

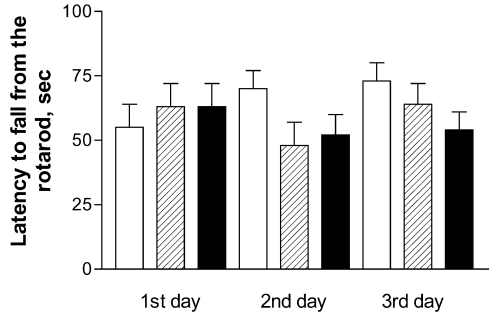

Fig. 3 The performance of CCK_2 receptor deficient mice in the rotarod test. The number of animals in each group was as follows: 30 wild-type, 28 heterozygous, and 28 homozygous animals. The study was repeated on 3 consecutive days. *White bars* wild-type; *striped bars* heterozygous; *black bars* homozygous

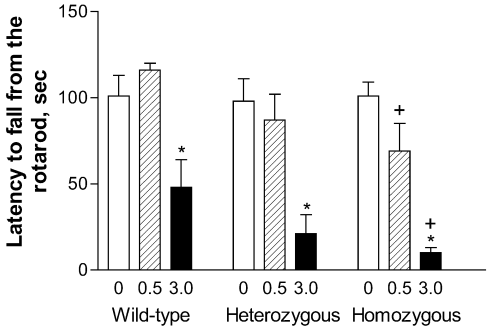

Fig. 4 The effect of diazepam (0.5 and 3 mg/kg) on the performance of CCK_2 receptor deficient mice in the rotarod test. The number of animals in each group was between 8 and 11. *White bars* vehicle; *striped bars* diazepam 0.5 mg/kg; *black bars* diazepam 3 mg/kg. *P<0.05 (compared to the respective vehicle-treated group, Tukey HSD test after significant one-way ANOVA); +P<0.05 (compared to diazepam-treated wild-type mice)

(respective values: P=0.15 and P=0.38). The application of two-way ANOVA did not demonstrate any difference between the genotypes if the action of diazepam (0.5–3 mg/kg) was studied on the ratio between the open and total arm entries [genotype $F(2,391)$=0.61, P=0.54, treatment $F(3,391)$=9.04, P<0.01; genotype×treatment $F(6,391)$=0.86, P=0.52]. The following post hoc analysis demonstrated that only in the wild-type mice (+/+), not in heterozygous (+/–) and homozygous (–/–) animals, did diazepam (3 mg/kg) induce a statistically significant (P<0.05) increase in this parameter of plus-maze exploration (Fig. 2).

Rotarod test

The performance of the wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice in the rotarod test did not differ on days 1, 2 and 3 (Fig. 3). Treatment with diazepam (0.5 and 3 mg/kg) on day 4 caused a dose-dependent impairment of motor co-ordination in all genotypes [genotype $F(2,77)$=4.94, P<0.01; treatment $F(2,77)$=38.04, P<0.01; genotype×treatment $F(4,77)$=0.99, P>0.25] (Fig. 4). However, further post hoc analysis

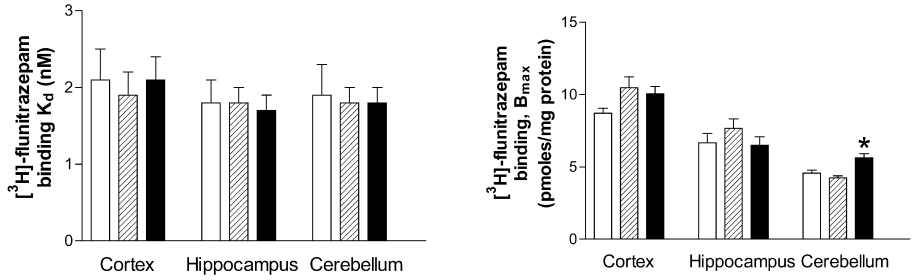

Fig. 5 The parameters of [^{3}H]-flunitrazepam binding in the brain structures of CCK_2 receptor deficient mice. The number of animals in each group was 24, the brains of six mice were pooled, and the mean is a result of four experiments. *White bars* wild-type; *striped bars* heterozygous; *black bars* homozygous. *$P<0.05$ (compared with wild-type mice, Student's *t*-test)

established that diazepam (0.5 and 3 mg/kg) caused a significantly greater impairment (Tukey HSD test: $P<0.05$) of motor co-ordination in the homozygous (–/–) mice compared to the wild-type (+/+) littermates.

Radioligand binding studies

The density of [^{3}H]-flunitrazepam binding sites (B_{max}) in the cerebellum was increased in homozygous (–/–) mice compared to their wild-type (+/+) littermates (Fig. 5). No such difference was established between the wild-type (+/+) and homozygous (–/–) mice in the cerebral cortex and hippocampus. The density of benzodiazepine binding sites tended to be higher in the cerebral cortex and hippocampus of the heterozygous (+/–) mice relative to the wild-type (+/+) animals. 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 the wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice (Fig. 5).

Discussion

According to the present study, the exploratory behaviour of CCK_2 receptor deficient female mice is higher compared to their wild-type (+/+) littermates. The homozygous (–/–) mice visited the open arms more often and spent a longer time on the open parts (open arms and central square) of an elevated plus-maze compared to the wild-type (+/+) mice. Interestingly, Daugé et al. (2001b) also established an increased number of open arm entries in CCK_2 receptor deficient mice, but this effect was not statistically significant. The ratio between the open and total arm entries, a classical measure of anxiety in the elevated plus-maze, was also increased in homozygous (–/–) mice, but this increase did not reach statistical significance. The exploratory activity of the heterozygous (+/–) mice, which had 50% of their CCK_2 receptors remaining (Nagata et al. 1996; Kõks et al. 2001), was different in the separate studies. In the experiment, where the effect of diazepam was analysed, it did not differ from that of homozygous (–/–) mice. However, in the experiment where the response of untreated mice was compared, the exploratory activity of heterozygous (+/-) mice did not differ from the wild-type (+/+) animals. The background of these differences in the exploratory behaviour of heterozygous (–/–) mice is not clear and remains to be established. The effect of targeted mutation of the CCK_2 receptor gene on the exploratory activity of mice is weaker compared with the effect of diazepam (0.5 mg/kg) in wild-type (+/+) mice. Accordingly, the increase of exploratory activity in homozygous (–/–) mice is not robust. The results of the present study to some extent resemble the findings obtained using CCK_2 receptor antagonists in rodents (Shlik et al. 1997; Nobel et al. 1999). The selective CCK_2 receptor antagonists L-365,260, CI-988 and LY262691 showed anxiolytic-like effects in several animal anxiety tests (Woodruff and Hughes 1991). However, in some laboratories, the anxiolytic effect of a CCK_2 receptor antagonist as a single treatment has not been evident (Harro and Vasar 1991a; Crawley 1992; Dawson et al. 1995). The potent anxiolytic effects of CCK receptor antagonists per se have been demonstrated using exploratory activity tests but not in other tests (Hughes et al. 1990; Powell and Barrett 1991). It has been suggested that this effect of CCK_2 receptor antagonists is due to the additional effect of the motivational systems that mediate curiosity (Harro and Vasar 1991b; Harro 1993). Therefore, it is not clear whether the increased exploratory activity of CCK_2 receptor deficient mice is related to reduced anxiety or to increased drive to explore the elevated plus-maze. Recent evidence suggests that the sensitivity of dopamine D_2 receptors is increased in mice lacking CCK_2 receptors (Daugé et al. 2001a; Kõks et al. 2001). Therefore, one could expect that the increased exploratory activity in the elevated plus-maze might be due to increased dopaminergic neurotransmission in the brain. Indeed, Daugé et al. (2001a) have demonstrated increased locomotor activity in mice without CCK_2 receptors. However, we have not been able to confirm this finding, because the locomotor activity of homozygous (–/–) mice is either reduced (Kõks et al. 2003) or unchanged compared to their wild-type (+/+) littermates (Kõks et al. 2001). We found that the administration of amphetamine, an indirect

dopamine agonist, induced significantly stronger stimulation of locomotor activity in the homozygous (–/–) mice (Kõks et al. 2001, 2003). The density of dopamine D_2 receptors was increased in the striatum of CCK_2 receptor deficient animals, but not in the mesolimbic structures (Kõks et al. 2001). Nevertheless, increased sensitivity of dopamine D_2 receptors was established in male, but not in female homozygous (–/–) mice (Vasar et al. 2000). Therefore, it is unlikely that dopamine D_2 receptors contribute to the increased exploratory behaviour established in female homozygous (–/–) mice.

The effect of diazepam, a benzodiazepine agonist, is dependent on the baseline exploratory activity of animals. The baseline activity of heterozygous (+/–) and homozygous (–/–) animals was higher and, therefore, the administration of a higher dose of diazepam was necessary to increase the exploratory activity of genetically modified mice. Even 0.5 mg/kg diazepam induced a significant increase of open arm visits in the wild-type (+/+) mice. A similar increase in the homozygous (–/–) mice was found after the administration of 1 mg/kg diazepam, whereas in the heterozygous (+/–) animals diazepam-induced increase of exploratory activity was not statistically significant. Consequently, the sensitivity of benzodiazepine receptors could be changed in the heterozygous (+/–) and homozygous (–/–) mice, and this might therefore mask the action of a lower dose of diazepam in the genetically modified mice. According to previous studies, where the animals were subjected to the stressful manipulations or the animals were pre-selected according to their exploratory behaviour, the most prominent changes in the parameters of benzodiazepine and CCK receptor binding were established in the cerebral cortex and hippocampus (Rägo et al. 1988; Harro et al. 1990). These are the brain structures where CCK is co-localized with GABA in the neurons (Hendry et al. 1984; Somogyi et al. 1984). However, we did not find any changes in the affinity of benzodiazepine binding sites in heterozygous (+/–) and homozygous (–/–) mice, showing that the sensitivity of benzodiazepine receptors was not affected. We were also unable to detect any differences in [^{3}H]-flunitrazepam binding in the cerebral cortex and hippocampus between the wild-type (+/+) and homozygous (–/–) mice. The highest dose of diazepam (3 mg/kg) also caused different effects in the wild-type (+/+) and homozygous (–/–) mice. In the wild-type (+/+) animals, this dose of diazepam induced a prominent anxiolytic-like action, including a significant increase in open arm entries, time spent on open arms, and ratio between the open and total arm entries. By contrast, in homozygous (–/–) mice, diazepam (3 mg/kg) significantly suppressed motor activity, including a reduced number of line crossings and closed arm visits. In heterozygous (+/–) mice, the highest dose of diazepam tended to reduce these parameters, reflecting locomotor activity, but these changes were not statistically evident. Accordingly, it is likely that the dose-response curve for diazepam is shifted to the left in homozygous (–/–) mice, and the lower dose of diazepam inhibits locomotor activity in homozygous (–/–) mice compared with the wild-type (+/+) animals. Classical benzodiazepines like diazepam act to enhance the effectiveness of GABA in a unique way, by lowering the concentration of GABA required for opening the chloride channel (Nutt and Malizia 2001). Benzodiazepine binding allosterically changes the $GABA_A$-benzodiazepine complex to increase the efficiency of GABA, so enabling the GABAergic circuits to produce a larger inhibitory effect. Recent evidence suggests that the behavioural effects of benzodiazepine agonists are mediated via different subtypes of $GABA_A$ receptors (Möhler et al. 2002). Möhler et al. (2002) have demonstrated that the sedative effect of benzodiazepine agonists is mediated via the α_1 subtype of $GABA_A$ receptors, whereas the α_2 subtype is responsible for the anxiolytic action. Therefore, it is tempting to speculate that the tone of the GABAergic system is increased in mice without CCK_2 receptors. This may explain the reduced anxiety and a shift of anxiolytic-like and motor suppressant effects of diazepam established in the homozygous (–/–) mice. However, the relevance of this hypothesis should be tested in further studies.

The performance of wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice in the rotarod test did not differ. This is different from previous studies performed with male CCK_2 receptor deficient mice, showing a significant impairment of motor co-ordination in the 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 C57BL/6J mice show much longer latencies to fall than 129SvJ mice in the rotarod test (Homanics et al. 1999). Therefore, it is possible that the targeted mutation of CCK_2 receptors induces the motor disturbances in mice predominately having the genes of a 129 Sv strain. This suggestion is supported by our studies on male mice showing that back-crossing of mice to the C57BL/6 background reverses the suppression of motor activity evident in our first studies with the homozygous (–/–) mice (Kõks et al. 2001, 2003). Nevertheless, the administration of diazepam revealed the difference between the wild-type (+/+) and homozygous (–/–) mice. Statistical analysis established that diazepam (0.5 and 3 mg/kg) induced significantly stronger impairment of motor co-ordination in homozygous (–/–) mice compared to their wild-type (+/+) littermates. This finding can also explain why the highest dose (3 mg/kg) of diazepam suppressed the motor activity of homozygous (–/–) mice in the elevated plus-maze. The cerebellum has a key role in the regulation of motor co-ordination (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 conclusion, the present study demonstrates that homozygous (–/–) mice display the reduced anxiety in a novel aversive environment in comparison with their wild-type (+/+) littermates. The diminished level of anxiety is a probable reason why the administration of a higher dose of diazepam is necessary to induce an anxiolytic-like action in the homozygous (–/–) mice compared to wild-type (+/+) animals. By contrast, motor suppression and impairment of motor co-ordination induced by diazepam are stronger in the genetically modified mice than in their wild-type (+/+) littermates. An elevated density of benzodiazepine receptors in the cerebellum is probably related to the increased effects of diazepam in CCK_2 receptor deficient mice. Altogether, the present study seems to be in favour of increased tone of the GABAergic system in mice lacking CCK_2 receptors.

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