DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
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Sex and environmental factors determine the behavioural phenotype of mice lacking CCK_2 receptors: implications for the behavioural studies in transgenic lines

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1. The author designed the study, took care of the different housing conditions, performed the behavioural experiments, carried out statistical analysis, and wrote most parts of the manuscript.
2. The author designed the study, performed the behavioural experiments, determined blood ethanol concentrations, carried out ethanol intake experiment and statistical analysis, and wrote the manuscript.
3. The author designed the study, took care of housing conditions, performed several behavioural experiments (the hot plate test, restraint-induced analgesia and the rota-rod), carried out all statistical analysis, and wrote the manuscript.
4. The author designed the study, took care of housing conditions, performed the behavioural experiments and statistical analysis, and wrote the manuscript.

ABBREVIATIONS

129 – 129S6/SvEv strain or, more generally, all 129 strains
5-HT – 5-hydroxytryptamine or serotonin
5-HT_2 – serotonin type 2 (receptor)
5-HT_{1B} – serotonin type 1B (receptor)
B6 – C57BL/6 (strain; a substrain to C57)
BEC – blood ethanol concentration
BEC_{RRR} – blood ethanol concentration at the regain of righting reflex
C57 – C57BL strains
CCK – cholecystokinin
CCK_1 – cholecystokinin type 1 (receptor)
CCK_2 – cholecystokinin type 2 (receptor)
CNS – central nervous system
D_2 – dopamine type 2 (receptor)
GABA – gamma-aminobutyric acid
$GABA_A$ – gamma-aminobutyric acid-A (receptor)
GIRK2 – G-protein-coupled inwardly rectifying potassium (channel)
HPA – hypothalamo-pituitary-adrenal (axis)
LORR – loss of righting reflex
mRNA – messenger ribonucleic acid
NMDA – N-methyl-D-aspartate
RRR – regain of righting reflex

INTRODUCTION

Over the past decade genetically manipulated mice have become as powerful tool for scientists in establishing molecular basis of psychiatric disorders as the pharmacological agents had been in the second half of the 20th century. However, soon after the first euphoria induced by the advent of transgenic technologies several problems were encountered. It was established that the effects of several mutations that provided nice and meaningful results could not be reproduced if back-crossed in a genetically distinct background strain (Kelly *et al.* 1998; Le Roy *et al.* 2000). Furthermore, it became obvious that there were no standard "wild-type" controls, so that depending on the strain (i.e. C57BL/6 or 129Sv/C57BL6) used as the "wild-type" the observed phenotype of the mutant mice could vary substantially. And yet, even a sufficient number of back-crosses in a certain inbred strain and the use of littermate wild-types did not turn out to guaranty the genetic identity of the two lines that should differ only in the properties of the mutated gene (Crusio 2004). In addition to the genetic matching issues, it was soon discovered that if a gene was manipulated and its product substantially altered, several compensatory changes could take place in the affected organism over-shadowing the effects of the mutation and producing either false positive or false negative results (Gerlai 2001). Thus, the initial expectation that by producing a new organism with a manipulated gene the functions of the gene could be easily revealed, turned out to be a mere illusion.

The issue of reproducibility of behavioural findings has accompanied the research in the field from the very beginning. The fact that several results could not be reproduced by a different research group or even by the same group led Crabbe *et al.* (1999) to design a study which aimed to take under control all possible environmental variation. What they found, however, was that despite obsessive standardisation of testing conditions, different laboratories still produced results that varied substantially. Although it was concluded that the major differences between the inbred strains had been more or less reproduced across laboratories (Crabbe *et al.* 1999; Wahlsten *et al.* 2003), it still sounded as a warning to those suffering from blind faith in the results of behavioural studies. The approach employing alternative environmental conditions within a laboratory was soon adopted in transgenic research, and it was discovered that in addition to various genetic factors, environmental factors could significantly contribute to the development of behavioural phenotypes in mutant mice (Rampon *et al.* 2000; van Dellen *et al.* 2000). To mess things up even more, several laboratories reported that sex was a prominent factor that affected the phenotypes of transgenic mice (Ramboz *et al.* 1998; Walther *et al.* 2000).

The present study was triggered by the urge of the authors to gain insight into possible sources of variation in the results reported on the mutant strain with invalidated cholecystokinin–2 (CCK_2) receptor gene. Although the functions of the CCK_2 receptors in the central nervous system have been described

earlier using pharmacological tools, the behavioural phenotype of mice lacking CCK_2 receptors has not always supported these findings. Moreover, as will be discussed in the course of the present thesis, the phenotype of CCK_2 receptor deficient mice has varied so dramatically across laboratories that it has sometimes seemed as if different studies had been performed using totally different lines. The present study was initially designed to establish the role of CCK_2 receptors in the responses to stressful stimuli. Therefore, the effect of social isolation was studied in mice lacking CCK_2 receptors. Since our unpublished observations indicated that sex was a prominent determinant of the behavioural phenotype in these mutants, experiments were carried out in both male and female mice. Due to the main author's infirm interest in the topic, the second part of the study was already devoted to the sex factor rather than the reward-related functions associated with CCK and ethanol. Furthermore, since conflicting reports on the phenotype of mice lacking CCK_2 receptors kept emerging with certain persistence (for instance, eight different reports regarding the anxiety in these mice were published over six years), the authors designed a study to assess the reproducibility of the behavioural findings in CCK_2 receptor deficient mice employing alternative pre-experimental housing conditions as the source of environmental variation. The final part of the study was carried out in 129S6/SvEv and C57BL/6 strains housed in different conditions to establish if this approach could provide a meaningful estimate of reproducibility of behavioural data within one laboratory.

REVIEW OF LITERATURE

1. Cholecystokinin and mice, lacking CCK_2 receptors

1.1. Functions of cholecystokinin in the central nervous system

CCK was first described in the mammalian central nervous system in 1975 (Vanderhaeghen *et al*. 1975). Cholecystokinin is one of the most prevalent neuropeptides in the brain (Rehfeld *et al.* 1992). Regional distribution of CCK varies widely with high levels in the cerebral cortex, hippocampus, amygdala and basal ganglia, moderate levels in the thalamus, hypothalamus and olfactory bulb, and low levels in the pons, medulla and spinal cord. In the cerebellum CCK is barely detectable (Beinfeld *et al.* 1981). Several biologically active forms of CCK derived from 115 amino-acid pre-proCCK exist (Rehfeld and Nielsen 1995). The most common form of CCK in the brain is the sulphated octapeptide, but also longer and shorter peptides as well as unsulphated forms occur. By now, two subtypes of CCK receptors have been identified (Noble *et al.* 1999). CCK_1 receptors (formerly CCK-A receptors) and CCK_2 receptors (formerly CCK-B/gastrin receptors) are both G-protein coupled receptors. CCK_1 receptors are located in the pancreas, gall-bladder, and in distinct brain regions, for example the hypothalamus, central amygdala, hippocampus and nucleus accumbens (Moran *et al*. 1986). However, the predominant CCK receptors in the brain are CCK_2 receptors. CCK_2 mRNA expression and binding have been described in the olfactory bulb, amygdala, the 3rd and 4th layer of the neocortex, nucleus accumbens, striatum, hippocampus, thalamus, hypothalamus, substantia nigra, raphe dorsales and periaqueductal grey matter (Beinfeld 1983; Gaudreau *et al*. 1983; Pelaprat *et al.* 1987; Niehoff 1989; Honda *et al.* 1993). It should be noted, though, that there is some variation in CCK_2 receptor distribution across different species (Sekiguchi and Moroji 1986).

CCK is involved in the regulation of various physiological functions in the brain such as modulation of anxiety and stress-related behaviours, regulation of feeding, nociception, memory, body temperature and reward-related behaviours (Crawley and Corwin 1994; Daugé and Lena 1998; Szelenyi 2001; Rotzinger and Vaccarino 2003; Moran 2004). Also, there is substantial evidence that CCK acts as a neurotransmitter and that it exerts a modulatory influence through several classic neurotransmitters, including dopamine, serotonin, GABA and opioid peptides (Crawley 1995).

CCK or its receptors are increased in various brain regions of rodents subjected to such stressors as restraint (Nevo *et al.* 1996; Giardino *et al.* 1999), social stress (Del Bel and Guimaraes 1997; Becker *et al.* 2001), chronic mild stress (Kim *et al.* 2003), immunological challenge (Juaneda *et al.* 2001), predator odour (Hebb *et al.* 2002) and anxiogenic drug administration (Pratt and Brett 1995). CCK receptor agonists stimulate hypothalamo-pituitary-adrenal (HPA) axis (Porter and Sander 1981; Reisine and Jensen 1986; Abelson and

Liberzon 1999) and, more specifically, CCK_2 receptor agonists induce anxiety-like behaviour in rodents (Belcheva *et al.* 1994; Vaccarino *et al.* 1997; Matto *et al.* 1997; Derrien *et al.* 1994). By contrast, antagonists of CCK_2 receptors attenuate anxiety-like behaviour (Hughes *et al*, 1990; Costall *et al.* 1991; Männistö *et al.* 1994; Adamec *et al.* 1997), and block the acquisition and expression of conditioned fear (Tsutsumi *et al.* 1999). Furthermore, it has been established that reducing experimental stress abolishes behavioural effects of compounds acting on CCK_2 receptors (Daugé *et al.* 1989; Lavigne *et al.* 1992; Kõks *et al.* 2000). The relevance of CCK and CCK_2 receptors in anxiety is also suggested by studies in genetically modified mice. Although data on anxiety-like behaviour in mice lacking CCK_2 receptors is variable (summarised below), mice with the up-regulation of CCK_2 receptors display an increase in anxiety (Chen *et al.* 2006b). However, contrarily to what could be expected, mice lacking the CCK gene also display increased anxiety-like behaviour as measured by the plus-maze test (Lo *et al.* 2008).

CCK participates in pain regulation and stress-induced analgesia, and is associated with chronic pain. CCK is localised in brain regions involved in pain regulation (Baber *et al.* 1989). While CCK antagonises stress- and morphine-induced analgesia (Faris *et al.* 1983), CCK receptor antagonists potentiate opioid analgesia (Watkins *et al*. 1984; Watkins *et al*. 1985; Lavigne *et al.* 1992; Noble *et al.* 1995; Nichols *et al.* 1996; Homayoun and Dehpour 2004). Also, CCK_2 receptor antagonists reverse mechanical allodynia in nerve-ligated rats (Kovelowski *et al.* 2000).

Cholecystokinin and its analogues have been shown to participate in learning and memory. Cholecystokinin accelerates habituation to novel environment, a form of non-associative learning (Crawley 1984; Voits *et al.* 1994), but it also improves spatial memory in aged rats (Voits *et al.* 2001) and in 6-hydroxydopamine-lesioned young rats (Rex and Fink 2004). Itoh *et al.* (1989) established that unselective CCK receptor agonist cerulein prolonged extinction of a learned task, and prevented electro-convulsive shock and scopolamine-induced amnesia. Furthermore, pBC264, a CCK_2 receptor agonist, improves cognitive performance in rats in two-trial recognition memory task (Taghzouti *et al.* 1999), whereas CCK_2 receptor antagonists impair memory in rats (Sebret *et al.* 1999). In a recent study, Lo *et al.* (2008) reported that mice lacking cholecystokinin show impaired performance in the passive avoidance and water maze tests. Also, cognitive impairments have been reported in rats lacking CCK_1 receptors (Li *et al.* 2002) and mice lacking CCK_2 receptors (Sebret *et al.* 1999; Daugé *et al.* 2001b). On the other hand, it has been suggested that CCK participates in memory impairment induced by stress (Daugé *et al.* 2003).

CCK is colocalised with dopamine in the mesolimbic pathways (Hökfelt *et al.* 1980) and in the nucleus accumbens (Lanca *et al.* 1998). There is also evidence of the functional interactions between cholecystokinin and dopamine in the mesolimbic structures. For instance, CCK_2 receptor stimulation reduces extracellular dopamine levels in the nucleus accumbens (Voigt *et al*. 1985),

opposes the postsynaptic effects of dopamine in the region (Yim and Mogenson 1991) and reduces dopamine-mediated behaviours (Crawley 1992). By contrast, CCK_2 receptor antagonists have been shown to induce behavioural sensitisation (Bush *et al.* 1999), to potentiate responding for conditioned rewards (Josselyn and Vaccarino 1995) and to modulate amphetamine-induced sensitisation (Wunderlich *et al*. 2000). CCK is also colocalised with endogenous opioids in various brain regions (Gall *et al.* 1987). CCK attenuates morphine-induced hyperactivity (Schnur *et al.* 1991), whereas CCK_2 receptor antagonists enhance conditioned place preference when administered with sub-threshold doses of opioid agonists (Higgins *et al.* 1992; Valverde *et al.* 1996). CCK has also been reported to suppress ethanol drinking and ethanol preference in rodents (Toth *et al.* 1990; Kulkosky 1996; DiBattista *et al.* 2003; Geary *et al.* 2004), and the role of CCK_1 receptors has been underlined (Crespi *et al.* 1997; Crespi 1998; Miyasaka *et al.* 2005). Studies concerning the role of CCK_2 receptors in ethanol preference have yielded contradictory results. Crespi (1998) described that pre-treatment with CCK_2 receptor antagonists did not affect ethanol drinking in rats. However, Little *et al.* (1999), and Croft *et al.* (2005) reported that CCK_2 antagonists decreased stress-induced ethanol preference in mice. On the other hand, chronic ethanol consumption has been shown to alter the brain CCK-ergic system (Weatherford *et al.* 1993; Harro *et al.* 1994) and CCK_2 receptors have been reported to have relevance in ethanol withdrawal-induced anxiety and convulsions (Wilson and Little 1998; Wilson *et al.* 1998).

Interestingly, CCK shows sex-specific distribution in the central nervous system (Fox *et al.* 1990; Phan and Newton 1999; Polston and Simerly 2003; Newton and Phan 2006). Estrogens have been shown to regulate pre-proCCK mRNA expression in the hypothalamus and limbic structures (Micevych *et al.* 1994). Also, estrogens reduce CCK receptor binding in the ventromedial nucleus of hypothalamus, an effect possibly reflecting ligand-induced down-regulation of receptors (Popper *et al.* 1996). Furthermore, reduction in cortical CCK release has been demonstrated in aged male but not female rats (Miyasaka *et al.* 1997). Dulawa and Vanderweele (1994) demonstrated that the ability of CCK to suppress food intake depended on the estrogen status of rats, but they were unable to detect sex differences in the effect of CCK. By contrast, Voits *et al.* (1996) established that the satiating effect of CCK–8 was stronger in male rats. In accordance with this finding, Strohmayer and Greenberg (1996) indicated that devazepide, a CCK_1 receptor antagonist, increased food intake in male but not in female rats. In addition to the interaction between CCK and estrogens in the regulation of food intake, CCK and estrogens interact in the regulation of sexual behaviour (Micevych *et al.* 2002). Taken together, these data indicate that CCK-ergic system shows anatomical and functional sex-dependent dimorphism, advocating the study of the system in both male and female subjects.

1.2. Mice, lacking cholecystokinin CCK_2 receptors

CCK_2 receptor deficient mice were first described in 1996 (Nagata *et al*. 1996). The mutant line was generated by replacing a part of exon 2, and exons 3–5 employing homologous recombination. This replacement deleted most of the seven membrane-spanning CCK_2 receptor loops except for the first 108 amino acids containing the first membrane-spanning region. The expression of CCK_2 receptor gene mRNA is absent in various structures of the central nervous system of mutant mice (Areda *et al.* 2006; Kurrikoff *et al.* 2008). Nagata *et al.* (1996) demonstrated that homozygous CCK_2 receptor deficient mice do not show ^{125}I-CCK–8 binding in the cell membrane fractions prepared from the whole brain. Kõks *et al.* (2001) have confirmed the absence of ^{3}H-pCCK–8 binding in the cerebral cortex and striatum of mutant mice used by our laboratory.

Since 1996, a number of studies have been published on the anatomical, behavioural, metabolic and neurochemical phenotypes of these mice. However, there have been inconsistencies in the results reported by different laboratories as well as differences in the results reported by the same laboratory across years. Homozygous mice, lacking CCK_2 receptors are fertile and show no abnormalities up to age of 24 months (Nagata *et al*. 1996). Kopin *et al.* (1999) reported no differences between CCK_2 receptor deficient mice and wild-type mice in food intake, weight gain and pancreatic function. Nevertheless, other groups have reported increased body weight, water intake and food consumption (Weiland *et al.* 2004; Chen *et al*. 2006a; Clerc *et al*. 2007), but both decrease (Chen *et al.* 2006a) as well as increase (Clerc *et al*. 2007) in body fat deposition in mice, lacking CCK_2 receptors. By contrast, Miyasaka *et al.* (2002a) demonstrated increased energy intake and expenditure, but unaltered body weight in mutant mice. It should be noted, though, that unlike the Miyasaka group (2002a), Weiland *et al.* (2004), Chen *et al.* (2006a) and Clerc *et al.* (2007) employed C57BL/6 mice as the "wild-type" controls for the mutant line. Also, Weiland *et al.* have established, that mice with genetic invalidation of CCK_2 receptors are hyperthermic (Weiland *et al.* 2004) and they do not develop sickness behaviour in response to lipopolysaccharide challenge (Weiland *et al*. 2007).

As shown by Daugé *et al.* (2001b) no differences in anxiety were observed in mice, lacking CCK_2 receptors. However, Raud *et al.* (2003; 2005) and Areda *et al.* (2006) demonstrated that female mice, lacking CCK_2 receptors, are less anxious than their wild-type littermates in the plus-maze and light-dark exploration tests. These findings were supported by a study from a different laboratory showing that CCK_2 receptor mutants (sex unspecified) displayed increased exploratory behaviour in the plus-maze and light-dark tests (Horinouchi *et al*. 2004). Conversely, Miyasaka *et al*. (2002b) reported that male mice with CCK_2 receptor gene invalidation show even higher levels of anxiety in the plus-maze test when compared to their wild-type littermates.

As contradictory as the reports on anxiety in mice, lacking CCK_2 receptors, have been reports on their locomotor activity. Daugé *et al.* (2001a; 2001b) and

Pommier *et al.* (2002) reported dramatically increased activity in mice, lacking CCK_2 receptors, in samples pooled for sex. Rünkorg *et al.* (2003) have partially replicated the hyperactive phenotype of male CCK_2 receptor deficient mice. However, in a recent study, no difference between wild-type and mutant mice in the activity level was noted (Rünkorg *et al.* 2006). Moreover, Kõks *et al.* (2001) and Weiland *et al.* (2004) have even reported reduced activity as a result of the genetic invalidation of CCK_2 receptors.

While Pommier *et al.* (2002) showed increased pain sensitivity in mutants, as assessed by the jump latency in the hot plate test, Veraksitš *et al.* (2003) revealed a decrease in pain sensitivity in male mutants, although confirming reduction in the hot plate jump latencies. Kurrikoff *et al*. (2004) have also established that male mice lacking CCK_2 receptors have lower mechanical sensitivity and they do not develop hyperalgesia after chronic constriction injury. Along the theme, CCK_2 receptor mutant mice display reduced footshock-induced analgesia and altered neurochemical basis of analgesia (Kurrikoff *et al.* 2008).

Impaired cognitive functions together with reduced rota-rod performance have been reported in CCK_2 receptor deficient mice. Sebret *et al.* (1999) and Daugé *et al.* (2001b) demonstrated impaired memory in mutant mice, using two-trial memory task and Y-maze test, respectively. Also, Daugé *et al.* (2001b), employing a pooled sample of male and female CCK_2 receptor deficient mice, and Kõks *et al.* (2001), employing male mice, established that CCK_2 receptor mutation induces an impairment in the rota-rod performance. Raud *et al.* (2003), though, did not find any alterations in the baseline rota-rod performance of female mice lacking CCK_2 receptors.

CCK_2 receptor invalidation would be expected to affect reward-related behaviours. Indeed, Daugé *et al.* (2001a) reported enhanced morphine sensitisation in CCK_2 receptor deficient mice. Also, Pommier *et al.* (2002) demonstrated increased severity of morphine withdrawal symptoms in mutant mice, but Rünkorg *et al.* (2003) did not find any difference in morphine-induced place preference. On the other hand, Rünkorg *et al.* (2006) have revealed increased sensitisation to amphetamine-induced locomotor effects, but reduced amphetamine-induced place preference in mice lacking CCK_2 receptors. However, no alterations in ethanol consumption have been found in male mice with genetic invalidation of CCK_2 receptors (Miyasaka *et al.* 2005).

From the neurochemical perspective, various groups have agreed that the mice lacking CCK_2 receptors show up-regulation of dopaminergic system (Daugé *et al.* 2001a; Kõks *et al*. 2001), endogenous opioid system (Pommier *et al.* 2002; Veraksitš *et al.* 2003; Rünkorg *et al.* 2003; Kurrikoff *et al.* 2004), and GABA-ergic system (Raud *et al.* 2003; Raud *et al.* 2005). On one hand, these neurochemical alterations suggest interactions between CCK-ergic and dopaminergic, opioid-ergic and GABA-ergic systems. On the other hand, such changes constitute mutation-induced secondary alterations that may be responsible for the observed false positive or false negative behavioural phenotypes.

2. Reproducibility of behavioural studies

Every laboratory has probably encountered the problem of being unable to reproduce certain behavioural findings. Moreover, different laboratories often report different results concerning the same species, strain or drug, producing confusion rather than the confidence needed for further research. In 1999, Crabbe *et al.* addressed the issue of reproducibility of behavioural studies across laboratories (Crabbe *et al.* 1999). In three different testing sites, the behavioural experiments were standardised to the best level that one can imagine. Still, significant interactions between the testing sites and the strains used were observed (Crabbe *et al*. 1999). Although the major differences between the strains had been reproduced in all three laboratories, as discussed by the authors (Crabbe *et al.* 1999) and later by Wahlsten *et al.* (2003), yet, several discrepancies in the results could have produced conflicting interpretations in different testing sites. It was concluded that the tests differed in their propensity to produce variable results with the plus-maze being one of the most problematic (Wahlsten *et al.* 2003). Also, an important determinant of reproducibility was the effect size of strain differences with the small and moderate differences being the most susceptible to environmental modifications (Wahlsten *et al.* 2003). This statement has been backed up by a recent study conducted by Wahlsten *et al.* (2006), which demonstrated that the gross behavioural differences between strains were generally stable both across laboratories and across years. There are several factors that influence the results of behavioural studies. The following is a selection of the most important factors from the scope of the present study.

2.1. Sex

Although the majority of psychiatric disorders show sex-dependent prevalence (Weissman and Klerman 1977; Bekker and van Mens-Verhulst 2007; Zahn-Waxler *et al.* 2008) and there are well-established behavioural differences between male and female rodents (Archer 1975), yet the basic research shows a clear preference toward the use of male subjects. There are several reasons that guide the choice of only male subjects (Cryan and Mombereau 2004). First, the pragmatic reasons: reduction of cost and of variability in results, which may stem from the biological cycles in female rodents. Second, the results of the behavioural research in rodents do not indicate clear prevalence of, for example, depression-like behaviours in female subjects as would be expected from studies in humans. Consequently, different species show distinct differences between male and female subjects. Therefore, the sex-related differences in one species (e.g. human) would not necessarily be represented in another species (e.g. mouse).

Nevertheless, sex is one of the most important factors that influence the results of rodent behavioural studies, using both the pharmacological and

genetic tools. For instance, studies on pain sensitivity in rodents have consistently reported differences between males and females. Analgesia as well as side effects produced by the opioid receptor agonists are generally stronger in male than in female rodents (Kest *et al.* 2000). Also, female rats experience lower levels of stress-induced analgesia (Romero and Bodnar 1986). Moreover, the neurochemical mechanisms of stress-induced analgesia differ between male and female rodents. Both dizocilpine (MK–801), an NMDA receptor antagonist, and naloxone, an opioid receptor antagonist, block the swim stress-induced analgesia in male mice, but neither compound has any effect on analgesia in normal female mice (Mogil *et al.* 1993). The differences between sexes in pain sensitivity and opioid-induced analgesia have been reported to be absent in mice lacking GIRK2 channel, thus, suggesting a contribution of GIRK2 in sex-related differences in nociception (Mitrovic *et al.* 2003). It should be emphasised, also, that the nociceptive thresholds in female rats depend on the phase of estrous cycle, with peak pain thresholds apparent in diestrus (Frye *et al.* 1993).

The reports concerning the sex influences on anxiety-like behaviour and on depression-like behaviour are less consistent. Johnston and File (1991) reported test-dependent anxiety levels in female hooded Lister rats. According to the classic interpretation of results, female rats were significantly more anxious in the social interaction test and the Vogel punished drinking test, while male rats were more anxious in the plus-maze test. Rodgers and Cole (1993) indicated that the male DBA/2 mice were more anxious than their female counterparts, but this sex-dependent difference was not observed in the T1 strain. Also, sex effects have been reported for the tail suspension test (Liu and Gershenfeld 2001), and sex by strain interaction has been observed in the learned helplessness paradigm in mice (Caldarone *et al.* 2000). Võikar *et al*. (2001) described sex-related differences in 129, B6 and FVB strains, but also in the two intercrosses between these strains, namely 129B6 and 129FVB. They found a number of sex-related differences in the anxiety-like behaviours, locomotor activity, depression-like behaviour, and coordination and balance. However, these differences were not uniform, depending substantially on the strain and on the test used (Võikar *et al.* 2001). Also, sex-related factors affect the effects of drugs (Caldarone *et al.* 2003) and substantially modify the phenotype of transgenic mice (Ramboz *et al.* 1998; Walther *et al.* 2000; Bale and Vale 2003).

The sex-dependent differences have been evident in mice, lacking CCK_2 receptors. Chen *et al.* (2006a) indicated that female mice, lacking CCK_2 receptors were significantly more overweight than their male counterparts. The impairment of rota-rod performance has been observed in male (Kõks *et al.* 2001), but not in female mutants (Raud *et al.* 2003). Although Daugé *et al.* (2001b) initially reported, that sex did not significantly affect the phenotype of mice, lacking CCK_2 receptors, several groups have thereafter clearly preferred subjects of certain sex depending on the aims of their study. For example, studies on anxiety and the properties of GABA-ergic system in CCK_2 receptor deficient mice have been conducted in female mice (Raud *et al.* 2003; Raud *et*

al. 2005; Areda *et al.* 2006). On the other hand, male subjects have been used to reveal the locomotor, pain-related and reward-related phenotypes of mice lacking CCK_2 receptors (Kõks *et al.* 2001; Rünkorg *et al.* 2003; Veraksitš *et al.* 2003; Kurrikoff *et al.* 2004; Miyasaka *et al.* 2005; Rünkorg *et al.* 2006; Kurrikoff *et al.* 2008). Thus, the regular preference of one sex over another in different studies has resulted in a puzzling situation where the effects of the genetic invalidation of CCK_2 receptors had not been fully covered, and, therefore, our understanding of the mutation-induced effects in the opposite sex has often been based on speculations.

2.2. Background strain

Most typically the genetic manipulation is carried out in the embryonic stem cells derived from the 129 or FVB strain. Thereafter, the mutant line is back-crossed to some other strain (e.g. C57BL/6) in order to obtain a line more suitable for behavioural studies. However, soon after the advent of transgenic technologies in the field of behavioural neuroscience, concerns regarding background strain rose (Gerlai 1996; Silva *et al.* 1997). Distinct mouse strains have been shown to behave differently to a considerable extent (Crawley *et al.* 1997; Crabbe *et al.* 1999; McIlwain *et al.* 2000; Võikar *et al*. 2001), and, thus, the modification of the mutation-induced effects could be expected by different background strains. Indeed, there is a bulk of evidence showing that mutation-induced effects can appear or disappear when back-crossed to a different strain. The anxious phenotype was observed when serotonin transporter null-mutants were back-crossed to B6 strain, but this phenotype disappeared when the mutant line was back-crossed to 129 strain (Holmes *et al.* 2003). Similar findings have been reported for dopamine D_2 receptor deficient mice (Kelly *et al.* 1998), serotonin $5\text{-}HT_{1B}$ receptor knock-out mice (Crabbe *et al.* 1999), neuronal oxide synthase knock-out mice (Le Roy *et al.* 2000) and dopamine transporter null-mutant mice (Morice *et al.* 2004). Also, genes flanking the targeted locus and originating from the embryonic stem cells have been shown to affect the behavioural phenotype of mutants (Bolivar *et al.* 2001). Schalkwyk *et al.* (2007) have recently reported a difference in expression of at least 10 genes flanking the CCK_2 receptor locus in mice, lacking CCK_2 receptors. Each of these genes could be responsible for the phenotypic alterations observed in CCK_2 receptor deficient mice. Moreover, several compensatory changes or secondary phenotypic alterations may occur as a result of a mutation (Gerlai 2001). Thus, the phenotypic changes may be masked by the altered functions of other genes (the false negative), or, conversely, an established phenotype may be caused by the changes in the functions of other genes rather than the mutation itself (the false positive).

In addition to the modifications of phenotypes by background strain in the transgenic studies, distinct strains react differently in pharmacological studies.

B6 strain is known for its high ethanol intake, while DBA strains for very low ethanol intake (Belknap *et al.* 1993). However, the DBA strain is one of the most sensitive to the locomotor effects of both ethanol and cocaine (Crawley *et al.* 1997; Lessov *et al.* 2001). The 129 strain, on the other hand, is quite sensitive to locomotor effects of cocaine but does not develop cocaine-induced place preference as easily as B6 strain (Miner 1997). B6 strain, again, is the most sensitive to the locomotor and analgesic effects of opiates (Crawley *et al.* 1997). Liu and Gershenfeld (2001) reported substantial differences in the responses to antidepressant imipramine between different strains in the tail suspension test. While DBA/2J and FVB/NJ strains showed prominent reduction of immobility in response to imipramine administration in the tail suspension test, B6 and 129 strains showed only modest drug effect. DBA/2J seems to be one of the most receptive strains to the antidepressant effects in the forced swim test as well (Lucki *et al.* 2001). Also, certain differences exist between the strains in their sensitivity to the anxiolytic effects of benzodiazepine drugs with B6 being a rather sensitive and DBA/2J an insensitive strain (Crawley *et al.* 1997; Griebel *et al.* 2000).

Importantly, distinct strains react differently to several environmental factors. For instance, Belzung *et al.* (2001) reported substantial differences between strains in their response to such natural stressor as cat odour. Similarily, Raud *et al.* (2007) demonstrated that while B6 strain clearly reacted to cat odour as evidenced by behavioural adaptation and changes in wolframin gene expression, 129 strain was completely unreactive. It has been speculated that the genetic influence of 129 strain might be responsible for the ameliorated response to cat odour in wild-type littermates of mice lacking CCK_2 receptors (Areda *et al.* 2006; Raud *et al.* 2007). Therefore, it is likely that the observed increase in sensitivity of female CCK_2 receptor deficient mice to cat odour is specific to the current genetic background.

2.3. Environmental conditions

Roughly speaking, environmental conditions can be divided into two categories: the conditions that the animals have experienced during their lifetime (e.g. aversive early life experience, environmental chemicals, social isolation and testing experience), and the conditions during the testing (e.g. test characteristics, odours, room temperature and seasonal factors).

Differences in the life history of experimental subjects may substantially interfere with the results of a study. For instance, stressors occurring during critical periods of development have long-term effects on the behavioural as well as physiological readouts. For instance, prenatal stress by means of restraining mothers has been shown to cause HPA axis hyper-reactivity and to increase immobility in the forced swim test in adult rats (Morley-Fetcher *et al.* 2003). Moreover, prenatal stress has sexually dimorphic effects on behaviour in

adult rats (Weinstock 2007). Zuena *et al.* (2008) reported, that in male rats, subjected to prenatal stress, increased anxiety and reduced neurogenesis were observed, whereas in stressed females, reduced anxiety, better water maze performance and no alteration in neurogenesis were evident. Also, compared to unstressed rats, prenatally stressed animals showed altered sensitivity to the effects of various psychoactive drugs (Darnaudery and Maccari 2008). Characteristics of maternal care during early life are a powerful determinant of behavioural and endocrine responses to stress in adult rodents (Liu *et al.* 1997; Caldji *et al.* 1998). Thus, the differences in maternal care induced, for instance, by a gene mutation, may significantly alter the behaviour of the experimental subjects. Several manipulations with the postnatal rodents by the experimenter have been shown to produce substantial effects that last into adulthood. For example, handling of rat pups for 3–15 minutes daily reduces fearfulness and stress responses in the adult rats (Meaney *et al.* 1991). Conversely, prolonged daily separation of pups from mothers increases stress responses and depressive-like behaviour in the offspring (Ladd *et al.* 2000; El Khoury *et al.* 2006). Additionally, stressing lactating mothers has been shown to have long-term effects on the behaviour of pups as well (Moles *et al.* 2004).

Rodents are social animals (van Loo *et al.* 2003). Yet, long-term social isolation or individual housing of rodents is often used in laboratories. Social isolation can substantially interfere with the results of the behavioural tests, though. Long-term individual housing leads to the so-called "social isolation syndrome" (Valzelli 1973) that is characterised by locomotor hyperactivity, enhanced behavioural responses to novel environments, deficits in prepulse inhibition, increase in anxiety and aggressiveness, and altered responses to drugs like barbiturates, benzodiazepines, opiates, and dopamine agonists and antagonists (Sahakian *et al.* 1975; Gentsch *et al.* 1982; Phillips *et al.* 1994; Wongwitdecha and Marsden 1996; Coudereau *et al.* 1997; Domeney and Feldon 1998; Miczek *et al.* 2001). Social isolation causes prominent neuro-chemical alterations in the GABA-ergic, dopaminergic and serotonergic systems (Bickerdike *et al.* 1993; Wongwitdecha and Marsden 1996; Fulford and Marsden 1998a; Fulford and Marsden 1998b). Also, the up-regulation of the CCK-ergic system in response to social isolation has been described (Vasar *et al.* 1993; Del Bel and Guimaraes 1997). Importantly, Võikar *et al.* (2005) established that social isolation produced strain-specific effects in mice of B6 and DBA strains. Body weight gain as well as the results of the novel object recognition, fear conditioning and forced swim tests were affected by social isolation in strain-specific manner (Võikar *et al.* 2005), indicating substantial interactions between the environmental manipulation and the strain.

Enriched environments have long been known to affect rodent behaviour (Hebb 1947). Environmental enrichment refers to housing conditions that facilitate enhanced sensory, cognitive and motor stimulation (Nithianantharajah and Hannan 2006). Enrichment can be applied by adding different objects to the housing cages of the experimental subjects. Alternatively, daily access of

subjects to cages with complex environments can be used. Henderson *et al.* (1976) demonstrated that even short exposure (6 hours) to enriched environment could substantially alter the performance of mice in a food seeking task. Environmental enrichment has been shown to significantly affect the phenotype in several mouse strains (van de Weerd *et al.* 1994; Tucci *et al.* 2006). Therefore, it could be expected that enrichment would alter the phenotypes in genetically modified rodents. Indeed, Rampon *et al.* (2000) reported that housing in enriched environment attenuated the deficits in non-spatial memory induced by the hippocampus CA1 region-specific mutation of the NMDA receptor 1 subunit. Similar effects of enriched housing conditions have been reported for the Huntington's disease R6/1 mice (van Dellen *et al.* 2000), the mice under-expressing the beta-amyloid precursor protein (Tremml *et al.* 2002) and phospholipase C-beta1 knock-out mice (McOmish *et al.* 2008). An apparent problem with the use of environmental enrichment is, that the protocols vary to a significant extent across laboratories (Fox *et al.* 2006; Nithianantharajah and Hannan 2006). Different laboratories use different cage sizes, objects, numbers of objects, frequencies of changing objects, group sizes per cage etc. Still, the majority of studies agree that enrichment produces a robust improvement of cognitive as well as fine motor abilities in rodents. These improvements are accompanied by increased brain weight, cortical thickness, neurogenesis and dendritic branching, and decreased apoptosis (Chapillon *et al.* 2002; Lewis 2004; Nithianantharajah and Hannan 2006). However, there are also several inconsistencies in the reports on enrichment, for example, those concerning emotional outcomes (Chapillon *et al.* 2002).

Pre-experimental stress and the novelty of testing situation significantly affect the behavioural readouts. Novelty has been reported to increase hot plate latencies in rodents, an effect similar to stress-induced analgesia (Siegfried *et al.* 1987). Daugé *et al.* (1989) demonstrated that CCK-8 decreased locomotion in rats only if the animals had not been habituated to the testing situation. Furthermore, an unselective CCK receptor agonist cerulein failed to produce anxiety-like behaviour if rats had been made familiar with the experimental room (Kõks *et al.* 2000). On the other hand, Lavigne *et al.* (1992) revealed that the CCK receptor antagonists potentiated morphine-induced analgesia only in rats that were not habituated to the testing situation.

Several researchers have promoted the use of testing batteries to meet the demands of high-throughput testing employing minimal numbers of animals as is often needed in transgenic studies (Crawley and Paylor 1997). However, repeated testing in either the same paradigm or in different paradigms has been shown to significantly influence the behavioural results. For example, repeated testing in the plus-maze leads to the reduction in exploratory activity and to the loss of anxiolytic effect of diazepam in rats and mice (File *et al.* 1993; Rodgers and Shepherd 1993). Moreover, repeated testing is deliberately used in the modified forced swim test in rats since the pre-conditioning swim 24 hours before the test swim enables to increase passive floating during the latter and to

improve the sensitivity of the test to detect antidepressive compounds (Cryan and Mombereau 2004). The use of testing batteries where different paradigms are combined slightly differs from simple repeated testing. While repeated testing in the same paradigm leads to habituation to the specific testing situation, testing in distinct paradigms results in habituation to the components of testing (e.g. novelty, transportation, handling, and cognitive or motor challenges). McIlwain *et al.* (2001) and Võikar *et al.* (2004) have reported that although the behavioural differences between distinct strains can be reproduced when employing testing batteries, yet, this approach significantly affects the results in tests like the plus-maze, hot plate, forced swim and locomotor activity.

Also, the diurnal variation of behaviour has to be taken into account when testing rodents. Hossain *et al.* (2004) demonstrated that the ability of behavioural tests to discriminate genetically distinct mouse strains depends much on the light-dark cycle phase. Interestingly, Weiland *et al.* (2004) reported that mice, lacking CCK_2 receptors, were hyperthermic and hypoactive, but these phenotypes depended on the phase of the light-dark cycle. Namely, hyperthermia was evident only during the photophase, whereas reduced locomotor activity was observed only during the scotophase (Weiland *et al.* 2004). In addition to the light-dark cycle, seasonal variables have been suggested to affect the behaviour of laboratory rodents. Kõks *et al.* (2000) demonstrated that the exploratory activity of rats was significantly higher in November when compared to the exploratory activity in July. This finding was accompanied by seasonal differences in the binding properties of CCK and serotonin receptors in the brain (Kõks *et al.* 2000).

Also, prominent differences in the results may arise from tests that may deceptively seem to measure the same behavioural processes. For example, tail suspension test has been sometimes regarded as simply the "dry" version of the forced swim test. Nevertheless, the tail suspension test lacks several characteristics of the forced swim test (for example, tail suspension produces hyperthermia instead of hypothermia) (Cryan *et al.* 2005). Different drugs are known to produce considerably different effects in the tail suspension test and in the forced swim test. Bai *et al.* (2001) reported that the dose-response curves of imipramine differed between the two tests. Also, the behavioural differences between two mouse strains in the forced swim test are not exactly reproducible in the tail suspension test (Bai *et al.* 2001; Mombereau *et al.* 2004). The same test-dependent variation in the results applies to tests measuring anxiety-like behaviours. Despite certain similarities between the tests (e.g. plus-maze, zero-maze and light-dark exploration test), substantial discrepancies in the results produced either by drug administration (Griebel *et al.* 2000; Mathiasen *et al.* 2008) or by genetic manipulation (Jacobson *et al.* 2007) have been reported depending on the test used. It is also important to bear in mind, that the same test can be carried out using rather different methodologies. For example, two laboratories reported quite different results when assessing sensitivity to

diazepam in mice with mutated $GABA_A$ receptor α_1 subunit. However, when the testing methods were standardised between the laboratories discrepancies in the results vanished (Crestani *et al.* 2000). Also, Haller *et al.* (2004) reported that the anxiogenic-like effect of cannabinoid CB1 receptor gene disruption in mice was context-specific. Namely, mutant mice differed from wild-type controls only in the plus-maze test performed under high illumination level (i.e. aversive condition) but not low illumination level (Haller *et al.* 2004).

3. Characterisation of C57BL/6 and 129S6/SvEv strains

The need for establishing reliable phenotypes of mutant lines has emerged in parallel with the progress in transgenic techniques. Due to the conflicting results regarding the same mutants back-crossed in different genetic backgrounds, the concerns about the contribution of the genetic background have arisen (Gerlai 1996; Silva *et al.* 1997; Beck *et al.* 2000). Numerous studies have been published on the phenotypes of the most prevalent strains used in transgenic studies. Since mice from 129 sub-strains are most commonly used in gene targeting, whereas mice from C57 sub-strains are most commonly used for backcrossing the mutant lines in order to improve behavioural performance, a lot of attention has been paid to these two strains (Crawley *et al*. 1997). Both 129 and C57 are inbred mouse strains (Beck *et al.* 2000) and both have several sub-strains that differ one from another considerably in genetic and behavioural terms (Threadgill *et al.* 1997; Crawley *et al.* 1997; Cook *et al.* 2002). Nevertheless, 129 strains have been consistently reported to display increased anxiety-like behaviour as well as reduced exploratory activity in the plus-maze test, reduced locomotor activity, longer hot plate latencies and larger proportion of time spent immobile in the forced swim test when compared to C57 strains (Crawley *et al.* 1997; McIlwain *et al.* 2001; Võikar *et al.* 2001; Rodgers *et al.* 2002; Bothe *et al.* 2004; Võikar *et al.* 2004). The differences between the 129 and C57 strains in open field behaviour and in ethanol consumption have been reliably reproduced by different laboratories (Crabbe *et al*. 1999). Moreover, the behavioural dissimilarities between the two strains have been generally reproducible independent of subjects' sex (Võikar *et al.* 2001) or study design (McIlwain *et al.* 2001; Võikar *et al.* 2004).

4. Concluding remarks

Although pharmacologic studies have clearly demonstrated that CCK_2 receptors in the CNS participate in the regulation of anxiety-like behaviour, the locomotor activity, the reward-related behaviours, the pain regulation and the cognitive functions, the studies employing mice with genetic invalidation of CCK_2 receptors have provided results that have not always supported these findings. Moreover, the phenotype of mice lacking CCK_2 receptors has varied across laboratories to a considerable extent. Importantly, though, the pharmacologic studies have also demonstrated that the effects of compounds acting on CCK receptors substantially depend on the sex of experimental subjects and on various environmental factors (e.g. habituation to testing situation). However, the contradiction of results across laboratories is certainly not confined to studies on CCK-ergic system, being rather a more general phenomenon. The behavioural results are significantly affected by many factors, including species, genetic background, sex and a number of environmental conditions. The impact of various factors has been more than clear in studies using genetically manipulated rodents. There is a large body of evidence suggesting that the resulting phenotype in transgenic animals is subject to a variety of influences other than the manipulation itself. These influences may lead to either false positive or false negative findings that can substantially confuse researchers trying to establish the functions of affected genes.

In the present study, an attempt was made to determine the contribution of sex and altered environmental conditions to the phenotype of mice lacking CCK_2 receptors. It was hypothesised that because the effects of compounds acting on CCK receptors depend on both sex- and environment-related factors, the behavioural findings in CCK_2 receptor deficient mice would also be affected by these factors. Also, it was taken into account that the sensitivity of the genetic manipulation to various factors *per se* may influence the expression of the behavioural phenotype. An indirect support to these assumptions has been provided by previous studies from different laboratories reporting variable phenotypes in mice lacking CCK_2 receptors, and thus indicating influences from factors other than the mutation itself.

AIMS OF THE STUDY

The general goal of the present study was to reveal the contribution of sex and pre-experimental housing conditions to the behavioural phenotype of cholecystokinin CCK_2 receptor deficient mice. For that purpose male and female mice were used in behavioural studies. Also, different environmental conditions were applied by means of social isolation and environmental enrichment. In order to compare the effects of environmental enrichment in CCK_2 receptor deficient mice to the effects of enrichment in genetically distinct mouse lines C57BL/6 and 129S6/SvEv/Tac strains were used. The more specific aims of the present study were as follows:

1. To study the effects of pre-experimental social isolation on the behavioural phenotype of mice lacking cholecystokinin CCK_2 receptors. Also, the contribution of sex to the phenotype in mutants housed in different conditions was identified. In addition to the behavioural phenotype, the sex-dependent parameters of dopamine D_2 and serotonin 5-HT_2 receptors were revealed in group-housed CCK_2 receptor deficient mice.
2. To establish the influence of sex as a factor on behavioural effects of ethanol in cholecystokinin CCK_2 receptor deficient mice. Also, ethanol intake and blood ethanol kinetics were investigated in mutant mice of both sexes.
3. To reveal the relation of the behavioural phenotype in mice lacking cholecystokinin CCK_2 receptors to environmental factors by means of pre-experimental environmental enrichment.
4. To study the reproducibility of behavioural phenotypes in two genetically distinct mouse strains C57BL/6 and 129S6/SvEv/Tac housed in different conditions. Environmental enrichment was used as the alternative pre-experimental housing condition to standard housing conditions.

MATERIALS AND METHODS

1. Animals

In Papers I, II and III, mice lacking CCK_2 receptors were used. Mutant mice were provided from the original background 129Sv/C57BL6 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 (Kõks *et al.* 2001). Male and female homozygous (-/-) CCK_2 receptor deficient and wild-type (+/+) mice (10–12 weeks old) were used in the behavioural experiments. Mutant mice were crossed back six times (Papers I, II) or more than fifteen times (Paper III) to the C57BL/6 background to minimise the possible genetic effects from the 129Sv strain. 8–11 mice were housed per cage (except for Papers I and II, where a part of subjects were individually housed). In Paper IV, C57BL/6 (B6, Scanbur) and 129S6/SvEv/Tac (129, Taconic) mice were used, since they have been shown to display substantially different behavioural profiles (Rodgers *et al*. 2002) even independent of previous testing history (McIlwain *et al*. 2001; Võikar *et al*. 2004) or sex factors (Võikar *et al*. 2001). Breeding was performed in the University of Tartu, Estonia. 7–8 mice were housed per cage. In all experiments, mice were kept in the animal house at 22±1 °C under a 12:12 h light/dark cycle (lights off at 19:00 h). Tap water and food pellets were available *ad libitum*. Behavioural testing was carried out between 13:00 and 19:00 of the light phase.

2. Environmental manipulations

2.1 Social isolation (Paper I)

At the age 90 days, half of mice were isolated into the cages (330 × 120 × 130 mm) for 21 days, while their age-, sex- and genotype-related littermates were kept in groups of 8–11 (cage size 425 × 266 × 155 mm). Weighing procedures were carried out on days 1, 7, 14 and 21 of isolation. All through 22 days of isolation plus testing the housing conditions were kept unchanged.

2.2 Environmental enrichment (Papers III, IV)

After weaning at 3 weeks half of the mice were randomly allocated to either standard or enriched conditions for 7 weeks before the start of experiments. 7–10 mice were housed per cage. Standard housing conditions consisted of standard laboratory cages (425 × 266 × 155 mm) with bedding. Mice in

environmentally enriched conditions were housed in larger cages (595 × 380 × 200 mm) containing bedding, nesting material, one stainless steel running-wheel, one swing, one house, one igloo, two ladders, three tubes or two labyrinths. The house, igloo, ladders, tubes and labyrinths were made of aspen. The setup of an enriched cage consisted of five different objects at a time, always including nesting material, a running wheel and a shelter (for instance, the nesting material, the running wheel and an igloo combined with two ladders and two labyrinths). The running-wheel was repositioned once a week during the maintenance procedures, but never removed from the cage. The nesting material was repositioned once a week, and new material was added to keep the bunch size constant. The remaining three objects were changed and repositioned once a week.

3. Behavioural studies

3.1. Elevated plus-maze test (Papers I, II, III, IV)

The apparatus consisted of two opposite open (175 × 50 mm) arms without sidewalls and two enclosed arms of the same size with 140 mm high sidewalls and an end wall. The entire plus-maze apparatus was elevated to a height of 300 mm and placed in brightly lit room (400 lx in open arms; Papers I and II) or dimly lit room (20 lx in open arms; Papers III and IV). Pre-experimental single housing for 15–30 min was employed in order to increase exploratory activity (Papers I, II, III). Testing began by placing a mouse on the central platform facing an open arm. Standard 5 min test duration was used, and the maze was thoroughly cleaned with damp and dry towels between the subjects. Test sessions were video-recorded and the videotapes were analysed by a trained observer unaware of testing conditions. The following parameters were observed: number of closed arm entries; total number of arm entries, entries on the open arms; ratio between open and total arm entries or % open entries of total entries; time spent exploring open arms or time spent on the open arms expressed as % of total time spent on open and closed arms; number of unprotected head-dips; number of attempts to exit closed arms; number of line crossings; number of stretched-attend postures; number of rearings; number of groomings.

3.2. Locomotor activity (Papers I, II, III, IV)

For the study of locomotor activity the animals were placed singly into sound-proof photoelectric motility boxes (448 mm × 448 mm × 450 mm) connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany) for 5 min (Paper I) or 30 min (Papers II, III, IV). The illumination level of the

transparent test boxes was ~400 lx. After removing the mouse from the box, the floor was cleaned with damp towels and dried thoroughly. Time in locomotion, distance travelled (m), number of rearings, number of corner entries and time (s) spent in the central part of the motility boxes were registered. In Paper IV, the test was repeated on 3 consecutive days to estimate habituation to the motility boxes.

3.3. Forced swim test (Paper III and IV)

The forced swim test is a method to estimate behavioural despair in stressful and inescapable situation (Porsolt *et al*. 1977). The mouse was placed for 6 min in a glass cylinder (diameter 120 mm, height 240 mm, water depth 150 mm) filled with water at 25±0.5 ºC (Paper III) or 23±0.5ºC (Paper IV, according to Võikar *et al*. 2004). Test sessions were video-recorded and the videotapes were subsequently analysed by a trained observer unaware of testing conditions. The time (s) of immobility (passive floating, when the animal was motionless or doing only slight movements with tail or one hind limb) was measured during the last 4 min of the test.

3.4. Hot plate test (Papers III and IV)

Hot plate test was carried out for the assessment of pain sensitivity. The plate was heated to 52ºC and the mouse was confined there by a plexiglass cylinder (diameter 150 mm, height 200 mm). Latency (s) to show hind paw response (licking or shaking) and latency (s) to jump from the plate were measured. The cut-off time was set at 120 s.

3.5. Resident-intruder test (Papers I and IV)

The subject was placed alone for 5 min (Paper I) or 30 min (Paper IV) in a small housing cage (267 × 207 × 140 mm) with clean bedding and cover made of transparent plexiglass. After habituation period an unfamiliar age- and strain-matched standard opponent (the "intruder") was exposed to the subject (the "resident"). Animal behaviours were videotaped during 5 min with camera positioned above the testing cage. In Paper I social (first attack latency, number of attacks and duration of social interaction) as well as non-social (number of diggings, rearings and groomings) behaviours were recorded during 5 min observation period. Total time in social interaction (s) and the number of attacks performed by the resident were scored in Paper IV.

3.6. Loss of righting reflex (Paper II)

Mice were given an intraperitoneal injection of 4.0 g/kg of ethanol (20 % v/v). At the onset of ethanol-induced sedation (the loss of righting reflex), each mouse was placed on its back in a V-shaped paper-trough. Time (s) between the injection and the loss of righting reflex and time (min) between the loss of righting reflex and the regain of righting reflex defined as the ability to right itself on all four paws 3 times within a 30 sec interval, were taken. Tail blood samples (5 µl) were collected at the regain of righting reflex to determine blood ethanol concentration. Blood samples were analysed immediately by enzymatic colour test using LKM 139 and miniphotometer LP 20 (Dr Bruno Lange GmbH, Germany) according to manufacturer's instructions.

3.7. Ethanol intake test (Paper II)

50 ml plastic tubes with tips cut off were used for ethanol intake and taste preference tests. Tubes were controlled for leakage for 7 days and subsequent intake measurements were adjusted for leakage. Throughout the experiment, total fluid and food intake, and body weight were measured every 7 days. Prior to testing mice were housed individually and were habituated to drinking from two tubes containing plain water for 7 days. Mice were then given 24 h access to two tubes, one containing plain water and the other containing ethanol in water. The concentration of ethanol (v/v) was increased every 7 days. Initially, mice received 3 %, followed by 6 % and finally 10 % ethanol solution. The positions of the tubes were counterbalanced between groups and changed every 2 days to control for position preference. Average ethanol consumption (calculated in grams per kilogram of body weight per day) was obtained for each ethanol concentration by weighing tubes at the beginning and end of the exposure. As a measure of relative ethanol preference, an ethanol preference ratio was calculated for each ethanol concentration by dividing total ethanol solution consumed by total fluid (ethanol plus water) consumption. Food intake was calculated weekly at every ethanol concentration (expressed as grams per kilogram of body weight per day) by weighing food granules at the beginning and end of the exposure to ethanol. All spillage was collected and included in calculations.

3.8. Taste preference (Paper II)

Ten days after the end of ethanol consumption testing, the same mice used in alcohol intake test were given *ad libitum* access to two tubes, one containing plain water and the other a solution of sucrose or quinine. The compounds were presented in the following order: sucrose solutions (1.7 % and 4.3 %) followed

by quinine solutions (0.03 mM and 0.10 mM). Mice had 48 h access to each solution, the position of solutions was counterbalanced between groups and switched 24 h after presentation. Millilitres of solution consumed per kilogram of body weight per day and preference for either compound were measured and calculated as described in the previous section.

3.9. Restraint-induced analgesia (Paper III)

Restraint stress was induced by placing a mouse in 50 ml plastic tube for 30 min. Adequate ventilation was provided by means of holes on the tip and the sides of the tube. Remaining space in the tube was filled with soft clean paper towel swab to restrict animal's movements. Every effort was made to spare animals of any stress but restraint itself. The nociceptive threshold was determined using radiant heat tail-flick system (Plantar test 7371, TSE Systems, Germany). The tail-flick response was tested immediately before the restraint procedure (baseline) and immediately after termination of the procedure. Cut-off time was set at 30 s to avoid tissue damage.

3.10. Rota-rod (Paper III)

The equipment consisted of a motor-driven drum (30 mm in diameter) rotating at fixed speed (18 rpm). Since in an earlier experiment a substantial proportion of mice held on to the drum and rotated around the rod, but did not fall down, producing a false negative result, the latency (s) to either fall from the drum or to make the first complete revolution was recorded as described by McIlwain *et al.* (2001). The time of maximal performance was set at 120 s. 2 h before the test trial animals were shortly habituated to the rod rotating at 9 rpm.

3.11. Water maze (Paper III)

The water maze (TSE Systems, Germany) consisted of a circular pool (1500 mm in diameter), escape platform (160 mm in diameter), video camera and computer. The pool was filled with tap water (22°C) to a depth of 380 mm. The water was made opaque with the addition of a small amount of non-toxic white putty. The escape platform, positioned in the centre of the Southwest quadrant (Q2), 200 mm from the wall, remained in a fixed position. The water level was 10 mm above the platform, making it invisible. Each trial, the animals were put into the water, facing the wall, at pseudo-randomly assigned starting positions (East, North, South or West). The acquisition phase of the experiment consisted of a series of 20 training trials, lasting up to 60 s each (5 trials per day for 4 consecutive days, inter-trial interval 1 hour). Mice were allowed to search

for the platform for a maximum of 60 s at which time the mice were gently guided to the platform by means of a metal sieve. Mice remained on the platform for 15 s. Posters and furniture around the maze served as visual cues. During testing, the room was dimly lit with diffuse white light (20 lux). The latency (s) to find the submerged platform, the distance (m) travelled during the trial and swim velocity (cm/s) were registered. The average value per day obtained by collapsing data on 5 trials for each animal was used. On day 4, one hour after the last training trial, the platform was removed for a probe trial. Mice were put into the water in the Northeast position (Q4) and were allowed to swim for 60 s. The time (s) spent in all 4 quadrants (Q1, Q2, Q3, Q4) was measured, with time spent in target quadrant (Q2) where the platform had been located serving as indicator of spatial memory.

4. [^{3}H]-spiperone binding studies (Paper I)

For the radioligand binding studies group-housed mice were used. These animals were not exposed to behavioural testing. After decapitation, brains were quickly dissected on ice. The frontal cortex and sub-cortical structures (including the striatum, nucleus accumbens, tuberculum olfactorium and septum pellucidum) were dissected. The brain structures from six mice were pooled and the experiment was repeated three times. The sub-cortical structures were used for studying of dopamine D_2 receptors, whereas in the frontal cortex serotonin 5-HT_2 receptors were studied. The radioligand binding studies were performed according to Kõks *et al*. (1997). For labelling of dopamine D_2 and 5-HT_2 receptors [^{3}H]-spiperone (specific activity 107 Ci/mmole, Amersham Radiochemicals) was used. The parameters of dopamine and serotonin receptors were determined in the presence of 0.06–2 nM [^{3}H]-spiperone at 37 °C for 30 min. Raclopride (Astra, 1 μM), an antagonist of dopamine D_2 receptors, was added to the incubation mixture to determine the non-specific binding at dopamine D_2 receptors. Ritanserin (RBI, 1 μM), an antagonist of 5-HT_2 receptors, was used to detect the non-specific binding of 5-HT_2 receptors. The brain tissue was homogenised in 20 ml of 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 for the appropriate binding assay. The protein content was measured according to the method of Bradford (1976). The saturation curves of [^{3}H]-spiperone binding were analysed using GraphPad Prism (Version 3.00) for Windows software.

5. Determination of blood ethanol concentrations (Paper II)

In a separate experiment, designed to determine the blood ethanol kinetics in mice, 5 μl of blood was taken from the tail vein 30, 60, 120 and 240 minutes after intraperitoneal injection of ethanol (2.0 g/kg or 4.0 g/kg 20 % v/v, diluted in physiological saline). Blood samples were analysed immediately by enzymatic colour test using LKM 139 and miniphotometer LP 20 (Dr Bruno Lange GmbH, Germany) according to manufacturer's instructions.

6. Statistical analysis

Results are expressed as mean values ± S.E.M. Statistica for Windows 6.0 (Papers I, II) and 7.0 software (Papers III, IV) were used for statistical analysis.

Paper I: the results of behavioural studies were analysed using three-way ANOVA (genotype × sex × isolation). The data of radioligand binding studies with [^{3}H]-spiperone were treated by means of two-way ANOVA (genotype × sex). Post hoc comparisons between individual groups were performed by means of Newman-Keuls test.

Paper II: the results of the elevated plus-maze and locomotor activity tests were analysed using three-way independent-groups ANOVA (genotype × sex × ethanol treatment). The loss of righting reflex and blood ethanol concentration at regain of righting reflex were analysed by means of two-way independent-groups ANOVA (genotype × sex). Three-way mixed-design ANOVA (genotype × sex × concentration) with 2 between-subjects variables (genotype, sex) and 1 within-subjects variable (concentration) was used to analyse the results of the ethanol intake test, and preference for sucrose and quinine. Finally, four-way mixed-design ANOVA (genotype × sex × ethanol dose × time) with 3 between-subjects variables (genotype, sex, ethanol dose) and 1 within-subjects variable (time) was applied to analyse blood ethanol concentrations. Post hoc comparisons between individual groups were performed by means of Tukey HSD test for either equal or unequal sample sizes.

Paper III: the results of the plus-maze, locomotor activity, forced swim test, hot plate and rota-rod were analysed using three-way independent-groups ANOVA (genotype × sex × housing condition). The probe trial of the water maze was analysed using four-way independent-groups ANOVA (genotype × sex × housing condition × quadrant). Restraint-induced analgesia as well as the acquisition phase of the water maze were analysed using mixed design four-way ANOVA with 3 between-subjects variables (genotype × sex × housing condition) and 1 within-subjects variable (restraint and day, respectively). Since in the plus-maze, restraint-induced analgesia and water maze no differences between male and female mice were found, data on male and female mice were

pooled to increase statistical power. Post hoc comparisons between individual groups were performed by means of Newman-Keuls test.

Paper IV: the results of the plus-maze, hot plate, forced swim and resident-intruder test were analysed using two-way independent-groups ANOVA (strain × housing condition). Locomotor activity across 3 days was analysed using mixed design three-way ANOVA with 2 between-subjects variables (strain × housing condition) and 1 within-subjects variable (day). Post hoc comparisons between individual groups were performed by means of Tukey HSD test.

RESULTS

1. Experiment 1: The anxiety-like behaviour in the plus-maze is affected by the genetic invalidation of CCK_2 receptors in sex- and environment-dependent manner

1.1. Weight gain

Three-week isolation induced different alterations in the weight gain of male and female mice (Figure 1). This effect was not significantly modified by the genotype. In the end of the first week changes related to sex and isolation were evident (three-way ANOVA: sex × isolation: isolation: $F(1,74)=10.5$, $p<0.01$; sex: $F(1,74)=20.1$, $p<0.01$). However, the application of post hoc comparisons did not establish differences between the individual groups. In the end of the second week the use of three-way ANOVA demonstrated that social separation had caused a significant sex-dependent effect on the weight gain (three-way ANOVA: sex × isolation: $F(1,74)=8.58$, $p<0.01$; isolation: $F(1,74)=15.1$, $p<0.01$; sex: $F(1,74)=7.62$, $p<0.01$; genotype: $F(1,74)=7.38$, $p<0.01$).

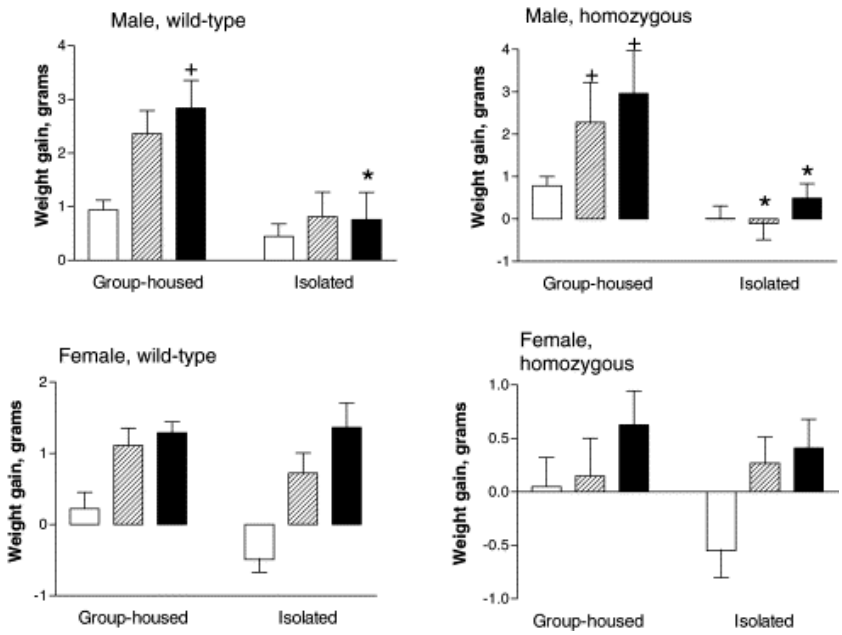

Figure 1. Effect of social isolation on the weight gain in male and female CCK_2 receptor deficient mice. White bars: the 7th day; striped bars: the 14th day; black bars: the 21st day, $^{*}p<0.05$: Newman–Keuls test after the significant MANOVA, compared to the respective group-housed male mice; $^{+}p<0.05$: compared to the respective group-housed female mice.

The differences became statistically significant between the groups of CCK_2 receptor deficient mice. Male group-housed mutant (-/-) mice gained weight significantly more than isolated male mutant (-/-) mice and group-housed female mutant (-/-) mice (Figure 1). On the 21st day of the individual housing differences related to sex and isolation became even stronger (three-way ANOVA: sex × isolation: $F(1,74)=12.0$, $p<0.01$; isolation: $F(1,74)=15.6$, $p<0.01$; sex: $F(1,74)=4.95$, $p<0.05$). Post hoc comparisons between the individual groups demonstrated that social isolation had caused a significant reduction of weight gain in both male wild-type (+/+) and male CCK_2 receptor deficient (-/-) mice. Isolation did not affect the weight gain in female mice. However, comparisons between male and female mice revealed that group-housed male wild-type (+/+) and mutant (-/-) mice had significantly higher weight gain compared to the respective groups of female mice.

1.2. Elevated plus-maze test

The data on the elevated plus-maze test are shown in Figure 2. Social isolation increased exploratory activity in all groups studied. In female wild-type mice, housed individually, increased anxiety was observed. However, social isolation failed to produce an increase in anxiety in female mice, lacking CCK_2 receptors. Number of open arm entries: social isolation induced sex-dependent changes in the exploratory behaviour of male and female mice in the elevated plus-maze. The application of three-way ANOVA demonstrated that social isolation caused different changes in the number of open arm entries in wild-type and mutant mice (sex × isolation × genotype: $F(1,74)=3.98$, $p<0.05$; genotype × sex: $F(1,74)=4.01$, $p<0.05$). The number of open arm entries tended to be higher in group-housed female mice compared to male group-housed animals. Post hoc comparisons between the groups established that social isolation increased the number of open arm entries in male wild-type (+/+) mice. In female wild-type (+/+) mice social isolation induced an opposite effect — a significant reduction of open arm entries. This reduction of open arm entries was not observed in female mutant (-/-) mice. Moreover, isolated female mutant mice made significantly more open arm entries than isolated female wild-type mice. By contrast, time spent exploring open arms was not significantly affected by genotype, isolation or sex.

Number of closed arm entries: social isolation caused a significant increase of closed arm visits in male mice compared to female animals, independent of their genotype (sex: $F(1,74)=14.2$, $p<0.01$; isolation: $F(1,74)=39.9$, $p<0.01$).

Ratio between the open and total arm entries: a significant genotype by sex interaction was established by three-way ANOVA (genotype × sex: $F(1,74)=4.46$, $p<0.05$). Post hoc comparisons revealed that social isolation induced a significant reduction of the ratio between open and total arm entries in female wild-type (+/+) mice. This behavioural change was not established in male mice

and female CCK_2 receptor deficient mice. Moreover, a substantially higher ratio to enter open arms was observed in isolated female mutant (-/-) mice compared to their isolated wild-type (+/+) littermates.

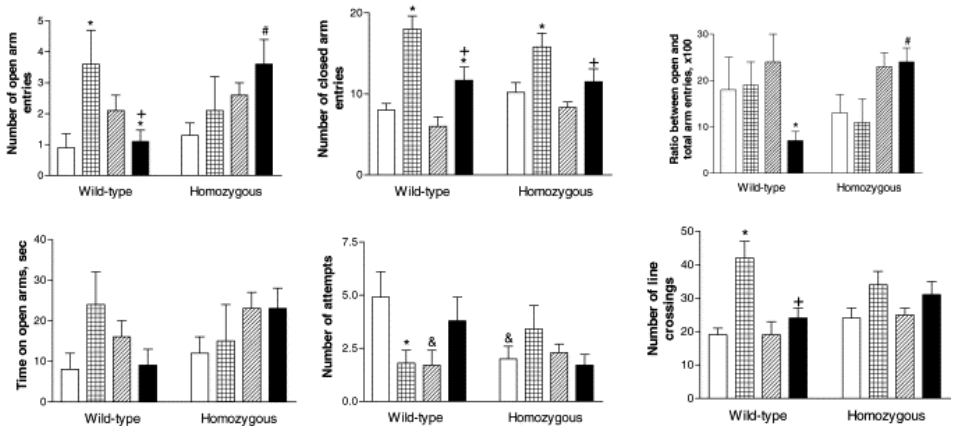

Figure 2. Effect of social isolation on the exploratory activity of male and female CCK_2 receptor deficient mice in the elevated plus-maze. White bars: male, group-housed; hatched bars: male, isolated; striped bars: female, group-housed; black bars: female, isolated; $^{*}p<0.05$: Newman–Keuls test after the significant MANOVA, compared to the respective group of group-housed mice; $^{+}p<0.05$: statistically significant difference between isolated female and male mice; $^{\#}p<0.05$: statistically significant difference between isolated mutant (-/-) and wild-type (+/+) female mice; $^{\&}p<0.05$: statistically significant difference with the group-housed wild-type (+/+) male mice.

Number of attempts to exit the enclosed arms: the number of attempts were affected by isolation in both sex- and genotype-dependent manner (sex × isolation × genotype: $F(1,74)=9.62$, $p<0.01$). The number of attempts made by group-housed male wild-type (+/+) mice was significantly higher compared to group-housed female wild-type (+/+) animals and to group-housed male mutant (-/-) mice. Individual housing significantly reduced the number of attempts in male wild-type (+/+) mice.

Number of line crossings: three-way ANOVA indicated significant effects of isolation ($F(1,74)=19.4$, $p<0.01$) and genotype × isolation interaction ($F(1,74)=4.32$, $p<0.05$). Isolation caused a more than two-fold increase in line crossings of male wild-type (+/+) mice. This effect was not evident in other groups. Post hoc comparisons established significant differences between group-housed and isolated male wild-type (+/+) mice and between isolated male and female wild-type (+/+) animals.

1.3. Locomotor activity

The data on the locomotor activity are shown in Figure 3. Social isolation increased locomotor activity in male, but not female mice. This effect was not significantly modified by genotype.

Time in movement: statistical analysis established sex- and isolation-dependent changes in the locomotor activity of mice (sex: $F(1,74)=29.9$, $p<0.01$; isolation: $F(1,74)=12.1$, $p<0.01$). Isolated male mutant (-/-) mice spent significantly more time in movement than their group-housed counterparts. Also, isolated male mice of both genotypes spent substantially more time in movement when compared to isolated female mice of the respective genotype.

Distance travelled: sex- and isolation-dependent changes in distance travelled were found (sex: $F(1,74)=18.2$, $p<0.01$; isolation: $F(1,74)=18.1$, $p<0.01$). Post hoc analysis revealed that male mice, independently from their genotype, travelled significantly longer distance compared to their female littermates. Again, in male mice lacking CCK_2 receptors (-/-) isolation significantly increased the distance travelled when compared to group-housed male mutant (-/-) mice.

Number of rearings: no effects of genotype, sex or isolation were observed.

Number of corner visits: significant effects of sex ($F(1,74)=20.6$, $p<0.01$), isolation ($F(1,74)=19.5$, $p<0.01$) and sex × isolation interaction ($F(1,74)=5.89$, $p<0.05$) were revealed. Social isolation induced a substantial increase of corner entries in both male wild-type (+/+) and male mutant (-/-) mice.

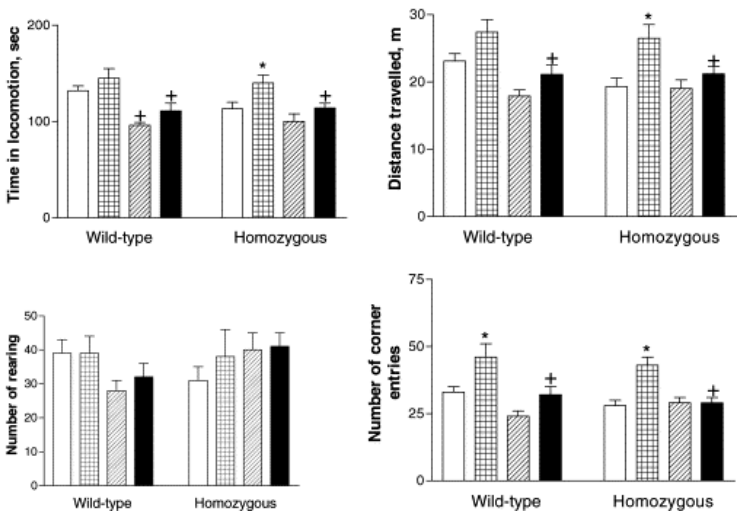

Figure 3. Effect of social isolation on the locomotor activity of male and female CCK_2 receptor deficient mice in the motility boxes. White bars: male, group-housed; hatched bars: male, isolated; striped bars: female, group-housed; black bars: female, isolated; $^{*}p<0.05$: Newman–Keuls test after the significant MANOVA, compared to the respective group-housed mice; $^{+}p<0.05$: compared to the respective groups of male mice.

1.4. Resident-intruder test

The data on the resident-intruder test are shown in Figure 4. Social isolation reduced non-social behaviours and increased aggressive encounters in male mice. Genotype did not significantly affect this finding. Reduced grooming and digging behaviour was observed in mutant mice, housed in groups. Also, isolated male mutant mice engaged more in social interaction when compared to their wild-type littermates, housed individually.

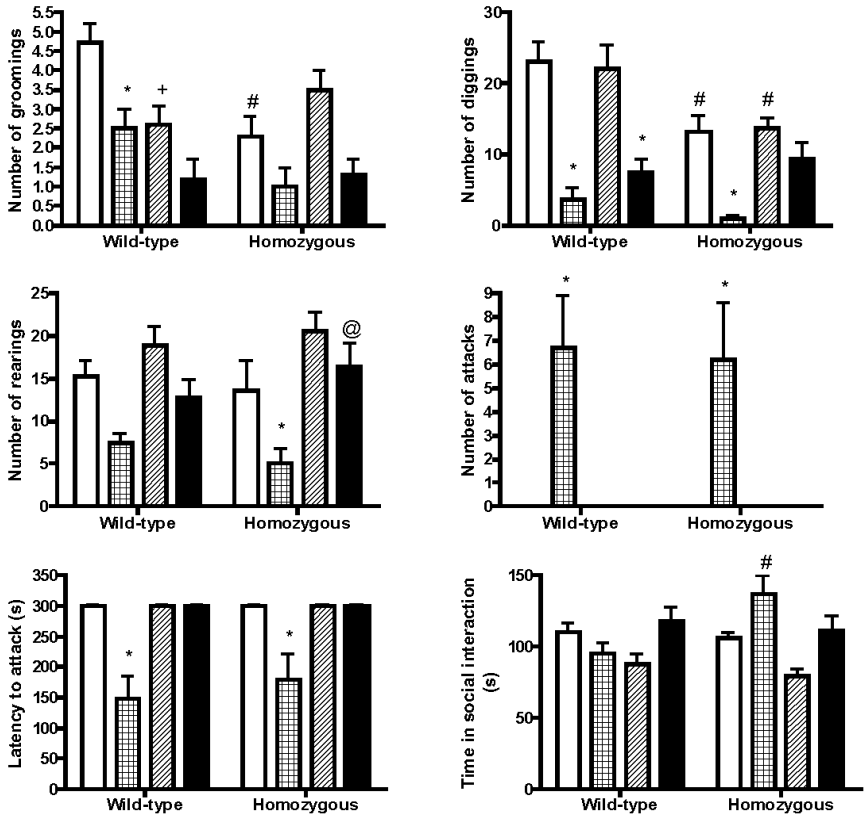

Figure 4. The effect of isolation on the behaviour of the male and female CCK_2 receptor deficient mice in the residen-intruder test. White bars: male, group-housed; hatched bars: male, isolated; striped bars: female, group-housed; black bars: female, isolated; $^{*}p<0.05$: Newman–Keuls test after the significant MANOVA, compared to the respective group of group-housed mice; $^{+}p<0.05$: statistically significant difference between female and male mice of respective housing condition; $^{\#}p<0.05$: statistically significant difference between mutant (-/-) and wild-type (+/+) mice housed in the same conditions; $^{@}p<0.05$: significant difference between male and female mice of the same genotype and housing condition.

Number of groomings: significant effects of genotype ($F(1,74)=4.87$, $p<0.05$), isolation ($F(1,74)=27.7$, $p<0.01$) and genotype × sex interaction ($F(1,74)=12.9$, $p<0.01$) were observed. Post hoc analysis demonstrated that the frequency of grooming of group-housed male wild-type (+/+) mice was significantly higher compared to group-housed female wild-type (+/+) mice and group-housed male homozygous (-/-) mice.

Number of diggings: three-way ANOVA revealed significant effects of genotype ($F(1,74)=9.05$, $p<0.01$), isolation ($F(1,74)=65.2$, $p<0.01$), sex × isolation interaction ($F(1,74)=3.99$, $p<0.05$) and genotype × isolation interaction ($F(1,74)=7.86$, $p<0.01$). The digging activity of wild-type (+/+) mice was significantly higher compared to homozygous (-/-) mice, and this effect was found in both male and female mice. Individual housing decreased the number of digging in male and female wild-type (+/+) mice, and in the male mutant (-/-) animals.

Number of rearings: effects of sex ($F(1,74)=18.0$, $p<0.01$) and isolation ($F(1,74)=17.3$, $p<0.01$) were found. Social isolation caused a statistically significant reduction of rearing in male homozygous (-/-) mice, and the difference between isolated male and female homozygous (-/-) mice was also significant.

Number of attacks: the number of attacks was significantly affected by sex ($F(1,74)=14.9$, $p<0.01$), isolation ($F(1,74)=16.3$, $p<0.01$) and sex × isolation interaction ($F(1,74)=14.9$, $p<0.01$). Social isolation increased the number of attacks in both male wild-type (+/+) and mutant (-/-) mice. In female mice no change in the aggressive behaviours was noted.

Attack latency: effects of sex ($F(1,74)=21.8$, $p<0.01$), isolation ($F(1,74)=26.6$, $p<0.01$) and sex × isolation interaction ($F(1,74)=21.8$, $p<0.01$) were established. Social isolation caused aggressive behaviour in male mice, independent of genotype.

Time in social interaction: three-way ANOVA revealed the effects of sex ($F(1,74)=4.18$, $p<0.05$), isolation ($F(1,74)=10.2$, $p<0.01$) and genotype × sex interaction ($F(1,74)=4.35$, $p<0.05$). Although post hoc comparison between groups did not show any significant alterations resulting from social isolation, a significant genotype-related difference was observed between isolated but not group-housed male mice. Socially isolated male mice lacking CCK_2 receptors (-/-) spent significantly more time in social interaction than their wild-type (+/+) littermates housed in the same conditions.

1.5. [^{3}H]-spiperone binding in the frontal cortex and sub-cortical structures

The affinity of [^{3}H]-spiperone binding in the frontal cortex was significantly increased in female CCK_2 receptor deficient (-/-) mice compared to their wild-type (+/+) littermates (genotype: $F(1,8)=6.55$, $p<0.05$; genotype × sex: $F(1,8)=5.52$, $p<0.05$). This genotype-dependent change in [^{3}H]-spiperone binding was not established in male mice. However, the density of [^{3}H]-spipe-

rone binding sites in the frontal cortex was not affected by the genetic invalidation of CCK_2 receptors. The affinity of [^{3}H]-spiperone binding sites in the sub-cortical structures was significantly increased in male, but not in female mice, lacking CCK_2 receptors (genotype: $F(1,8)=3.14$, $p<0.05$; genotype × sex: $F(1,8)=5.44$, $p<0.05$). Nevertheless, the density of [^{3}H]-spiperone binding sites in the sub-cortical structures was, again, not altered in mutant mice (Table 1).

Table 1. [^{3}H]-spiperone binding in the frontal cortex and sub-cortical structures of CCK_2 receptor deficient mice. K_d: the apparent dissociation constant; B_{max}: the apparent number of binding sites. $^+p<0.05$: significant difference between female wild-type (+/+) and female mutant (-/-) mice; $^*p<0.05$: significant difference between male wild-type (+/+) and male mutant (-/-) mice.

Group	Frontal cortex 5-hydroxytryptamine$_2$ (5-HT$_2$) receptors		Sub-cortical structures dopamine D_2 receptors	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Wild-type (+/+), male	0.33 ± 0.08	159 ± 15	0.16 ± 0.02	243 ± 15
Homozygous (−/−), male	0.28 ± 0.06	145 ± 13	0.08 ± 0.01*	235 ± 16
Wild-type (+/+), female	0.50 ± 0.08	194 ± 21	0.12 ± 0.03	264 ± 29
Homozygous (−/−), female	0.16 ± 0.03+	163 ± 15	0.13 ± 0.01	288 ± 35

2. Experiment 2: Sex-dependent behavioural effects of ethanol in mice, lacking CCK_2 receptors

2.1. Elevated plus-maze test

The anxiolytic effect of ethanol was significantly modified by the genetic invalidation of CCK_2 receptors. However, the altered effect of ethanol was only revealed in female, but not male mutant mice.

Number of entries on open arms. Significant effects of ethanol treatment ($F(3,129)=9.12$ $p<0.001$) and sex ($F(1,129)=5.94$ $p<0.05$) were established by the application of three-way independent-groups ANOVA (genotype × sex × ethanol treatment). Post hoc comparison did not indicate significant differences between groups. However, there was a tendency toward increase in the number of open entries induced by ethanol doses 1.0 g/kg ($p=0.09$) and 2.0 g/kg ($p=0.09$) in female mice, lacking CCK_2 receptors (-/-) (Fig. 5A).

Time spent exploring open arms. Significant effects of ethanol treatment ($F(3,129)=8.03$ $p<0.001$) and sex ($F(1,129)=9.57$ $p<0.01$) were revealed. The highest dose of ethanol (2.0 g/kg) induced a significant ($p<0.01$) increase in the time spent on open arms in female mice, lacking CCK_2 receptors (-/-), but not in their wild-type (+/+) littermates (Fig. 5B). This effect was not observed in male mice, independent of genotype, either.

Number of total arm entries. Three-way ANOVA indicated significant effect of sex ($F(1,129)=5.48$ $p<0.05$), but not of ethanol treatment. Ethanol did not alter the number of total arm entries in any group studied (data not shown).

Ratio between open and total arm entries. Significant effects of ethanol treatment ($F(3,129)=10.92$ $p<0.001$) and sex ($F(1,129)=4.50$ $p<0.05$) were observed. However, no significant differences between groups were established by post hoc comparison of means (data not shown).

Number of unprotected head-dips. The application of three-way ANOVA indicated significant effects of ethanol treatment ($F(3,129)=10.91$ $p<0.001$) and sex ($F(1,129)=8.81$ $p<0.01$), but also a tendency toward the effect of sex × ethanol treatment interaction ($F(3,129)=2.62$ $p=0.053$). Ethanol significantly increased the number of unprotected head-dips in female mutant mice (-/-) at doses 1.0 g/kg ($p<0.01$) and 2.0 g/kg ($p<0.01$) (Fig. 5C). This effect of ethanol was not observed in any other group studied.

Number of lines crossed. Significant effects of ethanol treatment ($F(3,129)=11.21$ $p<0.001$) and sex ($F(1,129)=7.10$ $p<0.01$) were established. Again, ethanol 2.0 g/kg increased the number of lines crossed significantly ($p<0.05$) only in female mice, lacking CCK_2 receptors (-/-), but not in their wild-type (+/+) littermates or male mice of either genotype (Fig. 5D).

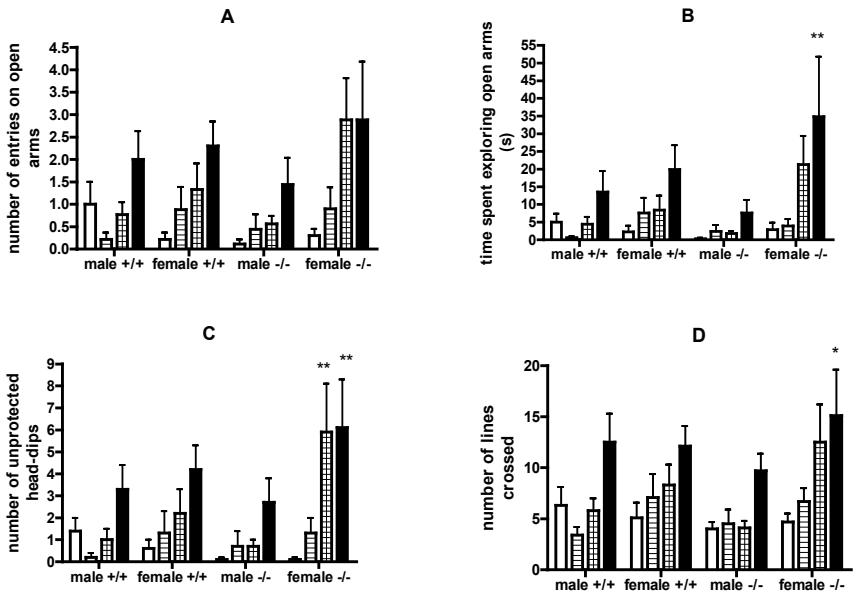

Figure 5. Effect of ethanol (0.5, 1.0 and 2.0 g/kg) on the exploratory activity of male and female CCK_2 receptor deficient mice in the elevated plus-maze. White bars: saline; striped bars: ethanol 0.5 g/kg; hatched bars: ethanol 1.0 g/kg; black bars: ethanol 2.0 g/kg. $^{*}p < 0.05$, $^{**}p < 0.01$: Tukey's HSD test, ethanol compared to saline treatment of the respective genotype.

2.2. Locomotor activity

Ethanol (2.0 g/kg) suppressed locomotor activity in all groups studied. The locomotor effect of ethanol was altered in male, but not female mice, lacking CCK_2 receptors. Only in male mutant mice, the vertical activity was suppressed by the small dose of ethanol (0.5 g/kg).

Time in movement. Three-way independent-groups ANOVA (genotype × sex × ethanol treatment) established significant effects of genotype ($F(1,167)=20.46$ $p<0.001$), ethanol treatment ($F(3,167)=13.85$ $p<0.001$) and genotype × sex × ethanol treatment interaction ($F(3,167)=2.82$ $p<0.05$). At the highest dose (2.0 g/kg), ethanol induced a significant suppression of locomotor activity only in female wild-type mice ($p<0.01$) (Fig. 6A). It should be noted, though, that the baseline activity of female mutant mice (-/-) tended to be lower compared to their wild-type littermates (+/+) ($p=0.07$). In male mice, lacking CCK_2 receptors (-/-), but not in their wild-type littermates (+/+), ethanol tended to suppress time in movement at doses 0.5 g/kg ($p=0.08$) and 2.0 g/kg ($p=0.07$) (Fig. 6A).

Distance travelled. Significant effects of genotype ($F(1,167)=15.50$ $p<0.001$), ethanol treatment ($F(3,167)=9.49$ $p<0.001$) and genotype × sex × ethanol treatment interaction ($F(3,167)=2.94$ $p<0.05$) were demonstrated. Significant decrease in locomotor activity after administration of the highest dose of ethanol (2.0 g/kg) was observed only in female wild-type mice (+/+) ($p<0.01$) (Fig. 6B). Again, the baseline activity of female mutant mice (-/-) tended to be lower compared to their wild-type littermates (+/+) ($p=0.09$). In male mice, ethanol administration did not significantly affect the distance travelled.

Number of rearings. Three-way ANOVA indicated significant effects of genotype ($F(1,167)=12.76$ $p<0.001$), ethanol treatment ($F(3,167)=35.40$ $p<0.001$), genotype × ethanol treatment interaction ($F(3,167)=4.11$ $p<0.01$) and genotype × sex × ethanol treatment interaction ($F(3,167)=3.22$ $p<0.05$). Ethanol significantly suppressed rearing at the highest dose used (2.0 g/kg) in all groups, except for female mutant (-/-) mice. The same effect was induced in female wild-type mice (+/+) by dose 1.0 g/kg ($p<0.05$) and in male mutant mice (-/-) by dose 0.5 g/kg ($p<0.01$) (Fig. 6C). The suppression of rearing behaviour induced by the small dose of ethanol (0.5 g/kg) in male mice lacking CCK_2 receptors (-/-) was significant when compared to the respective treatment in their wild-type littermates (+/+) ($p<0.001$) (Fig. 6C).

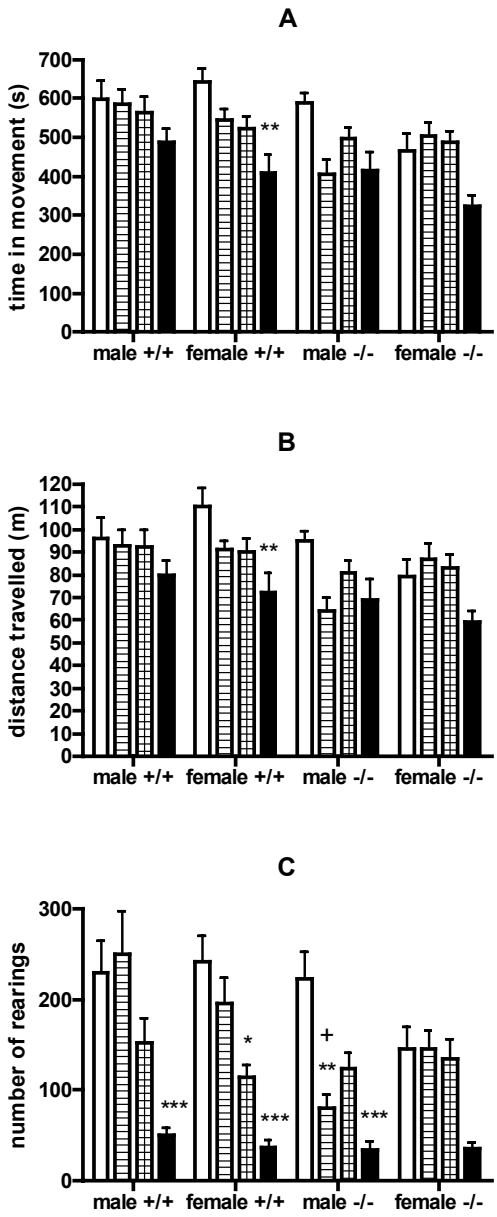

Figure 6. Effect of ethanol (0.5, 1.0 and 2.0 g/kg) on the locomotor activity of male and female CCK_2 receptor deficient mice. White bars: saline; striped bars: ethanol 0.5 g/kg; hatched bars: ethanol 1.0 g/kg; black bars: ethanol 2.0 g/kg. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$: Tukey's HSD test, ethanol compared to saline treatment of the respective genotype. $^{+}p < 0.001$: statistically significant difference between male wild-type (+/+) and male CCK_2 receptor deficient mice (-/-) of ethanol dose 0.5 g/kg.

2.3. Loss of righting reflex

Although the parameters of the loss of righting reflex were not significantly affected by the genotype, blood ethanol concentrations at the regain of righting reflex were significantly lower in female mice, lacking CCK_2 receptors, when compared to their wild-type littermates.

Time to loss of righting reflex. Two-way independent-groups ANOVA (genotype × sex) revealed no significant effects in the time to loss of righting reflex.

Time to regain of righting reflex. No genotype- or sex-related differences were observed in the time to regain of righting reflex.

Blood ethanol concentration at regain of righting reflex. Significant effects of genotype ($F(1,54)=22.82$ $p<0.001$) and genotype × sex interaction ($F(1,54)=4.47$ $p<0.05$) were demonstrated by two-way ANOVA. Blood ethanol concentrations at the regain of righting reflex were significantly lower in female mice lacking CCK_2 receptors (-/-), than in their wild-type (+/+) littermates ($p<0.001$). No genotype-related difference was established in male mice. The data are summarised in Table 2.

Table 2. Time to loss of righting reflex (LORR, s), time to regain of righting reflex (RRR, min) and blood ethanol concentrations at the regain of the righting reflex (BEC_{RRR}, g/l) in wild-type mice (+/+) and in mice, lacking CCK_2 receptors (-/-) after administration of ethanol 4.0 g/kg. *** $p<0.001$: Tukey HSD test, significant difference between female wild-type (+/+) and female mutant (-/-) mice.

Group	LORR (s)	RRR (min)	BEC_{RRR} (g/l)
Male (+/+)	74 ± 2.8	56 ± 7.9	3.4 ± 0.08
Female (+/+)	75 ± 2.0	40 ± 4.4	3.6 ± 0.07
Male (-/-)	75 ± 2.0	50 ± 7.4	3.2 ± 0.05
Female (-/-)	79 ± 3.0	51 ± 7.9	$3.1 \pm 0.07^{***}$

2.4. Blood ethanol concentrations (BECs)

Four-way mixed-design ANOVA (genotype × sex × dose × time) established significant effects of ethanol dose ($F(1,37)=671.53$ $p<0.001$), time ($F(3,111)=943.84$ $p<0.001$), genotype × time interaction ($F(3,111)=4.18$ $p<0.01$), sex × time interaction ($F(3,111)=5.33$ $p<0.01$), dose × time interaction ($F(3,111)=40.29$ $p<0.001$), genotype × sex × time interaction ($F(3,111)=3.48$ $p<0.05$) and genotype × dose × time interaction ($F(3,111)=3.35$ $p<0.05$). Blood ethanol concentrations were significantly lower in female mice, lacking CCK_2 receptors (-/-), when compared to female wild-type mice (+/+), 30 min and 120 min after the injection of ethanol at a dose 4.0 g/kg ($p<0.01$ and $p<0.05$, respectively)

(Fig. 7). In male mutant mice (-/-) BECs were also significantly lower than in their wild-type littermates (+/+) 30 min after injection of ethanol 4.0 g/kg ($P<0.05$) (Fig. 7). There were no genotype- or sex-related differences after injection of ethanol 2.0 g/kg.

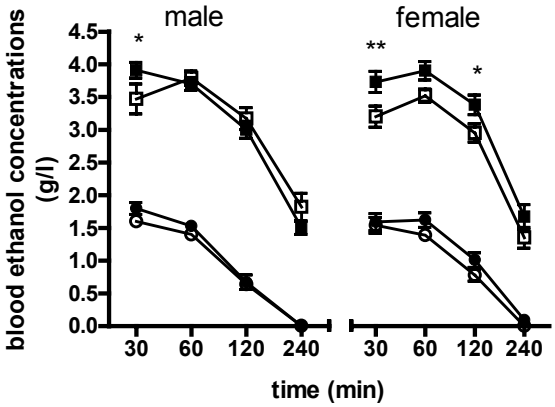

Figure 7. Blood ethanol concentrations (g/l) in male and female mice, lacking CCK_2 receptors (-/-), after injection of ethanol 2.0 g/kg or 4.0 g/kg. Filled squares: wild-type mice (+/+), ethanol dose 4.0 g/kg; open squares: mutant mice (-/-), ethanol dose 4.0 g/kg; filled circles: wild-type mice (+/+), ethanol dose 2.0 g/kg; open circles: mutant mice (-/-), ethanol dose 2.0 g/kg. * $p<0.05$, ** $p<0.01$: Tukey HSD test, wild-type mice (+/+) compared to CCK_2 receptor deficient mice (-/-) at respective time point after injection of ethanol 4.0 g/kg.

2.5. Ethanol preference and intake

Preference for ethanol did not significantly differ between wild-type and mutant mice. Yet, female mutant mice consumed significantly more 10% ethanol solution per body weight than their wild-type counterparts. This genotype-dependent difference was not observed in male mice.

Ethanol preference. Three-way mixed-design ANOVA (genotype × sex × ethanol concentration) indicated significant effects of sex ($F(1,56)=15.34$ $p<0.001$), ethanol concentration ($F(2,112)=30.03$ $p<0.001$) and sex × ethanol concentration interaction ($F(2,112)=9.39$ $p<0.001$). Ethanol preference at concentrations 6 % and 10 % was significantly higher in both female wild-type mice (+/+) ($p<0.05$ and $p<0.001$, respectively) and female mutant mice (-/-) ($p<0.01$ and $p<0.001$, respectively) when compared to their male littermates (Fig. 8A).

Ethanol intake. Significant effects of genotype ($F(1,56)=6.55$ $p<0.05$), sex ($F(1,56)=44.88$ $p<0.001$), ethanol concentration ($F(2,112)=212.28$ $p<0.001$), genotype × ethanol concentration interaction ($F(2,112)=5.12$ $p<0.01$) and sex × ethanol concentration interaction ($F(2,112)=34.34$ $p<0.001$) were established. Post hoc comparison between groups demonstrated significant sex- and genotype-related differences in ethanol consumption at concentration 10 %. Female mice, independent of genotype, consumed significantly more ethanol than their male littermates (6 % ethanol: $p<0.05$ for wild-type (+/+) mice and $p<0.01$ for mutant (-/-) mice; 10 % ethanol: $p<0.001$ for both wild-type (+/+) and mutant (-/-) mice) (Fig. 8B). Moreover, female mice, lacking CCK_2 receptors (-/-), consumed significantly more 10 % ethanol solution than their wild-type littermates (+/+) ($p<0.001$) (Fig. 8B).

2.6. Total fluid and food consumption, and body weight changes during ethanol intake test

Total fluid consumption. Significant effects of genotype ($F(1,56)=6.64$ $p<0.05$), sex ($F(1,56)=56.47$ $p<0.001$) and ethanol concentration ($F(2,112)=3.16$ $p<0.05$) were established by three-way mixed-design ANOVA (genotype × sex × ethanol concentration). Total liquid intake per body weight at ethanol concentration 10 % was significantly higher ($p<0.001$) in female mice, lacking CCK_2 receptors (-/-) compared to their wild-type littermates (+/+) (Fig. 8C). Genotype-related difference in total fluid intake was not observed in male mice.
Food consumption. Significant effects of sex ($F(1,56)=52.28$ $p<0.001$), ethanol concentration ($F(2,112)=68.56$ $p<0.001$), genotype × ethanol concentration interaction ($F(2,112)=3.46$ $p<0.05$) and sex × ethanol concentration interaction ($F(2,112)=10.29$ $p<0.001$) were demonstrated. Male mice, lacking CCK_2 receptors (-/-), consumed significantly more food per body weight than their wild-type (+/+) littermates at ethanol concentrations 3 % ($p<0.001$) and 6 % ($p<0.01$) (Fig. 8D). No such difference was observed in female mice.

Body weight changes. Three-way ANOVA indicated significant effects of sex ($F(1,56)=158.11$ $p<0.001$) and sex × ethanol concentration interaction ($F(2,112)=16.29$ $p<0.001$). While there were significant differences between male and female mice, no genotype-related differences were observed (data not shown).

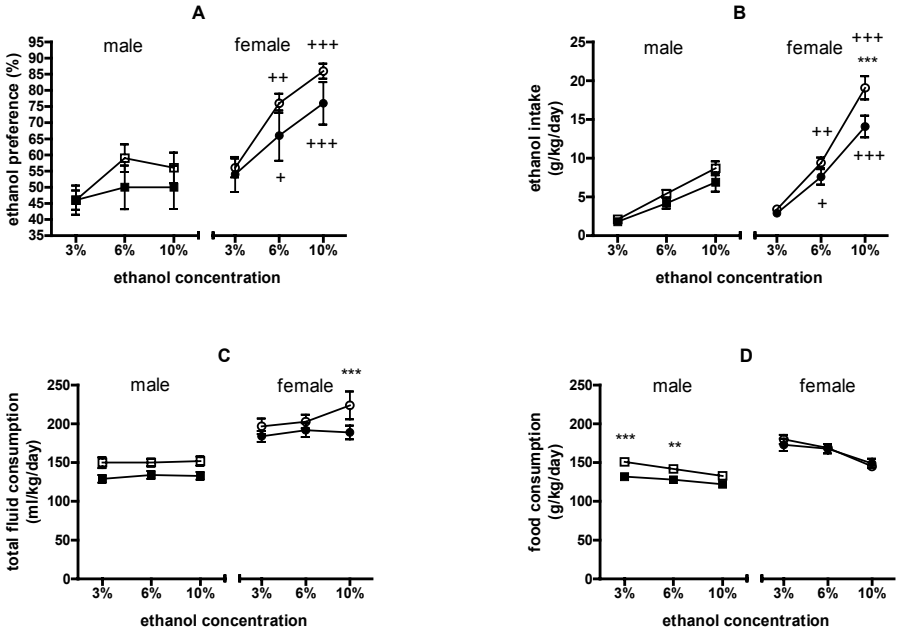

Figure 8. Ethanol preference and intake, and total liquid and food intake in CCK_2 receptor deficient mice during voluntary ethanol consumption. Filled squares: male wild-type (+/+) mice; open squares: male mutant mice (-/-); filled circles: female wild-type (+/+) mice; open circles: female mutant mice (-/-). ** $p<0.01$, *** $p<0.001$: Tukey HSD test, wild-type mice (+/+) compared to mice, lacking CCK_2 receptors (-/-) at respective ethanol concentration. + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$: female mice compared to male mice of respective genotype at respective ethanol concentration.

2.7. Taste preference

Sucrose preference. Three-way mixed-design ANOVA (genotype × sex × sucrose concentration) revealed significant effect of sucrose concentration ($F(1,56)=132.85$ $p<0.001$). Post hoc comparison did not reveal any differences between the groups.

Quinine preference. Significant effect of quinine concentration ($F(1,56)= 47.77$ $p<0.001$) was demonstrated. No significant genotype- or sex-related differences were observed. The data on taste preference are summarised in Table 3.

Table 3. Sucrose and quinine preference (%) in mice, lacking CCK_2 receptors (-/-). No significant alterations were observed in taste preference of mice, lacking CCK_2 receptors.

Group	Sucrose 1.7 %	Sucrose 4.3 %	Quinine 0.03 mM	Quinine 0.10 mM
Male (+/+)	89 ± 1.6	97 ± 0.6	55 ± 4.7	43 ± 5.6
Female (+/+)	89 ± 1.5	97 ± 0.5	55 ± 5.0	39 ± 5.2
Male (-/-)	90 ± 1.1	97 ± 0.3	56 ± 4.1	42 ± 4.0
Female (-/-)	90 ± 1.4	96 ± 0.5	61 ± 4.4	49 ± 5.8

3. Experiment 3: Housing in environmentally enriched conditions alters the behavioural phenotype of mice, lacking CCK_2 receptors

3.1. Elevated plus-maze test

Significant genotype × housing condition interaction was observed in the total number of arm entries ($F(1,149)=4.34$ $p<0.05$) and % entries on open arms ($F(1,149)=4.03$ $p<0.05$), and a tendency to genotype × housing condition interaction ($F(1,149)=3.07$ $p=0.08$) was revealed in the attempts to exit closed arms. Mice, lacking CCK_2 receptors (-/-), reared in standard conditions, made significantly less total arm entries ($p<0.05$) and open arm entries ($p<0.005$) than their standard-housed wild-type (+/+) littermates (Fig. 9A, 9B). However, CCK_2 receptor mutant mice (-/-) housed in enriched conditions made significantly more total and open entries than standard-housed mutants (-/-) ($p<0.005$), and did not differ from wild-type (+/+) mice housed in enriched conditions (Fig. 9A, 9B). Mice, lacking CCK_2 receptors (-/-), housed in standard conditions, also spent significantly less time exploring open arms ($p<0.05$) and made more attempts to exit closed arms ($p<0.005$) than standard-housed wild-type (+/+) mice (Fig. 9C, 9E). In mice reared in enriched conditions significantly increased exploratory activity and reduced anxiety was observed, as evidenced by increased total arm entries, % open entries, % time open and increased number of unprotected head-dips, and reduced number of attempts (Fig. 9A-E). The difference between standard-housed and enrichment-housed mice was especially prominent in mutants (-/-). Moreover, in enriched mice no genotype-dependent differences in behaviour were observed. Thus, the anxiety-like phenotype of CCK_2 receptor mutant mice was clearly dependent on the rearing conditions.

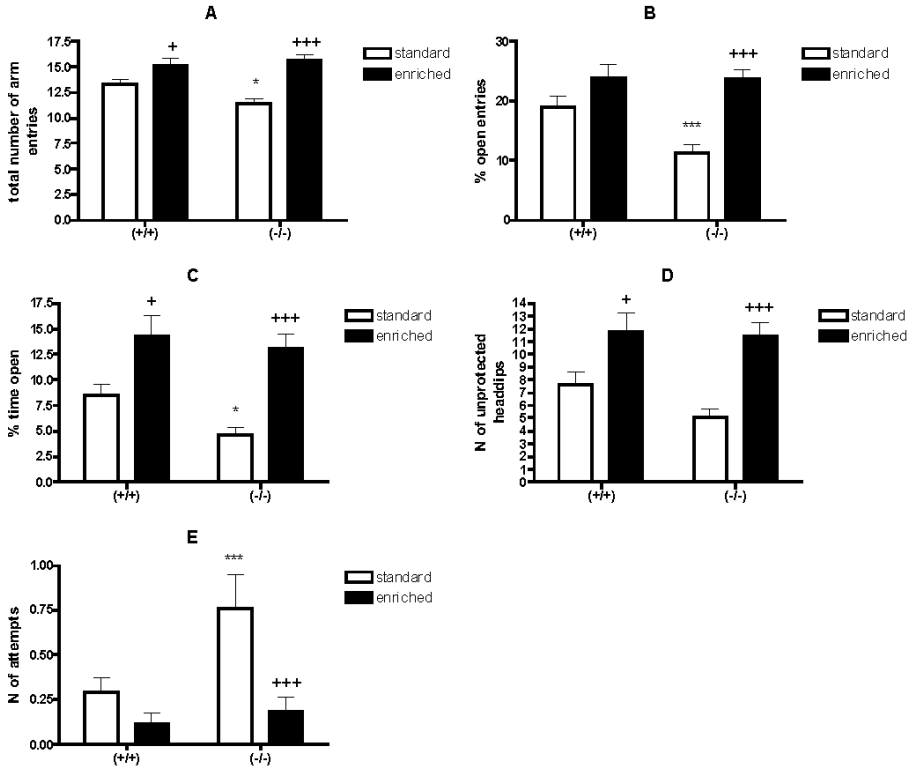

Figure 9. Plus-maze test in mice, lacking CCK_2 receptors (-/-), and their wild-type (+/+) littermates, housed in different environmental conditions. (A) total number of arm entries; (B) % open entries; (C) % open time; (D) number of unprotected head-dips; (E) number of attempts to exit closed arms. * $p<0.05$, *** $p<0.005$: Newman-Keuls test after the significant MANOVA, mutant mice (-/-) compared to their wild-type (+/+) littermates housed in the same conditions; + $p<0.05$, +++ $p<0.005$: mice housed in enriched conditions compared to mice of respective genotype housed in standard conditions.

3.2. Locomotor activity

Housing condition had a significant effect on distance travelled ($F(1,142)=6.13$ $p<0.05$) and a sex-dependent effect on time spent in the central part (sex × housing condition interaction $F(1,142)=10.46$ $p<0.005$), but did not affect rearing. Although post hoc analysis did not confirm significant effect of housing condition in groups studied, female mice, lacking CCK_2 receptors (-/-), housed in enriched conditions travelled significantly longer distance ($p<0.05$) and spent significantly more time in the central part ($p<0.01$) than corresponding male mutant mice (-/-) (Fig 10A and 10B). This sex-dependent difference was absent in wild-type (+/+) mice independent of housing conditions, and also in mutant (-/-) mice, housed in standard conditions.

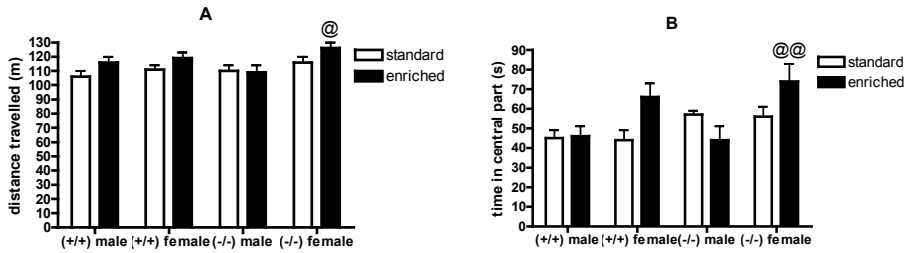

Figure 10. Locomotor activity in mice, lacking CCK_2 receptors (-/-), and their wild-type (+/+) littermates, housed in different environmental conditions. (A) distance travelled; (B) time in central part. @ $p<0.05$, @@ $p<0.01$: Newman-Keuls test after the significant MANOVA, female mutant mice (-/-) compared to male mutant mice (-/-) housed in the same conditions.

3.3. Forced swim test

Although a significant genotype × sex interaction effect ($F(1,144)=5.05$ $p<0.05$) was established by ANOVA, post hoc comparison between groups revealed no significant differences. The immobility time was not affected by the housing conditions in any of the groups studied (data not shown).

3.4. Hot plate test

Housing condition was found to significantly affect the latency to hind paw shake or lick ($F(1,149)=16.39$ $p<0.001$) and a significant sex × housing condition interaction was established ($F(1,149)=8.36$ $p<0.005$). In both male wild-type (+/+) mice and male mutant mice (-/-) enriched housing resulted in significantly lower nociceptive thresholds when compared to respective standard-housed groups ($p<0.05$ and $p<0.005$, respectively) (Fig. 11A). In

female mice there was no effect of the housing condition on the latency to hind paw shake or lick. Latency to jump was significantly affected by genotype ($F(1,149)=5.01$ $p<0.05$), sex ($F(1,149)=22.33$ $p<0.001$), housing condition ($F(1,149)=14.13$), sex × housing condition ($F(1,149)=6.66$ $p<0.05$) and genotype × sex × housing condition ($F(1,149)=4.80$ $p<0.05$). In female mice, lacking CCK_2 receptors (-/-), reared in enriched conditions, significantly longer latencies to jump were observed when compared to enriched female wild-type mice (+/+), enriched male mutant mice (-/-) and standard-housed female mutant mice (-/-) ($p<0.005$, for all) (Fig. 11B). No such genotype-dependent difference was observed in female mice, housed in standard conditions.

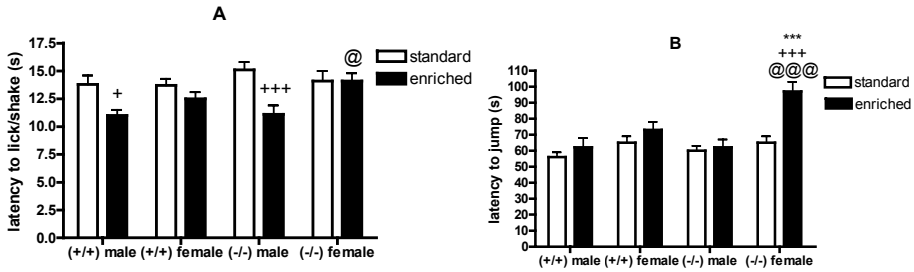

Figure 11. Hot plate test in mice, lacking CCK_2 receptors (-/-), and their wild-type (+/+) littermates, housed in different environmental conditions. (A) latency to lick/shake; (B) latency to jump. *** $p<0.005$: Newman-Keuls test after the significant MANOVA, mutant mice (-/-) compared to their wild-type (+/+) littermates of respective sex and housing condition; + $p<0.05$, +++ $p<0.005$: mice housed in enriched conditions compared to mice of respective genotype and sex housed in standard conditions; @ $p<0.05$, @@@ $p<0.005$: female mutant mice (-/-) compared to male mutant mice (-/-) reared in the same housing conditions.

3.5. Restraint-induced analgesia

Mixed design ANOVA revealed significant effects of housing condition ($F(1,154)=18.01$ $p<0.001$), restraint ($F(1,154)=284.37$ $p<0.001$), restraint × housing condition ($F(1,154)=4.36$ $p<0.05$) and genotype × restraint × housing condition ($F(1,154)=6.43$ $p<0.05$). In mutant mice (-/-), reared in standard conditions, restraint-induced tail-flick latencies were significantly longer when compared to standard-housed wild-type (+/+) mice ($p<0.005$) and to mutant (-/-) mice, housed in enriched conditions ($p<0.005$) (Fig. 12). While there was a robust genotype-dependent difference in mice, housed in standard conditions, no such difference was observed in mice, housed in enriched conditions.

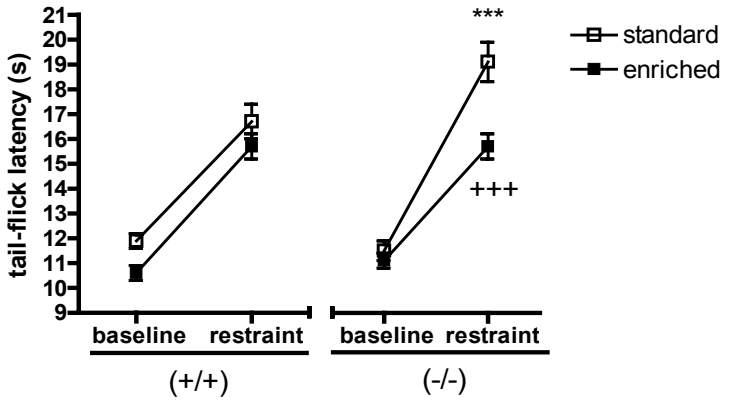

Figure 12. Restraint-induced analgesia in mice, lacking CCK_2 receptors (-/-), and their wild-type (+/+) littermates, housed in different environmental conditions. *** $p<0.005$: Newman-Keuls test after the significant MANOVA, restraint-induced tail-flick latencies in mutant mice (-/-) compared to their wild-type (+/+) littermates, reared in the same housing conditions; +++ $p<0.005$: restraint-induced latencies in mutant mice (-/-), housed in enriched conditions, compared to mutant mice, housed in standard conditions.

3.6. Rota-rod

Significant effects of housing condition ($F(1,150)=64.85$ $p<0.001$), sex ($F(1,150)=15.91$ $p<0.001$), genotype × sex ($F(1,150)=4.47$ $p<0.05$), sex × housing condition ($F(1,150)=8.30$ $p<0.005$) and genotype × sex × housing condition ($F(1,150)=4.71$ $p<0.05$) were established. In all groups, except for male mice, lacking CCK_2 receptors (-/-), enriched housing significantly improved rota-rod performance as evidenced by longer latencies to fall when compared to mice housed in standard conditions (Fig. 13). Moreover, male mutant mice (-/-), reared in enriched conditions, showed significantly shorter latencies to fall, when compared to enriched male wild-type (+/+) mice ($p<0.05$), and enriched female mutant (-/-) mice ($p<0.005$) (Fig. 13).

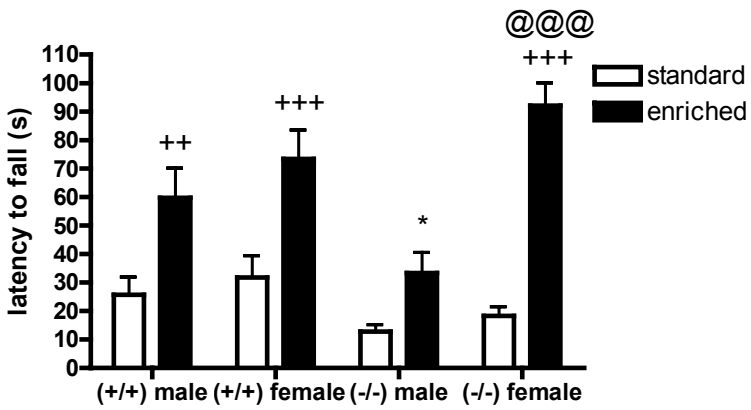

Figure 13. Rota-rod test in mice, lacking CCK_2 receptors (-/-), and their wild-type (+/+) littermates, housed in different environmental conditions. * $p<0.05$: Newman-Keuls test after the significant MANOVA, mutant mice (-/-) compared to their wild-type (+/+) littermates of respective sex and housing condition; ++ $p<0.01$, +++ $p<0.005$: mice, housed in enriched conditions, compared to mice of respective genotype and sex, housed in standard conditions; @@@ $p<0.005$: female mutant mice (-/-) compared to male mutant mice (-/-), reared in the same housing conditions.

3.7. Water maze

In the acquisition phase, latency to locate the platform was significantly affected by housing condition ($F(1,148)=70.86$ $p<0.001$) and day ($F(3,444)=444.25$ $p<0.001$), but only tendencies were observed regarding the effects of genotype and genotype × housing condition interaction. However, distance travelled to locate platform was significantly affected by genotype × housing condition interaction ($F(1,148)=10.31$ $p<0.005$) as well as housing condition ($F(1,148)=35.50$ $p<0.001$) and day × housing condition interaction ($F(3,444)=5.18$ $p<0.005$). This discrepancy between latency (s) and distance travelled (m) may have resulted from significant effect of housing condition ($F(1,148)=32.13$ $p<0.001$) and tendency to effect of genotype × housing condition interaction ($F(1,148)=2.91$ $p=0.09$) on swim velocity (cm/s). Since mice housed in enriched conditions swam significantly faster than their standard-housed counterparts (Fig. 14C), latencies to locate platform may not serve as an ideal indicator of spatial learning, because in this case reduced latencies in enriched mice result from not only improved spatial navigation, but also from better swimming ability.

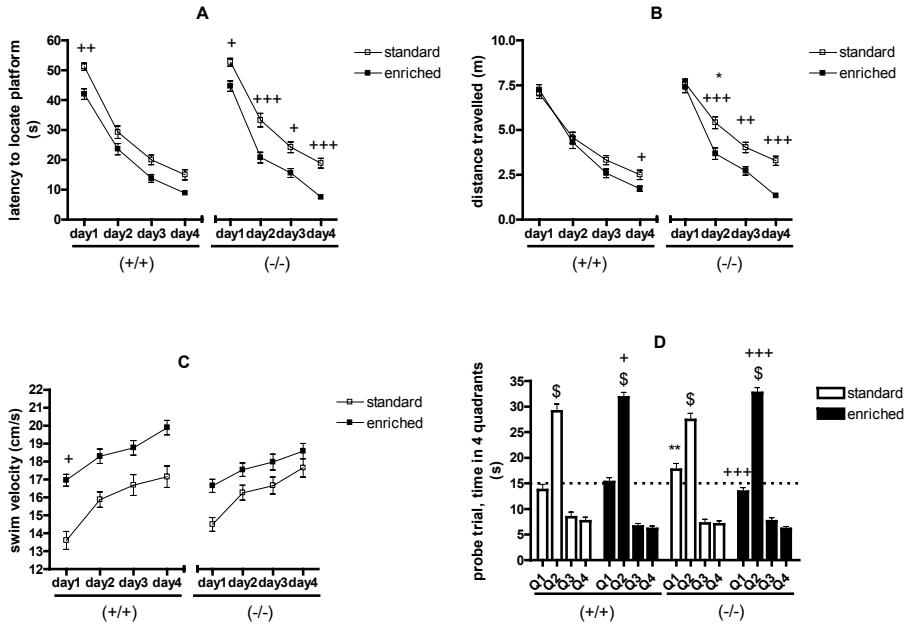

Figure 14. Water maze performance in mice, lacking CCK_2 receptors (-/-), and their wild-type (+/+) littermates, housed in different environmental conditions. (A) acquisition phase, latency to locate platform across days 1–4; (B) acquisition phase, distance travelled to locate platform across days 1–4; (C) acquisition phase, swim velocity across days 1–4; (D) probe trial, time in 4 quadrants. Quadrant Q2 is the target quadrant and dotted line denotes chance (15 s). * $p<0.05$, ** $p<0.01$: Newman-Keuls test after the significant MANOVA, mutant mice (-/-), housed in standard conditions, compared to their wild-type (+/+) littermates, housed in the same conditions, on the same day of acquisition phase and in the same quadrant in the probe trial; + $p<0.05$, ++ $p<0.01$, +++ $p<0.005$: mice, housed in standard conditions, compared to mice of respective genotype, housed in enriched conditions, on the same day; \$ $p<0.005$: time in the target quadrant Q2 compared to time in any other quadrant of the respective group.

Therefore, we considered distance travelled (in metres) instead of latency to locate platform (in seconds) across days as better indicator of spatial learning. In all groups, learning criterion (the significant difference between days 1 and 4, $p<0.001$, not shown in Fig. 14 by extra asterisks) was fulfilled for latency to find platform, distance travelled and swim velocity. Nevertheless, mice, lacking CCK_2 receptors (-/-), reared in standard conditions, travelled significantly longer distance to reach the platform than standard-housed wild-type mice (+/+) on day 2 ($p<0.05$, Fig. 14B). While enriched housing only slightly improved the water maze acquisition in wild-type mice (+/+), evidenced by shorter distance travelled on day 4 ($p<0.05$), enrichment robustly enhanced the water maze acquisition in mutant mice (-/-) (Fig. 14B;

day 2: $p<0.005$, day 3: $p<0.01$, day 4: $p<0.005$). Again, although, in mice housed in standard conditions a genotype-dependent phenotype was observed during the acquisition phase of the water maze, no such phenotype was observed in mice, housed in enriched conditions. In the probe trial significant effects of quadrant ($F(3,592)=570.17$ $p<0.001$), housing × quadrant interaction ($F(3,592)= 7.67$ $p<0.001$) and genotype × housing condition × quadrant interaction ($F(3,592)= 4.65$ $p<0.005$) were revealed. All groups spent significantly more time in the target quadrant than in any other quadrant ($p<0.005$, Fig. 14D). However, standard-housed mutant mice (-/-) spent significantly more time exploring irrelevant quadrant (Q1) than standard-housed wild-type (+/+) mice ($p<0.01$) and enrichment-housed mutant mice ($p<0.005$) (Fig. 14D). Both wild-type (+/+) and CCK_2 receptor deficient (-/-) mice, housed in enriched environment, spent significantly more time in the target quadrant than their counterparts, housed in standard environment ($p<0.05$ for wild-type, and $p<0.005$ for mutant mice, Fig. 14D). The results of the water maze test indicate minor but detectable spatial learning and memory deficits in CCK_2 receptor deficient mice (-/-) that are completely reversed by housing in complex environmental conditions.

4. Experiment 4: The behavioural differences between C57BL/6 and 129S6/SvEv/Tac strains are reproducible independent of pre-experimental housing conditions

4.1. Elevated plus-maze test

In the plus-maze test significant effect of strain was observed in the total entries on all arms ($F(1,120)=48.82$ $p<0.005$), unprotected head-dips ($F(1,120)=15.38$ $p<0.005$), stretched-attend postures ($F(1,120)=14.46$ $p<0.005$), number of rearings ($F(1,120)=158.41$ $p<0.005$) and number of groomings ($F(1,120)=81.12$ $p<0.005$). Housing condition substantially affected the number of rearings ($F(1,120)=8.92$ $p<0.005$), and significant interaction between strain and the housing condition was revealed in the total entries on all arms ($F(1,120)=12.88$ $p<0.005$), unprotected head-dips ($F(1,120)=5.60$ $p<0.05$), stretched-attend postures ($F(1,120)=4.41$ $p<0.05$) and the number of rearings ($F(1,120)=9.61$ $p<0.005$). B6 strain, housed in enriched conditions, made significantly more total arm entries ($p<0.05$) and unprotected head-dips ($p<0.05$) but less rearing ($p<0.005$) than their standard-housed counterparts (Fig. 15A, 15C, 15D). Independent of housing conditions, 129 strain performed rearing and grooming significantly less than B6 strain (Fig. 15E, 15F). Also, the exploratory activity of 129 strain, housed in enriched conditions, was significantly lower than that of enrichment-housed B6 strain's, evidenced by a smaller number of total arm entries ($p<0.005$) and unprotected head-dips ($p<0.005$, Fig. 15A, 15C). There was also a significant difference between strains, housed in enriched conditions,

in stretched-attend postures ($p<0.005$), indicating a higher level of anxiety in 129 strain, whereas no difference was observed between strains, housed in standard conditions (Fig. 15D). Moreover, the % open entries (Fig. 15B) and % open time (data not shown), which are important indicators of anxiety in the plus-maze, were neither affected by the strain nor housing condition.

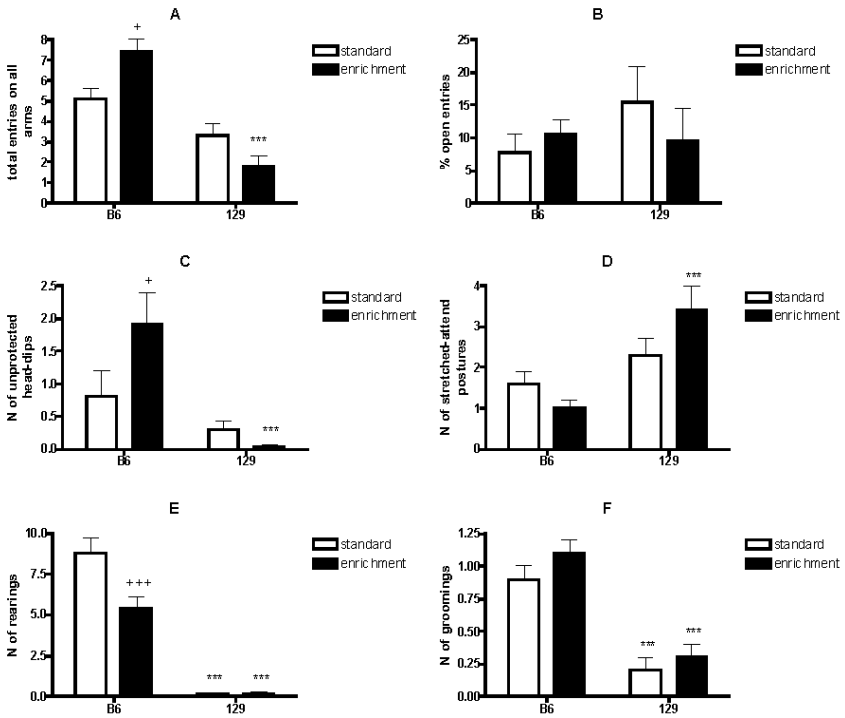

Figure 15. Plus-maze test in B6 and 129 strains, housed in different environmental conditions. (A) total number of arm entries; (B) % open entries; (C) number of unprotected head-dips; (D) number of stretched-attend postures; (E) number of rearings; (F) number of groomings. *** $p<0.005$: Tukey HSD test after the significant MANOVA, 129 strain compared to B6 strain housed in the same conditions; + $p<0.05$, +++ $p<0.005$: mice, housed in enriched conditions, compared to mice of the respective strain, housed in standard conditions.

4.2. Locomotor activity

Locomotor activity was significantly affected by strain ($F(1,120)=1716.90$ $p<0.005$), housing condition ($F(1,120)=24.31$ $p<0.005$), strain × housing condition interaction ($F(1,120)=5.19$ $p<0.05$), housing condition × day interaction ($F(2,240)=7.31$ $p<0.005$) and strain × housing condition × day interaction ($F(2,240)=3.63$ $p<0.05$). Independent of housing conditions and day,

B6 mice travelled significantly longer distances than 129 mice ($p<0.005$, Fig. 16A). Enriched housing significantly affected the locomotor activity in B6 strain. While standard-housed B6 mice clearly habituated to the test arena as evidenced by significant reduction of locomotor activity on days 2 and 3, compared to day 1 ($p<0.005$, Fig. 16A), no habituation was observed in enrichment-housed B6 mice. Moreover, enrichment-housed B6 strain remained significantly more active on day 3 of testing than their standard-housed counterpart ($P<0.005$, Fig. 16A).

4.3. Hot plate test

In the hot plate test the latency to lick or shake the hind paw was significantly affected by strain ($F(1,120)=176.08$ $p<0.005$), housing condition ($F(1,120)= 7.15$ $p<0.01$) and strain × housing condition interaction ($F(1,120)=8.41$ $p<0.005$). The latency to jump was significantly affected by strain ($F(1,120)= 148.83$ $p<0.005$), but not housing condition. Independent of housing conditions, 129 strain displayed significantly longer latencies to lick or shake hind paw, and longer latencies to jump when compared to B6 strain ($p<0.005$, Fig. 16C, 16D). Housing in enriched conditions even increased the latency to lick or shake in 129 mice ($p<0.005$, Fig. 16C), accentuating the difference between the two strains.

4.4. Forced swim test

In the forced swim test significant effects of strain ($F(1,120)=41.03$ $p<0.005$) and strain × housing condition interaction ($F(1,120)=6.36$ $p<0.05$) were revealed. 129 strain spent significantly more time immobile than B6 strain irrespective of housing conditions ($p<0.05$ for standard-housed mice, and $p<0.005$ for enrichment-housed mice; Fig. 16B).

4.5. Resident-intruder test

In the resident-intruder test total time in social interaction was significantly affected by strain ($F(1,58)=72.00$ $p<0.005$). Both standard and enrichment-housed 129 mice spent significantly more time in social interaction than B6 mice housed in respective conditions ($p<0.005$, Fig. 16E). In the number of attacks, significant effects of strains ($F(1,58)=4.15$ $p<0.05$) and housing condition ($F(1,58)=4.15$ $p<0.05$), but also tendency to strain × housing condition interaction effect ($F(1,58)=3.51$ $p=0.066$) were observed. 129 strain, housed in standard conditions, performed significantly more attacks on intruders than B6 strain, housed in standard conditions ($p<0.05$), but this strain-related difference was not observed in mice, housed in enriched conditions (Fig. 16F).

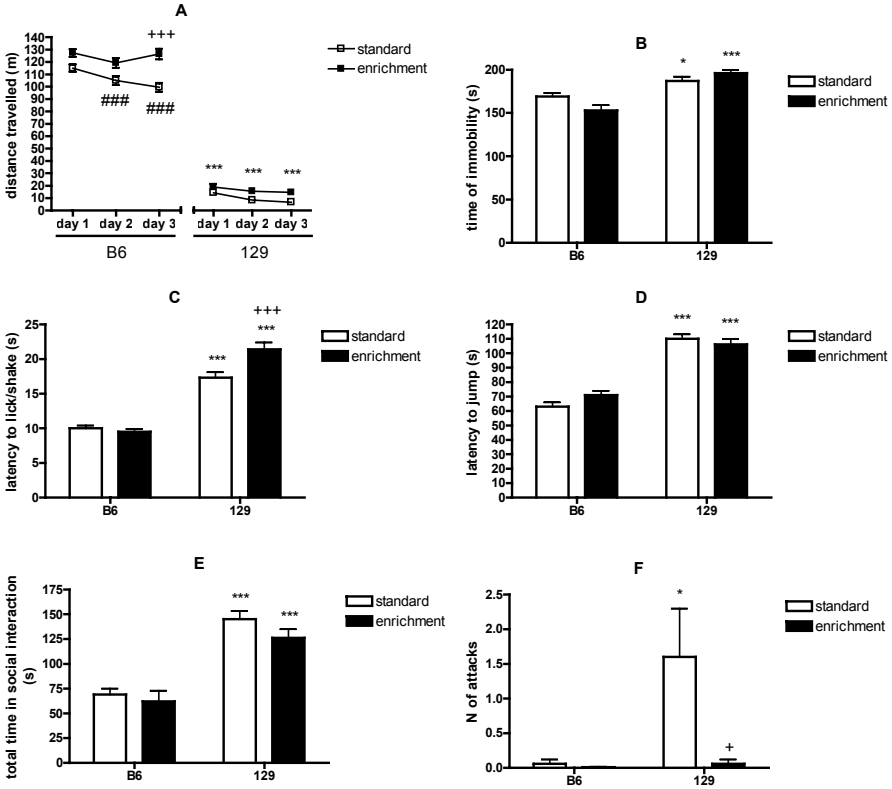

Figure 16. Results of the behavioural test battery in B6 and 129 strains, housed in different environmental conditions. (A) locomotor activity: distance travelled; (B) forced swim test: immobility; (C) hot plate test: latency to lick or shake; (D) hot plate test: latency to jump; (E) resident-intruder test: total time in social interaction; (F) resident-intruder test: number of attacks. * $p<0.05$, *** $p<0.005$: Tukey HSD test after the significant MANOVA, 129 strain compared to B6 strain housed in the same conditions; + $p<0.05$, +++ $p<0.005$: mice housed in enriched conditions compared to mice of respective strain housed in standard conditions; ### $p<0.005$: locomotor activity in B6 mice housed in standard conditions on days 2 and 3, compared to day 1.

DISCUSSION

1. Sex-dependent differences in the phenotype of mice, lacking CCK_2 receptors

Sex-related factors were found to affect the phenotype of CCK_2 receptor deficient mice in Papers I–III. It was observed that in addition to the altered behavioural phenotype, neurochemical findings as well as ethanol-induced effects differed in male and female mutant mice. Previous studies have established that sex has prominent influence on the behavioural phenotypes of inbred mouse lines (Rodgers and Cole 1993; Võikar *et al.* 2001; Caldarone *et al.* 2000). Therefore, it is not surprising that in mouse lines that are genetically less distinct compared to inbred strains (e.g. transgenic mice and their wild-type controls), sex significantly alters the behavioural phenotype. For example, Ramboz *et al.* (1998) observed sex-dependent phenotype of the serotonin 5-HT_{1A} receptor knock-out mice in the open field test. Walther *et al.* (2000) showed that while male Mas-deficient mice displayed increased anxiety in the plus-maze test, female mutant mice did not significantly differ from wild-type controls. That the phenotype of mice, lacking CCK_2 receptors, could be sex-dependent was suggested by previous findings that there are differences between males and females in the distribution patterns (Fox *et al.* 1990; Phan and Newton 1999) and the effects of CCK (Strohmayer and Greenberg 1996; Voits *et al.* 1996). Moreover, although Daugé *et al.* (2001b) reported that male and female CCK_2 receptor deficient mice did not significantly differ from each other, the unpublished observations of our laboratory indicated otherwise. For example, alterations in anxiety-like behaviour have only been observed in female mutant mice (Raud *et al.* 2003; Raud *et al.* 2005; Areda *et al.* 2006), whereas changes in the locomotor, pain-related and reward-related behaviour have been found in male mutants (Kõks *et al.* 2001; Rünkorg *et al.* 2003; Veraksitš *et al.* 2003; Kurrikoff *et al.* 2004; Rünkorg *et al.* 2006; Kurrikoff *et al.* 2008). Indeed, a prominent influence of sex on the behavioural, physiological, neurochemical and ethanol-induced phenotype of mice lacking CCK_2 receptors was observed in the present study.

1.1. Behavioural studies

In Paper I, we demonstrated, using the elevated plus-maze test, that isolated female mice, lacking CCK_2 receptors, were significantly less anxious than their wild-type littermates, housed in the same conditions. The anxiety-level did not significantly differ between male wild-type and male CCK_2 receptor deficient mice, although group-housed male mutant mice made significantly less attempts to exit closed arms than their group-housed wild-type littermates. Moreover, we established that in the resident-intruder test, male but not female mice, lacking

CCK_2 receptors, groomed significantly less than wild-type mice. On the other hand, significantly reduced digging behaviour in the resident-intruder test was found in both male and female mice, lacking CCK_2 receptors. The most clear sex-related finding in the Paper I was the slower weight gain in female mice, but this difference was not modified by the genetic invalidation of CCK_2 receptors.

In Paper III, we demonstrated that female mice, lacking CCK_2 receptors, if housed in environmentally enriched conditions, showed longer jump latencies in the hot plate test when compared to their wild-type littermates. On the other hand, male but not female enrichment-housed mutant mice performed significantly worse on the rota-rod than their wild-type counterparts. Sex-related differences were also observed in the latency to lick or shake hind paw in the hot plate test and in the locomotor activity test. While there were no differences between male and female wild-type mice, enrichment-housed female mutant mice showed significantly longer latencies in the hot plate test as well as higher locomotor activity than male mutant mice. However, several behavioural differences between CCK_2 receptor deficient and wild-type mice were not affected by sex. For example, sex did not modify the mutation-induced alterations in the plus-maze behaviour, restraint-induced analgesia and water maze performance.

On one hand, the sex-specific behavioural phenotype in mice, lacking CCK_2 receptors, could be viewed as a phenotype that is prone to variation due to sex-related factors. This interpretation could undermine the applicability of genetic invalidation of CCK_2 receptors for the behavioural study of the functions of these receptors. On the other hand, though, the sex-dependent phenotype possibly indicates the sex-specific functions of the mutated gene. Indeed, CCK has been shown to have sex-specific functions. It has been demonstrated, that the satiating effect of CCK is stronger in male rats (Voits *et al.* 1996) and that the antagonism of CCK_1 receptors increases food intake only in male rats (Strohmayer and Greenberg 1996). Moreover, it has been demonstrated that estrogens regulate the neurochemical properties and functions of the CCK-ergic system (Dulawa and Vanderweele 1994; Micevych *et al.* 1994; Popper *et al.* 1996; Micevych *et al.* 2002).

However, simple sex-based interpretations of the present findings should rather be avoided, because both Paper I and Paper III used an additional, environmental variable, and both studies indicated a more complex interplay between genotype, sex and environmental factors. For example, the finding that CCK_2 receptor deficient female mice were less anxious than their wild-type littermates was specific for mice, housed in isolated conditions. This behavioural difference was not found in mice, housed in standard conditions (Paper I), or in mice, housed in enriched conditions (Paper III). The interactions between genetic, sex-related and environmental factors are discussed below.

1.2. Radioligand binding

Radioligand binding study with [^{3}H]-spiperone, an antagonist of dopamine D_2 and serotonin 5-HT_2 receptors, also demonstrated differences between male and female CCK_2 receptor deficient mice. In male mutant mice the affinity of dopamine D_2 receptors was increased in the sub-cortical structures. This is in good accordance with the previous studies showing that the sensitivity of dopamine D_2 receptors is elevated in male mice due to the targeted invalidation of CCK_2 receptors (Daugé *et al.* 2001a; Kõks *et al.* 2001). By contrast, dopamine D_2 receptors were not affected in female mutant mice, whereas the affinity of serotonin 5-HT_2 receptors in the frontal cortex was increased three-fold compared to female wild-type mice. CCK has been shown to interact with both dopaminergic and serotonergic systems in the brain (Crawley 1995), thus the alterations in the binding of D_2 and 5-HT_2 receptors resulting from CCK_2 receptor invalidation could be anticipated. However, interestingly there was a clear sex-specific alteration in the sensitivity of those receptors. Although the functional significance of these sex-dependent alterations remained obscure, this was another finding advocating the study of both male and female animals in transgenic research.

1.3. Ethanol-induced behavioural effects and ethanol consumption

In the present study we demonstrated that the behavioural effects of ethanol are altered in mice, lacking CCK_2 receptors, and that these alterations are sex-specific. Ethanol induced a significant reduction in anxiety, as evidenced by an increase in time spent on open arms, the number of unprotected head-dips and lines crossed, in the plus-maze test in female mice, lacking CCK_2 receptors. Moreover, this effect was observed at two doses (1.0 g/kg and 2.0 g/kg), indicating shift in sensitivity to anxiolytic properties of ethanol in female mutant mice. This finding was coupled with an increased sensitivity to the sedative effect of ethanol (4.0 g/kg) in female mutant mice. Although the duration of the loss of righting reflex was not significantly altered, female homozygous mice had significantly lower blood ethanol concentrations at the regain of the righting reflex compared to their wild-type littermates. Given the fact that alcohol exerts many of its effects in the CNS through the GABA-ergic system (Davies 2003; Criswell 2005) our current results were in accordance with the evidence in favour of altered activity of GABA-ergic system in female CCK_2 receptor deficient mice (Raud *et al.* 2003; Raud *et al.* 2005). According to the study of Raud *et al.* (2003), female mice, lacking CCK_2 receptors, have an increased tone of GABA-ergic system, a reduced basal anxiety and an increased sensitivity to diazepam-induced impairments in motor coordination. Moreover, Raud *et al.* (2005) demonstrated 1.6-fold increase in the expression

of the α2 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). While certain genotype-related differences in the anxiolytic and sedative effects of ethanol were established in female mice, none of these were observed in male mice. By contrast, in male mice, ethanol induced a significant genotype-dependent suppression of locomotor activity. While in male wild-type mice ethanol decreased the frequency of rearing only at the largest dose used (2.0 g/kg), a significant suppression of rearing in male mutant mice was observed at doses 0.5 g/kg and 2.0 g/kg. Acute administration of ethanol has been reported to increase striatal dopamine at lower doses, and to decrease it at higher doses (Blanchard *et al.* 1993; Budygin *et al.* 2001). According to previous reports, male but not female mice, lacking CCK_2 receptors, display altered properties of striatal dopamine neurotransmission and altered effects of dopaminergic drugs on locomotor activity (Daugé *et al.* 2001a; Kõks *et al.* 2001, Rünkorg *et al.* 2006). The reduced expression of dopamine D_2 receptors in the nucleus accumbens and mesencephalon has been demonstrated in male CCK_2 receptor deficient mice (Miyasaka *et al.* 2005; Rünkorg *et al*. 2006). We have previously hypothesised that in male mutant mice there is an increase in sensitivity of pre-synaptic dopamine D_2 receptors that might account for the reduction of locomotor activity after administration of small doses of apomorphine or amphetamine (Kõks *et al.* 2001). It is therefore possible that the altered dopaminergic neurotransmission was responsible for the modified locomotor effects of ethanol that was observed in male but not female mutant mice.

Blood ethanol concentrations after injection of ethanol 2.0 g/kg were not significantly affected by either genotype or sex, implicating that the behavioural results reported here were not due to altered ethanol metabolism. However, significantly lower blood ethanol concentrations were observed after injection of ethanol 4.0 g/kg in mice, lacking CCK_2 receptors. While in male mutant mice blood ethanol concentrations were significantly lower only 30 min after ethanol injection, in female mice significant differences were found 30 min and 120 min after ethanol administration. Altered blood ethanol kinetics after dose 4.0 g/kg may have confounded the loss of righting reflex test. Although the duration of the loss of righting reflex was not affected by genotype or sex, the blood ethanol concentrations at the regain of righting reflex were significantly lower in female CCK_2 receptor deficient mice. This finding likely reflected the higher sensitivity of female mutant mice to the sedative action of ethanol, but this effect could not be demonstrated by measuring the loss of righting reflex because of accelerated ethanol kinetics in female mutant mice.

Female mice, independent of genotype, preferred and consumed ethanol significantly more than their male counterparts, especially at higher concentrations (6 % and 10 %). This was in line with other studies reporting increased ethanol preference in female compared to male rodents (Lancaster and Spiegel

1992; Middaugh *et al.* 1999), making studies in both sexes preferable to the common practice of using only male rodents. Indeed, female, but not male mice, lacking CCK_2 receptors, consumed significantly more 10 % ethanol than their wild-type littermates. This finding was in good accordance with the study of Miyasaka *et al.* (2005) reporting unaltered ethanol consumption in male CCK_2 receptor deficient mice. However, higher ethanol intake in female mutant mice was accompanied by a significant increase in total fluid intake at alcohol concentration 10 %, resulting in unaltered ethanol preference. Provided that female mice, lacking CCK_2 receptors, displayed increased sensitivity to anxiolytic and sedative effects of ethanol, increased ethanol intake was a somewhat unexpected finding. To exclude alterations in taste preference that could have influenced ethanol intake, preference to non-alcoholic tastants sucrose and quinine was measured in mice, lacking CCK_2 receptors. Yet, no genotype or sex-related alterations were observed in taste preference. Further studies are encouraged to determine if the development of tolerance to ethanol has been affected by the invalidation of CCK_2 receptors in female mice. It has to be mentioned that the background strain of mutant and wild-type mice in the current study was 129Sv/C57BL/6 back-crossed more than six times to C57BL/6. C57BL/6 is known for its high spontaneous ethanol preference and consumption (Belknap *et al.* 1993; Middaugh *et al.* 1999). Thus, the phenotype reported here might have been specific to the current background strain and could be modified in strains with lower ethanol intake.

2. Different environmental conditions altered the behavioural phenotype in CCK_2 receptor deficient mice

Both environmental manipulations (social isolation and environmental enrichment), used in the present study, significantly altered the behaviour of mice. Interestingly, alternative environmental conditions substantially modulated the behavioural phenotype of mice, lacking CCK_2 receptors, indicating that the mutation-induced phenotype resulted from both genetic and environmental factors. Moreover, as was mentioned earlier, several behavioural domains were affected by the complex interplay between the mutation, sex and environmental factors.

2.1. Social isolation-induced differences in the phenotype of mice, lacking CCK_2 receptors

Social isolation reduced weight gain in male, but not in female mice. Similar sex-specific reduction of weight gain has been reported by Ness *et al.* (1995). The weight gain of CCK_2 receptor deficient mice did not differ from that in wild-type

mice. This was in good accordance with the study of Kopin *et al*. (1999) showing that mice, lacking CCK_2 receptors, do not exhibit any alterations in weight gain under normal housing conditions. In male mice, social isolation produced a substantial increase in exploratory activity, evident in both plus-maze and locomotor activity test. Võikar *et al.* (2005) reported a similar finding in C57 and DBA strains. However, in female mice only a slight increase of exploratory activity was observed. While in male mice, independent of genotype, anxiety was not significantly affected by social isolation, in female wild-type mice increased anxiety-like behaviour was established. By contrast, in female mutant mice social isolation failed to produce an increase in anxiety. This was an interesting genotype by sex by environmental condition interaction. Palanza (2001) reported that in CD–1 strain social isolation produced opposite effects in male and female mice. Isolated male mice were less anxious, whereas isolated females showed signs of increased anxiety when compared to their group-housed counterparts. Moreover, it has been shown that social isolation increases CCK mRNA expression and CCK receptor density in the frontal cortex in rats (Vasar *et al.* 1993; Del Bel and Guimaraes 1997). It is therefore suggested, that the attenuated social isolation-induced anxiety in female mice, lacking CCK_2 receptors, indicated the sex-specific role of CCK_2 receptors in the development of anxiety by individual housing. On the other hand, though, it should be stressed that the reduced anxiety, which had been anticipated to result from the genetic invalidation of CCK_2 receptors, was only evident in socially isolated female mice. The anxiety-like behaviour was not modified in male mutant mice, independent of housing conditions, and in female mutant mice housed in groups. This was in contradiction with the studies of Raud *et al.* (2003; 2005) and Areda *et al.* (2006), who demonstrated that standard-housed female CCK_2 receptor mice display reduced anxiety in both the plus-maze and the light-dark exploration test. Therefore, from the transgenic point of view, the credibility of anxiety-related findings in mice, lacking CCK_2 receptors, is low, because this phenotype strongly depends on sex- and environment-related factors. This interpretation would suggest, that the role of CCK_2 receptors in the anxiety-like behaviours is rather middling. The same applies to the locomotor activity-related phenotypes. Previous studies have established that CCK_2 receptor deficient mice show increased locomotor activity (Daugé *et al.* 2001b) and increased sensitivity of the dopaminergic system (Daugé *et al.* 2001a; Kõks *et al.* 2001). However, in the present study no differences in the locomotor activity between wild-type and mutant mice were found. Social isolation increased locomotor activity in male mice, independent of genotype. Furthermore, the increase of aggressive behaviours produced by individual housing, which has been associated with the alterations in the dopaminergic system (Poshivalov 1981), did not differ between CCK_2 receptor deficient mice and their wild-type littermates. Therefore, it can be concluded that the effects of CCK_2 receptor mutation were rather context-specific and the validity of results beyond a specific context (i.e. species, strain, sex and environmental conditions) may be pretty scanty.

2.2. Environmental enrichment-induced differences in the phenotype of mice, lacking CCK_2 receptors

Environmental enrichment produced a substantial reduction of anxiety and increased exploratory activity in mice. In the hot plate test reduced latency to hind-paw response was established in male mice. This seeming increase in pain sensitivity could have been related to a reduction in stress-induced analgesia caused by testing itself in naïve animals (Siegfried *et al.* 1987). Most notable effects of enrichment were, however, observed in the water maze and rota-rod tests. In line with previous studies (Lewis 2004; Nithianantharajah and Hannan 2006), these tests revealed substantially better cognitive abilities and coordination in mice, housed in enriched conditions, when compared to their littermates, housed in standard conditions.

In the plus-maze test, an increase of anxiety-like behaviours was observed in mice, lacking CCK_2 receptors. However, this phenotype was only observed in mutant mice, housed in standard, but not enriched conditions. The phenotype reported in the present study was in notable contradiction with previous findings from our own laboratory. Raud *et al*. (2003) and Areda *et al.* (2006) reported that female mice, lacking CCK_2 receptors, were significantly less anxious in the plus-maze. It should be noted, though, that this phenotype has varied from study to study and has not been consistently reproduced even by our own laboratory. Similarly, other laboratories have reported different anxiety-related phenotypes in mice, lacking CCK_2 receptors (Daugé *et al.* 2001b; Miyasaka *et al.* 2002b; Horinouchi *et al*. 2004). Of interest is the study conducted by Miyasaka *et al.* (2002b), which also demonstrated increased anxiety-like behaviour in CCK_2 receptor deficient mice. Although authors did not specify the level of illumination of the plus-maze, they reported handling of mice prior to the experiment. Since altering aversion of the testing situation has been shown to substantially influence plus-maze results (Hogg 1996), we believe that the increased anxiety-like behaviour of mutant mice in the present study could be attributed to the use of dimly lit plus-maze, instead of the brightly lit plus-maze, used in previous studies (Raud *et al.* 2003; Areda *et al.* 2006). It is possible, that the modified plus-maze test revealed the inability of CCK_2 receptor deficient mice to adapt to lower levels of stress. Still, concerning the main goal of the present study, the plus-maze test result clearly demonstrated that the phenotype of mice, lacking CCK_2 receptors, could significantly differ within one laboratory, depending on the housing conditions of the subjects.

The reports on the locomotor activity of mice, lacking CCK_2 receptors, have been as contradictory as the reports on their anxiety. While Daugé *et al.* (2001a; 2001b) and Pommier *et al.* (2002) reported dramatically increased activity in CCK_2 receptor deficient mice, our laboratory has reported all possible alterations: slight increase and impaired habituation (Rünkorg *et al.* 2003), slight decrease (Kõks *et al.* 2001), and no difference at all (Rünkorg *et al.* 2006). In the present study locomotor activity was not modified by genetic

invalidation of CCK_2 receptors in mice, independent of sex or housing condition. Although environmental enrichment itself had only a minor effect, a sex-related difference in mutant mice was observed depending on the rearing conditions. Namely, female mutant mice were significantly more active and spent more time in the centre of the apparatus than male mutant mice, when housed in enriched, but not in standard conditions. This sex-dependent difference was not observed in wild-type mice in either environmental condition.

Pommier *et al.* (2002) reported reduced jump latencies in CCK_2 receptor deficient mice as assessed by the hot plate test, suggesting tolerance in the pain inhibiting endogenous opioid system. By contrast, Veraksitš *et al.* (2003) reported increased nociceptive thresholds in male mice, lacking CCK_2 receptors, as indicated by longer latencies to hind paw reaction in the hot plate test. None of these findings were reproduced in the present study, though. The CCK_2 receptor gene invalidation did not alter either the latency to hind paw reaction or latency to jump in the hot plate test in mice, housed in standard conditions. On the other hand, housing in enriched environment significantly modified the hot plate results. While the latency to hind paw reaction was affected by housing condition in sex-, but not genotype-dependent manner, the latency to jump was significantly affected by both genotype and sex. Female mice, lacking CCK_2 receptors, housed in enriched environment, showed significantly longer latencies to jump than any other group, independent of housing condition. Furthermore, a significant environment-dependent phenotype was observed in the restraint-induced analgesia, which was substantially increased in CCK_2 receptor deficient mice, housed in standard, but not enriched conditions. Unlike hot plate latencies in unstressed mice, however, restraint-induced analgesia was not affected by sex. Previously, it has been reported that the CCK_2 receptor gene invalidation leads to the up-regulation of the endogenous opioid system (Pommier *et al.* 2002; Veraksitš *et al.* 2003; Kurrikoff *et al.* 2004), thus serving as a possible explanation for the potentiation of stress-induced analgesia in mice, lacking CCK_2 receptors. However, this interpretation was not supported by the finding in mutant mice, housed in enriched conditions, which totally lacked the phenotype observed in standard-housed mice.

As reported by Daugé *et al.* (2001b) employing a pooled sample of male and female mice, CCK_2 receptor deficient mice displayed impaired rota-rod performance. This finding was replicated by Kõks *et al.* (2001) using male mice, but not by Raud *et al.* (2003) using female mice. In the present study, significant genotype- and sex-dependent differences were found in mice, housed in enriched conditions. Male mice, lacking CCK_2 receptors, showed significantly shorter latencies to fall from rota-rod than their wild-type littermates. This genotype-dependent difference was not observed in female mice, thus confirming the phenotype published before. Yet, the impairment in the rota-rod performance was not observed in CCK_2 receptor deficient mice, housed in standard conditions. In order to differentiate standard-housed mice from mice, housed in enriched conditions, we used a rather high rotation speed, possibly

reducing the sensitivity of the test to detect alterations in standard-housed mice. Sebret *et al.* (1999) and Daugé *et al.* (2001b), in the only studies describing cognitive functions in mice, lacking CCK_2 receptors, showed that mutant mice made significantly more errors than their wild-type littermates in the two-trial memory task and in the Y-maze. In the present study, employing the water maze, a test to assess spatial learning and memory, we confirmed impaired cognitive functions in standard-housed CCK_2 receptor deficient mice. Mutant mice, housed in standard conditions, travelled significantly longer distance to platform on day 2 of acquisition phase and spent significantly more time exploring an irrelevant quadrant in the probe trial than their wild-type littermates, these differences indicating impairments in spatial learning and memory. In CCK_2 receptor deficient mice, though, enrichment produced a robust improvement in learning and memory, resulting in no detectable differences between the genotypes.

The reproducibility issue of behavioural phenotypes has been raised by several studies before (Crabbe *et al.* 1999; Wahlsten *et al.* 2003; Wahlsten *et al.* 2006; Lewejohann *et al.* 2006). In a study initially published by Crabbe *et al*. (1999) and later discussed in more detail by Wahlsten *et al.* (2003) a major conclusion was drawn that the phenotypic differences with large effect size could be reproduced in different laboratories, whereas the phenotypes with small effect size would be more prone to modification by laboratory environment. Wahlsten *et al.* (2006) also demonstrated that prominent phenotypes of inbred mouse strains were generally stable even over decades. In case of significant genotype by laboratory interactions, however, conflicting interpretations of the results could emerge. It is possible, that the mice, lacking CCK_2 receptors, represent a strain with small effect size phenotype that has a propensity to vary across studies, resulting in controversial allegations of the functions of the given gene. Among behavioural tests, especially the plus-maze results may markedly vary across laboratories (Crabbe *et al.* 1999; Wahlsten *et al.* 2003; Lewejohann *et al.* 2006), as has been the case with CCK_2 receptor deficient mice (Daugé *et al.* 2001b; Miyasaka *et al.* 2002b; Raud *et al.* 2003; Horinouchi *et al.* 2004; Raud *et al.* 2005; Areda *et al.* 2006). It should be stressed, though, that the results of the current study could not be precisely compared to the results of previous studies employing CCK_2 receptor deficient mice, due to modifications in the behavioural tests, differences in the innate laboratory conditions and probable alterations in the genetic background of mice. Rather, the present study aimed to show that the phenotype of mutants could substantially differ depending on pre-experimental housing conditions within the same laboratory, warning against immediate conclusions about the functions of the mutated gene.

Results that can not be easily reproduced in different environmental conditions, similarly to those discussed in the present study, might be misleading and viewed as disadvantageous by many scientists. However, such results may represent an opportunity to broaden our understanding of the functions of genes

by revealing complex interactions between genes and environmental factors (Würbel 2002). Indeed, it has been suggested that controlled variation of environment, for example by means of environmental enrichment, would only enhance studies on gene functions and could become an integral part of transgenic studies (van der Staay and Steckler 2002; Würbel 2002). Environmental enrichment could serve as a simple method to dissect a phenotype into effects arising mainly from the genetic manipulation (reproducible effects) and effects arising from the interplay between the manipulated gene and environment (non-reproducible effects) (Würbel 2002). In this context, the phenotype of CCK_2 receptor deficient mice, which was not reproducible in mice reared in different environmental conditions, could not be attributed to the mutation itself but rather to the interaction between the mutation and environment. For example, the results of the water maze reported here could be viewed as a reproduction of cognitive impairment in mice, lacking CCK_2 receptors, by a different laboratory, but this held true only for mutants housed in standard conditions. Thus, the more relevant interpretation of this finding would be, that the invalidation of CCK_2 receptor gene resulted in cognitive impairment in only specific environmental conditions (i.e. standard housing conditions), indicating not an absolute effect of the gene but rather an interaction between the gene and environmental factors. This implies that unless CCK_2 receptor mutation has been studied in different environmental conditions (as well as in different sexes and in different genetic backgrounds), any generalisations are hardly justified and only assertions regarding the functions of the gene in specific environmental conditions (i.e. standard housing), sex (i.e. male) and genetic background (i.e. C57/BL6) can be made.

3. Environmental enrichment did not significantly alter the behavioural phenotype of C57BL/6 and 129S6/SvEv strains

The present findings regarding the behavioural profiles of B6 and 129 strains were in accordance with those published before (Crawley *et al.* 1997; McIlwain *et al.* 2001; Võikar *et al.* 2001; Rodgers *et al.* 2002; Bothe *et al.* 2004; Võikar *et al.* 2004). We demonstrated that independent of pre-experimental housing conditions 129 strain displayed reduced number of rearings and groomings in the plus-maze test, lower locomotor activity across 3 days, increased latencies to lick or shake hind paw as well as to jump in the hot plate test, more time spent immobile in the forced swim test and more time engaging in social interaction when compared to B6 strain. Housing in enriched environment significantly altered the behaviour of both strains when compared to their counterparts, housed in standard conditions. Enrichment-housed B6 mice displayed higher exploratory activity in the plus-maze test as well as reduced habituation in the locomotor activity test, whereas enrichment-housed 129 strain

showed longer latencies to lick or shake hind paw in the hot plate test and reduced aggression in the resident-intruder test. Nevertheless, the major behavioural differences between the strains were reliably reproduced independent of pre-experimental housing conditions. On the other hand, differences in exploration- and anxiety-related behaviours in the plus-maze (i.e. total entries on all arms, number of unprotected head-dips and stretched-attend postures) and aggressive encounters in the resident-intruder test depended on pre-experimental housing conditions, suggesting a substantial contribution of environmental factors to the development of these phenotypes and, therefore, lower reproducibility. Interestingly, in the plus-maze, locomotor activity, hot plate and forced swim test, behavioural differences between B6 and 129 strains, housed in enriched conditions, were even larger when compared to differences between strains housed in standard conditions. A similar finding has been reported for BALB/c and B6 mice. Van de Weerd *et al.* (1994) demonstrated that housing in enriched environment increased anxiety in BALB/c mice, a strain known for high levels of anxiety, but not in B6 mice. These results suggest that environmental enrichment might facilitate emotional development and even accentuate behavioural differences between strains that are very distinct in genetic as well as behavioural terms.

Although it is well accepted that genetic factors (i.e. background genes) substantially affect the development of phenotypes in genetically manipulated animals (Gerlai 1996; Crusio 2004), the contribution of environmental variation is usually either neglected or viewed as undesirable (Würbel 2002). The present study together with other studies that have reported significant interactions between genotypes and enriched environment (Rampon *et al.* 2000; van Dellen *et al.* 2000; Tremml *et al.* 2002; McOmish *et al*. 2008) advocates the application of alternative housing conditions within a laboratory. This approach could be used to assess the reproducibility of results in transgenic studies, but also to reveal the extent to which environmental factors affect the development of behavioural phenotypes (van der Staay and Steckler 2002; Würbel 2002).

The routine use of environmental enrichment in addition to standard housing conditions has several caveats, however. Certainly, this approach is in contradiction with the reduction principle of the 3 R's (replacement, refinement, reduction). Furthermore, the regular application of an additional environmental condition is expensive and labour consuming. And yet, probably the most important issue is that there are virtually endless possibilities to enrich environment in rodents (Nithianantharajah and Hannan 2006). Thus, theoretically, a large variation of behavioural phenotypes can be produced within a single mouse line by endless alterations in environmental conditions. However, at present, our ability to draw meaningful conclusions from such enormous amount of behavioural information seems pretty low.

4. Concluding remarks and future prospects

The present study demonstrates that sex and environmental factors substantially contribute to the expression of the behavioural phenotype in cholecystokinin CCK_2 receptor deficient mice. Sex significantly modifies the effects of ethanol in mutant mice but also determines several behavioural differences from wild-type mice. To name just a few, male mice, lacking CCK_2 receptors, display impaired rota-rod performance whereas female mice show altered anxiety-like behaviours and nociceptive thresholds. Moreover, sex affects the alterations in the phenotype of mutant mice subjected to environmental manipulations by means of social isolation and environmental enrichment. Female, but not male mice, lacking CCK_2 receptors, differ from their wild-type controls in anxiety-like behaviours when housed individually but not when housed in groups. These results suggest that the CCK_2 receptors may have a sex-specific role in the regulation of behaviour. This statement is backed up by previous findings, indicating sex-specific effects of CCK in rodents. Therefore, to reveal possible sex-specific functions of gene products, more attention should be paid to sex as a factor in transgenic research.

The present study also demonstrates that various pre-experimental environmental conditions affect the expression of the behavioural phenotype in mice, lacking CCK_2 receptors. Social isolation produces a marked increase in the anxiety level in female wild-type mice, but fails to affect female mutant mice or male mice of either genotype. On one hand, this finding implies that social isolation produces sex-dependent effects and that these effects can be counteracted by the targeted mutation of CCK_2 receptors in female mice. On the other hand, this result demonstrates that the expected low-anxiety profile in the mutant line can be revealed only in one sex and in certain environmental conditions. The results of the study employing environmental enrichment as an alternative housing condition are especially intriguing. We demonstrate that the behavioural profile of mice, lacking CCK_2 receptors, cannot be reproduced in mice housed in alternative conditions. The authors view this finding as the reproduction of controversial results regarding this mutant line published over a period of almost ten years. It is therefore suggested that parallel use of substantially different housing conditions within one laboratory may enable prediction of the reproducibility of behavioural results. To test this hypothesis, we performed basically the same study but employed two genetically distinct mouse lines. Indeed, in the lines with substantially different behavioural profiles and good reproducibility of results over years, the altered housing conditions fail to substantially affect the behavioural differences.

The authors view the present findings as a strong indication for more sophisticated approach to behavioural studies in transgenic research. The often-used approach of studying the effects of a gene mutation in only one sex or under certain environmental conditions is reductionist in its nature and does not allow drawing any far-reaching assertions of the functions of the affected gene.

It seems that the reductionist approach is quite prone to producing conflicting rather than reproducible results. Unless an enormous variety of additional factors to the mutation have been studied in detail, authors would like to question any far-reaching implications (e.g. from mouse to man) based on the results that cannot be applied even to other strains of the same species, to other sex of the same strain or to virtually the same subjects housed in different conditions.

Unfortunately, it is impossible to conclude from this study, whether the low reproduciblity of results in mice, lacking CCK_2 receptors, stems from the innate properties of the CCK-ergic system, from the more general problem with reproducibility in transgenic research or from the combination of both. With a ray of optimism but the bitter knowledge that a more complex and labour-consuming approach might render higher reliability of behavioural findings, the author plans to carry on with studies in novel transgenic lines that are available at our laboratory. At present there is substantial body of evidence demonstrating that the behavioural effects of drugs can be significantly modified by environmental factors. Therefore, in contrast to pharmacogenomic studies that explore mainly the contribution of the genetic factors, we are also interested in further expanding the knowledge about the contribution of environment-related factors to the effects of drugs.

CONCLUSIONS

1. Pre-experimental social isolation for three weeks induces sex-specific behavioural alterations in mice. In male mice, reduced weight gain, increased locomotor activity and aggressive behaviours are observed, whereas in female mice, increase in anxiety-like behaviour is evident. The genetic invalidation of CCK_2 receptors does not significantly modulate the effect of social isolation in male mice. However, in female CCK_2 receptor deficient mice social isolation fails to produce an increase in anxiety, which is observed in isolated female wild-type mice. Therefore, CCK_2 receptors likely participate in the development of social isolation-induced anxiety in female mice. Consequently, the pre-experimental housing conditions as well as sex-related factors significantly modify the behavioural phenotype of mice, lacking CCK_2 receptors. Also, sex affects the neurochemical alterations induced by CCK_2 receptor invalidation. In male group-housed mutant mice the sensitivity of subcortical dopamine D_2 receptors is increased. By contrast, in female mutant mice the sensitivity of serotonin 5-HT_2 receptors in the frontal cortex is increased.
2. Ethanol induces sex-specific effects in mice lacking CCK_2 receptors. Female mutant mice are more sensitive to the anxiolytic as well as sedative effects of ethanol when compared to their wild-type littermates. On the other hand, the locomotor effects of ethanol are modified only in male mutant mice. Female, but not male mice, lacking CCK_2 receptors, consume ethanol significantly more than their wild-type littermates.
3. The behavioural phenotype of mice, lacking CCK_2 receptors, is significantly modified by pre-experimental housing conditions. With the exception of the forced swim test, none of the behavioural phenotypes in CCK_2 receptor deficient mice can be reproduced in alternative housing conditions. Standard-housed mutant mice are more anxious, show stronger restraint-induced analgesia and perform worse in the water maze when compared to their wild-type littermates. The behavioural profile of enrichment-housed mutant mice is substantially different, though. Male mutant mice housed in enriched conditions display poor rota-rod performance and female mutant mice show increased jump latency from the hot plate when compared to wild-type mice, housed in enriched conditions.
4. In accordance with the previous studies, 129 strain shows substantially reduced exploratory behaviour, increased hot plate latencies, more time spent immobile in the forced swim test and engages more in social interaction when compared to B6 strain. Although environmental enrichment significantly modifies the behavioural profiles of both strain, the major behavioural differences between the two strains are reproducible in mice housed in alternative conditions.

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SUMMARY IN ESTONIAN

Sugu ja keskkondlikud tegurid määravad CCK_2 retseptorite puudulikkusega hiirte käitumusliku fenotüübi: soovitused käitumuslikeks uuringuteks transgeensetel hiireliinidel

Sissejuhatus

Geneetiliselt modifitseeritud hiired on muutunud tänapäeval sama oluliseks uurimisobjektiks selgitamaks psüühiliste seisundite baasmehhanisme nagu olid psühhofarmakonid pool sajandit tagasi. Paraku on paljude mutantsete hiireliinide puhul osutunud võimatuks korrata esmapilgul veenvaid ja "ilusaid" (hüpoteesiga kooskõlas olevaid) katsetulemusi. On ilmnenud, et geneetiliste manipulatsioonide tagajärjel tekkivaid fenotüüpe mõjutavad mitmed tegurid. Nimetatud teguritest on olulisemad geneetilised tegurid (s.h. kasutatav taustaliin ja tüvirakkudest pärinevad geenid), sugu ning erinevad keskkondlikud faktorid. Käesolevaks tööks andsid ajendi mitu tähelepanekut. Esiteks, meie laboris koletsüstokiniini funktsioonide uurimisel kasutatav koletsüstokiniini CCK_2 retseptori puudulikkusega hiireliinil näis esinevat katseloomade soost sõltuv fenotüüp, mis oli erinevatel põhjustel jäetud tähelepanuta. Teiseks, nimetatud mutantse hiireliini kohta olid enam kui viis erinevat laborit üle maailma avaldanud kohati diametraalselt vastukäivaid tulemusi. Ning kolmandaks, vasturääkivaid tulemusi saadi ka erinevate teadlaste poolt meie enda laboris. Seetõttu seati käesoleva töö eesmärgiks uurida tegureid, mis võivad mõjutada CCK_2 retseptorite puudulikkusega hiirte käitumuslikku fenotüüpi ning vähendada tulemuste korratavust.

Uurimuse põhieesmärgid

Käesoleva uurimuse põhieesmärgiks oli selgitada katselooma soo ja katse-eelsete keskkondlike tegurite mõju koletsüstokiniini CCK_2 retseptorite puudulikkusega hiirte käitumuslikule fenotüübile. Selleks kasutati emaseid ja isaseid CCK_2 retseptorite puudulikkusega hiiri. Alternatiivsete katse-eelsete keskkonnatingimustena kasutati sotsiaalset isolatsiooni ja keskkonna rikastamist. Et võrrelda mutantsetel hiirtel saadud keskkonna rikastamise tulemusi kahel geneetiliselt erineval hiireliinil saadud tulemustega, kasutati lisaks 129S6/SvEv ja C57BL/6 hiireliine. Uurimuse täpsemad eesmärgid olid järgnevad:

1. Uurida katse-eelse sotsiaalse isolatsiooni efekti CCK_2 retseptorite puudulikkusega hiirte käitumuslikule fenotüübile. Samuti selgitati katseloomade soo mõju erinevates tingimustes majutatud mutantide käitumuslikule fenotüübile. Lisaks käitumuslikule fenotüübile uuriti tavatingimustes majutatud katseloomadel CCK_2 retseptorite mutatsioonist tingitud dopamiini D_2 ja

serotoniini 5-HT_2 retseptorite parameetrite muutuste sõltuvust loomade soost.
2. Selgitada, kas katseloomade sugu modifitseerib etanooli käitumuslikke efekte, etanooli tarvitamist ning etanooli kineetikat CCK_2 retseptorite puudulikkusega hiirtel.
3. Kasutades alternatiivse majutustingimusena keskkonna rikastamist, uurida katse-eelsete majutustingimuste mõju CCK_2 retseptorite puudulikkusega hiirte käitumuslikule fenotüübile.
4. Kasutades alternatiivse majutustingimusena keskkonna rikastamist, hinnata käitumiskatsete tulemuste korratavust kahel geneetiliselt erineval hiireliinil, 129S6/SvEv/Tac ja C57BL/6.

Katseloomad ja meetodid

Kasutati isaseid ja emaseid CCK_2 retseptorite puudulikkusega hiiri. Lisaks kasutati isaseid ja emaseid 129S6/SvEv/Tac ja C57BL/6 liini hiiri.

Sotsiaalse isolatsiooni teostamiseks majutati pooled mõlemast soost ja genotüübist katseloomad üksikpuuridesse (330 × 120 × 130 mm). Kontrollloomadena kasutati ühispuurides majutatud loomi (8–11 hiirt puuris; puuri suurus 425 × 266 × 155 mm). Majutustingimuste muutmise hetkel oli loomade vanus kolm kuud. Sotsiaalse isolatsiooni kestvus oli kolm nädalat.

Keskkonna rikastamise teostamiseks majutati pooled katseloomad suurematesse puuridesse (595 × 380 × 200 mm), milles olid peale tavapärase allapanu, toidu ja vee ka pesamaterjal, haavapuidust torud, maja, iglu, redelid või labürindid, ning roostevabast terasest jooksuratas või kiik. Kord nädalas muudeti objektide asetust või vahetati need uute vastu välja. Standard-majutustingimustel majutatud loomad elasid tavapuurides (425 × 266 × 155 mm), milles oli ainult allapanu, toit ja vesi. Keskkonna rikastamise alustamise hetkel olid loomad kolm nädalat vanad ning keskkonna rikastamine kestis seitse nädalat enne katseid ja lisaks kaks nädalat katsete vältel.

Katseloomade käitumuslikuks iseloomustamiseks kasutati tõstetud plusspuuri, automatiseeritud liikumisaktiivsuse testi, residendi-sissetungija katset, püstamisrefleksi kaotamise katset, sundujumise katset, rota-rodi, vesipuuri testi, kuuma plaadi katset ning saba jõnksatuse testi immobiliseerimisest tingitud analgeesia hindamiseks. Lisaks hinnati CCK_2 retseptorite puudulikkusega loomadel alkoholi tarbimist ja maitse-eelistust sahharoosile ja kiniinile. Etanooli kineetika mõõtmiseks hinnati vere etanooli kontsentratsioone erinevatel ajahetkedel. Dopamiini D_2 ja serotoniini 5-HT_2 retseptorite parameetrite uurimiseks ajukoores ja ajukoorealustes struktuurides kasutati sidumiskatseid [^{3}H]-spiperooniga.

Peamised tulemused

Sotsiaalne isolatsioon põhjustas isastel hiirtel olulise kaaluiibe alanemise ning uudistamisaktiivsuse ja agressiivsuse tõusu. Emastel hiirtel põhjustas individuaalne majutus seevastu genotüübist sõltuva ärevuse tõusu. Erinevalt metsikut-tüüpi emastest hiirtest ei ilmnenud isoleeritud emastel CCK_2 retseptorite puudulikkusega hiirtel olulist ärevuse-taolise käitumise tõusu pluss-puuris. Samuti leiti mutantsetel hiirtel olulised soost sõltuvad erinevused dopamiini ja serotoniini retseptorite parameetrites. Isastel CCK_2 retseptorite puudulikkusega hiirtel oli märkimisväärselt tõusnud subkortikaalsete dopamiini D_2 retseptorite afiinsus, mida ei esinenud emastel mutantsetel hiirtel. Seevastu emastel CCK_2 retseptorite puudulikkusega hiirtel leiti kortikaalsete serotoniini 5-HT_2 retseptorite afiinsuse tõus.

Emased CCK_2 retseptorite puudulikkusega hiired olid märkimisväärselt tundlikumad etanooli ärevusvastasele toimele pluss-puuris ning etanooli uinutavale toimele. Sellist genotüübist tingitud erinevust ei ilmnenud isastel hiirtel. Seevastu isastel CCK_2 retseptorite puudulikkusega hiirtel oli muutunud etanooli toime liikumisaktiivsusele. Nimelt põhjustas suur etanooli annus (2.0 g/kg) liikumisaktiivsuse langust kõigis rühmades, kuid erandina tekkis liikumisaktiivsuse supressioon isastel mutantsetel hiirtel ka etanooli vähima annuse (0.5 g/kg) manustamise järgselt. Etanooli annuse 4.0 g/kg kineetika oli CCK_2 retseptori puudulikkusega hiirtel muutunud, kuid sugu seda muutust oluliselt ei mõjutanud. Küll aga mõjutas katseloomade sugu oluliselt etanooli tarvitamist. Emased hiired tarvitasid ja eelistasid etanooli märkimisväärselt enam kui isased loomad. Enamgi veel, emased CCK_2 retseptori puudulikkusega hiired tarvitasid märkimisväärselt rohkem etanooli kui nende metsikut-tüüpi pesakonnakaaslased. Sellist genotüübist sõltuvat erinevust alkoholi tarbimises ei esinenud isastel loomadel.

Katse-eelne majutamine rikastatud keskkonnas mõjutas oluliselt käitumusliku profiili avaldumist CCK_2 retseptorite puudulikkusega hiirtel. Standardsetes tingimustes majutatud mutantsed hiired oli ärevamad kui nende metsikut-tüüpi pesakonnakaaslased, neil esines tugevam immobilisatsioonist tingitud valutundlikkuse langus ning vesipuuri testis ilmnes neil langenud ruumilise õppimise võime ja kehvem mälu. Rikastatud tingimustes majutatud mutantsetel loomadel selliseid erinevusi metsikut-tüüpi pesakonnakaaslastest ei leitud. Seevastu rikastatud tingimustes elavatel CCK_2 retseptorite puudulikkusega hiirtel ilmnes soost sõltuv fenotüüp kuuma plaadi katses ning rota-rodi testis. Nimelt täheldati emastel mutantsetel hiirtel märkimisväärselt pikenenud hüppelatentsi kuuma plaadi katses, samas kui isastel mutantsetel hiirtel olid oluliselt häiritud tasakaal ja koordinatsioon rota-rodi testis.

Rikastatud majutustingimused mõjutasid oluliselt 129S6/SvEv (129) ja C57BL/6 (B6) hiireliinide käitumist. B6 hiireliinil tõusis oluliselt eksploratiivne aktiivsus pluss-puuris ning halvenes kohanemine liikumisaktiivsuse testis. 129 hiireliinil seevastu põhjustas elamine rikastatud tingimustes käpa raputamise

latentsi pikenemise kuuma plaadi katses ning agressiivsuse vähenemise residendi-sissetungija katses. Vaatamata märkimisväärsele rikastatud keskkonna efektile olid olulised käitumuslikud erinevused kahe liini vahel korratavad sõltumata majutustingimustest. B6 liin oli statistiliselt tunduvalt kõrgema uudistamisaktiivsusega pluss-puuris ja liikumisaktiivsuse testis ning märksa aktiivsem sundujumise katses. 129 liinile olid iseloomulikud pikemad latentsid kuuma plaadi katses ning suurem sotsiaalne aktiivsus residendi-sissetungija katses.

Järeldused

1. Katse-eelne sotsiaalne isolatsioon kolme nädala jooksul tekitab olulise soost sõltuva füsioloogilise ja käitumusliku muutuse hiirtel. Isastel hiirtel esineb isolatsioonist tingitud kaaluiibe langus, tõuseb liikumisaktiivsus ning agressiivsus. Emastel hiirtel seevastu ilmneb genotüübist sõltuv ärevuse tõus. CCK_2 retseptorite geneetiline väljalülitamine ei mõjuta oluliselt sotsiaalse isolatsiooni efekti isastel hiirtel. Seevastu emastel CCK_2 retseptorite puudulikkusega hiirtel ei tekita sotsiaalne isolatsioon ärevuse tõusu. Seega, CCK_2 retseptorid tõenäoliselt osalevad sotsiaalsest isolatsioonist tingitud ärevuse tekkimises emastel hiirtel. Samuti mõjutab katseloomade sugu CCK_2 retseptorite puudulikkusest tingitud neurokeemilisi muutusi. Isastel mutantsetel hiirtel on võrreldes nende metsikut-tüüpi pesakonnakaaslastega tõusnud subkortikaalsete dopamiini D_2 retseptorite afiinsus, samas kui emastel mutantsetel hiirtel on tõusnud serotoniini 5-HT_2 retseptorite afiinsus frontaalkoores.
2. Etanool põhjustab katseloomade soost sõltuvaid efekte CCK_2 retseptorite puudulikkusega hiirtel. Emased mutantsed hiired on võrreldes metsikut-tüüpi pesakonnakaaslastega tundlikumad etanooli ärevusvastasele ja sedatiivsele toimele. Isastel CCK_2 retseptori puudulikkusega hiirtel on seevastu muutunud etanooli motoorikat pärssiv toime. Emased, kuid mitte isased mutantsed hiired tarbivad etanooli oluliselt rohkem kui nende metsikut tüüpi pesakonnakaaslased.
3. Katse-eelne keskkonna rikastamine muudab märkimisväärselt CCK_2 retseptorite puudulikkusega hiirte käitumuslikku fenotüüpi. Kui välja arvata sundujumise katse, ei ole üheski katses saadud mutantsete hiirte käitumuslik fenotüüp korratav alternatiivsetes tingimustes majutatud katseloomadel. Standardsetes tingimustes elavate CCK_2 retseptori puudulikkusega hiirtel on tõusnud ärevus, neil esineb tugevam stressist tingitud analgeesia ning nende kognitiivne võimekus vesipuuri katses on kehvem kui samades tingimustes elavatel metsikut-tüüpi hiirtel. Rikastatud keskkonnas majutatud mutantsetel hiirtel seevastu esineb soost sõltuv käitumise muutus kuuma plaadi ja rota-rodi katses. Nimelt on rikastatud tingimustes elavatel emastel CCK_2 retseptori puudulikkusega hiirtel pikem hüppelatents kuuma plaadi katses,

kuid samades tingimustes elavatel isastel mutantidel ilmneb kehvem tasakaal ja koordinatsioon rota-rodi katses.

4. Katse-eelsete majutustingimuste muutmine geneetiliselt ja käitumuslikult väga erinevatel hiireliinidel (129S6/SvEv ja C57BL/6) ei mõjuta märkimisväärselt nende käitumuslike erinevuste avaldumist. Kuigi keskkonna rikastamine muudab märkimisväärselt mõlema hiireliini käitumist, on nende liinide erinevused eksploratiivses aktiivsuses, kuuma plaadi katses, sundujumise katses ja sotsiaalse interaktsiooni katses sõltumata katse-eelsetest majutustingimustest üldiselt korratavad.

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PUBLICATIONS

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Professional employment

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2001–2003	Tartu University, Department of Physiology, laboratory technician
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Special courses

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2005 Eumorphia/EMBO lecture course 'Mouse models for human disease', Bischoffsheim, France
2006 6th Core Maudsley Forum, Institute of Psychiatry, King's College, London, UK
2006 Introduction to family psychology and therapy, Meritus, Tartu, Estonia

Scientific work

My scientific work has focused on the behavioural and neurochemical phenotyping of mice, lacking cholecystokinin CCK_2 receptors. The contribution of sex as well as environment-related factors to the expression of the behavioural phenotype in mouse lines has been of special interest.

Publications

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2005 Laboriloomateadus: C-kategooria kursus, Tartu, Eesti

2005 Eumorphia/EMBO loengukursus “Mouse models for human disease”, Bischoffsheim, Prantsusmaa
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Teadustöö

Teadustöö on keskendunud koletsüstokiniini CCK-2 retseptorite puudulikkusega hiirte käitumusliku ja neurokeemilise fenotüübi uurimisele. Oluliseks huviks on soo ja keskkondlike tegurite mõju käitumusliku fenotüübi avaldumisele.

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DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
2. **Kati Koido.** Single-nucleotide polymorphism profiling of 22 candidate genes in mood and anxiety disorders. Tartu, 2005.
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4. **Tarmo Areda.** Behavioural and neurogenetic study of mechanisms related to cat odour induced anxiety in rodents. Tartu, 2006.
5. **Aleksei Nelovkov.** Behavioural and neurogenetic study of molecular mechanisms involved in regulation of exploratory behaviour in rodents. Tartu, 2006.
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