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CHARACTERIZATION OF BEHAVIOURAL AND BIOCHEMICAL PHENOTYPE OF CHOLECYSTOKININ–2 RECEPTOR DEFICIENT MICE: CHANGES IN THE FUNCTION OF THE DOPAMINE- AND ENDOPIOIDERGIC SYSTEM

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ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
5-HIAA	5-hydroxyindoleacetic acid, metabolite of serotonin
5-HT_2	5-hydroxytryptamine receptor, type 2
ANOVA	analysis of variance
CCK	cholecystokinin
CCK_1 or CCK_A	cholecystokinin receptor of alimentary/peripheral subtype
CCK_2 or CCK_B	cholecystokinin receptor of brain/central subtype
CCK-4	cholecystokinin tetrapeptide
CCK-8	cholecystokinin octapeptide
CCK-8s	sulphated form of cholecystokinin octapeptide
CNS	central nervous system
DOPAC	dihydroxyphenylacetic acid, metabolite of dopamine
GABA	γ-aminobutyric acid
HPLC	high-performance liquid chromatograph
HVA	homovanillic acid, metabolite of dopamine
L-DOPA	levodopa, (–)-3-(3, 4-dihdroxyphenyl) L-alanine
MANOVA	multiple analysis of variance
MAPK	mitogen-activated protein
NMDA	N-methyl-D-aspartic acid
S.E.M	standard error of mean

INTRODUCTION

Cholecystokinin (CCK) belongs to the family of brain-gut peptides, which are found in both the digestive tract and the central nervous system (CNS). The presence of CCK in mammalian CNS was suggested by the discovery of a gastrin-like peptide in the rat brain, subsequently identified as CCK octapeptide (CCK-8) (Vanderhaeghen et al., 1975; Dockray, 1976). CCK is present in different biologically active molecular forms cleaved from a 115-amino-acid precursor molecule (pre-pro-CCK), including CCK-58, CCK-39, CCK-33, CCK-22, CCK-8 with its sulphated form CCK-8s, but also CCK-7, CCK-5, and CCK-4 (Rehfeld and Nielsen, 1995, table 1). CCK peptides are abundant throughout the brain, with the highest levels in the cerebral cortex, limbic structures, and basal ganglia (Beinfeld et al., 1981; Savasta et al., 1988; Vanderhaeghen et al., 1992; Lindefors et al., 1993). CCK acts as a neurotransmitter and exerts a neuromodulatory influence on several classical transmitters, including dopamine, serotonin (5-hydroxytryptamine, 5-HT), noradrenaline, γ–aminobutyric acid (GABA), glutamate and endopioid peptides (Shlik et al., 1997; Noble et al., 1999). CCK receptors have been characterised (Hays et al., 1980; Innis et al., 1980) and cloned (Kopin et al., 1992; Wank et al., 1992). CCK interacts with two receptor subtypes: CCK_A (peripheral or alimentary type) receptors, mainly located in the gastrointestinal tract, and CCK_B (brain subtype) receptors that are abundantly found in the central nervous system (CNS) (Wank, 1995). Recently it has been proposed that CCK_A and CCK_B receptors should be renamed CCK_1 and CCK_2, respectively (Noble et al., 1999). CCK-related peptides vary in their affinity to the subtypes of CCK receptors. CCK-8s and its amphibian analogue, caerulein, are non-selective agonists of CCK receptors, whereas unsulphated CCK-8, gastrin, pentagastrin (CCK-5), and CCK-4 are selective CCK_2 receptor agonists.

CCK_2 receptors are widely distributed in the brain and have been found to participate in the mediation of various physiological functions, including pain processing, learning, memory, motivated behaviour, locomotor activity, and anxiety (Singh et al., 1991; Costall et al., 1991; Shlik et al., 1997). Recently studies on the physiological role of CCK and CCK_2 receptors have reached a new level because the targeted mutation of the CCK_2 receptor gene has been achieved in mice (Nagata et al., 1996). CCK_2 receptor-deficient mice are fertile. Despite the atrophy of gastric mucosa the oldest animals of this line have reached the age of 24 months and their general appearance is similar to their wild-type (+/+) littermates (Nagata et al., 1996; Kopin et al., 1999). According to the study of Kopin et al. (1999), the genetic invalidation of CCK_2 receptors does not affect the weight gain and feeding behaviour in mice. Miyasaka et al. (2002) however reported that the energy metabolism and turnover are increased in these animals. When we started our experiments in 1998, very little was known about the behavioural and biochemical phenotype of CCK_2 receptor-

deficient mice. Therefore, the main task of the present work was to study the behavioural and biochemical phenotype of mice lacking CCK_2 receptors. It is well known that CCK is located in the same neurons with dopamine and endopioid peptides in the mammalian brain (Hökfelt et al., 1980a,b; Gall et al., 1987). This is an apparent reason why CCK closely interacts with the dopamine- and endopioidergic system in the regulation of various forms of behaviour (Shlik et al., 1997; Noble et al., 1999). Thus, a specific goal for the present work was to study, using the behavioural, biochemical, and pharmacological tools, the changes in the function of the dopamine- and endopioidergic system due to the genetic invalidation of CCK_2 receptors.

REVIEW OF LITERATURE

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

Neuropeptide CCK was first described in the mammalian nervous system in 1975. Vanderhaeghen et al., (1975) discovered a gastrin-like peptide in the rat brain subsequently identified as CCK (Dockray 1976; Rehfeld 1985). CCK belongs to the group of peptides found in the digestive tract and the central nervous system (CNS). The biochemical studies have shown that the majority of neuronal CCK is expressed in the form of sulphated (in position 7 from the –COOH terminal — table 1) octapeptide — CCK-8s. The unsulphated form (CCK-8) of this peptide does exist, and also a larger (CCK-58, CCK-33), and smaller fragments (CCK-5, CCK-4) have been detected (Rehfeld and Nielsen 1995). These isoforms are cleaved from N terminus of pre-pro-CCK, a 115-amino-acid precursor molecule (Dockray 1992). Another peptide — gastrin, has an identical –COOH terminal pentapeptide sequence with CCK. CCK peptides are abundant throughout the brain, with the highest levels in the cerebral cortex, hippocampus, basal ganglia, hypothalamus, and periaqueductal grey matter (Beinfeld et al., 1981; Savasta et al., 1988; Vanderhaeghen et al., 1992; Lindefors et al., 1993). It has been proposed that the brain contains at least three subpopulations of CCK neurones with different post-translational processing pathways (Rehfeld and Hansen 1986, Rehfeld 1992b). According to this view it is possible that different forms of CCK function independently in distinct neuronal settings. Pre-pro-CCK has been cloned in the rat and human (Deschenes et al., 1984; Takahashi et al., 1985), and mapping studies of pre-pro-CCK mRNA using *in situ* hybridisation histochemistry have been conducted (Savasta et al., 1988 and 1990; Ingram et al., 1989; Schalling et al., 1989; Vanderhaeghen and Schiffmann 1992). Certain discrepancies between the density of CCK immunoreactivity and CCK mRNA-containing neurones occur in some brain areas. This difference might come from the greater sensitivity of *in situ* hybridisation histochemistry compared to immunohistochemistry, because it allows to detect the putative neurones with long projections that synthesise CCK but transport it rapidly to the nerve terminals (Vanderhaeghen and Schiffmann 1992). High levels of CCK like immunoreactivity are present in synaptosomal preparations (Pinget et al., 1978; Emson et al., 1980), and CCK is synthesized *de novo* in the brain (Golterman et al., 1980). CCK is released from the brain slices or synaptosomes exposed to depolarising stimuli in a calcium-dependent manner (Pinget et al., 1979; Dodd et al., 1981; Emson et al., 1980; Verhage 1991). Furthermore, the specific high-affinity binding sites for CCK are widely distributed throughout the CNS (Inns and Snyder 1980; Saito et al., 1980). CCK has been shown to induce excitation of central neurones (Dodd and Kelly 1979, 1981; Ishibashi et al., 1979; Boden and Hill., 1988). However,

inhibitory postsynaptic effects have also been recorded (Ishibashi et al., 1979; MacVicar et al., 1987; Lopes da Silva et al., 1990). This is in accordance with the morphological studies suggesting that CCK is present in both excitatory and inhibitory neurones (Peters and al. 1983). Mechanisms terminating the action of CCK are less clear, but recently selective uptake into synaptosomal fraction *in vitro* has been demonstrated (Migaud et al., 1993).

Two types of high-affinity binding sites, initially termed as peripheral (alimentary) and central (brain) were characterised in 1980 by several groups (Hays et al., 1980; Innis and Snyder 1980; Saito et al., 1980; Sankaran et al., 1980). Based on the autoradiographical studies of Moran and colleagues (1986) CCK receptors were originally named according to their preferential localisation: the peripheral or alimentary type (CCK_A) and the central or brain type (CCK_B). CCK_B receptors predominate in the brain (Wank, 1995) while CCK_A receptors are present in the visceral organs and in some discrete brain nuclei (Hill et al., 1990). CCK_A and CCK_B receptors were cloned and the localisation of their mRNA in the brain was established (Kopin et al., 1992; Wank et al., 1992; Lee et al., 1993; Ulrich et al., 1993). According to the nomenclature created by the authorised Committee on Receptor Nomenclature and Drug Classification of the International Union of Pharmacology, the receptors were renamed (Table 1): CCK_1 (formerly CCK_A) and CCK_2 (formerly CCK_B) receptors according to their affinity to the different CCK analogues. CCK-8s, like its amphibian analogue caerulein, is a non-selective agonist for CCK receptors. Unsulphated CCK-8, pentagastrin (CCK-5), and CCK-4 are selective CCK_2 receptor agonists (Woodruff and Hughes 1991). CCK_1 and CCK_2 receptors possess seven trans-membrane domains and belong to the G-protein linked receptor superfamily with considerable amino acid sequence similarities to the other members of this family.

CCK_1 receptors (Table 1) are located mainly in peripheral tissues. On the other hand, it has been shown that CCK_1 receptors occur in certain brain regions including the area postrema, nucleus of the solitary tract, and interpeduncular nucleus (Moran et al., 1986). Further radioligand and electrophysiological studies revealed that the distribution of CCK_1 receptors was even more widespread in the brain. These receptors have been identified in the dorsal raphe, nucleus accumbens, substantia nigra, and ventral tegmental area (Hill et al., 1990; Barrett et al., 1989; Gerhard et al., 1989; Vickroy and Bianchi 1989). CCK_1 receptors, at least in the gastrointestinal tract, are coupled to a guanine nucleotide binding regulatory protein — G-protein, which activates phospholipase C, inducing the breakdown of inositol phospholipids, mobilisation of intracellular calcium, and activation of the protein kinase C (Jensen et al., 1989).

In 1993 Song and colleagues described the gene for CCK_2 receptors in humans (Table 1). It consists of 5 exons and 4 introns and is conformed similarly in humans, mice (Nagata et al., 1996), and rabbits (Blandizzi et al., 1994). Three splice variants of CCK_2 gene [long form, short form (Song et al., 1993)

and Δ form (Miyake 1995)] have been identified. The mRNA for CCK_2 receptor was also found to express a wide range of splice diversity and is located also in the regions of CNS without the presence of functional receptors (Pélaprat et al., 1987; Jagerschmidt et al., 1994). CCK_2 receptors are widely distributed in the brain, with the highest concentration in the striatum, cerebral cortex, and limbic system (Beinfeld 1983), but these receptors are also present in the gastrointestinal tract. For a long time the CCK_2 receptor has caused confusion for its similarity to the gastrin receptor. Recently it was revealed, that the canine parietal cell gastrin receptor and brain CCK_2 receptor are highly homologous if not identical (Kopin et al., 1992). Indeed, the reported Southern-blot hybridisation analysis of human genomic DNA indicates that a single gene encodes both CCK_2 and gastrin receptors (Lee et al., 1993). CCK_2 receptor (belonging to the G-protein coupled receptor superfamily (Wank et al., 1992)) is linked to two effector pathways through pertussis toxin-sensitive and -insensitive G-proteins (Roche et al., 1990; Pommier et al., 1999). MAP kinase pathway has also been shown to be activated via the stimulation of CCK_2 receptors (Taniguchi et al., 1994). Radioligand binding studies have also revealed the heterogeneity among CCK_2 binding sites giving the possibility that there exist at least two subtypes of these receptors (Noble et al., 1999).

Table 1. Characterisation of subtypes of CCK receptors

Receptor	**CCK_1** CCK_A /Alimentary / Peripheral	**CCK_2** CCK_B / Brain / Central
Structure – human	428 — aminoacid sequence (P32238 7TM)	447 — aminoacid sequence (P32239 7TM)
Gene and location Human: Mouse:	*CCK_1* chromosome 4 chromosome 5	*CCK_2* Chromosome 11 Chromosome 7
Splice variants		Yes (long form, short form, Δ form)
Genetically induced disruption of gene in mice	Kopin et al., 1999	Nagata et al., 1996

Distribution	Gall bladder, pancreas, pylorus, intestine, spinal cord, vagus nerve, limited brain areas (nucleus tractus solitarius, area postrema, nucleus interpeduncularis, posteromedial part of nucleus accumbens)	Throughout the brain (with the highest densities in the cerebral cortex, nucleus caudatus, anterolateral part of nucleus accumbens), vagus nerve, stomach, pancreas
Endogenous ligands according to their affinity to specific receptor CCK-8s: (**HOOC**)F–D–M–W–G–M–Y(**SO_3H**)–D CCK-8: (**HOOC**)F–D–M–W–G–M–Y–D CCK-4: (**HOOC**)F–D–M–W gastrin: (**HOOC**)F–D–M–W–G–Y(**SO_3H**)–A–E–E–E–E–E–L–W–P–G–pQ	CCK-8s >> CCK-8 > gastrin ≥ CCK-4	CCK-8s > CCK-8 ≥ gastrin = CCK-4
Agonist	Caerulein (amphibian CCK analogue)	Caerulein
Selective agonists	A 71623; A 70874; JMV–180	CCK-4; Boc–CCK-4; BC 197; BC 264
Antagonists	Proglumide; Lorglumide; Devazepide; Lintitript (SR 27897)	Proglumide; L–365,260; L–740,093; LY 288513; CI–988
Intracellular activation	G-protein q/11	G-protein q/11
Functional role	Mediates CCK actions on gall bladder contraction, secretion of pancreatic enzymes, gastric emptying, inhibits feeding and respiration, potentiates dopamine-mediated behaviours and dopamine release in shell of nucleus accumbens	Mediates CCK actions on increases in neuronal firing rates, nociception, anxiety, respiration, inhibits dopamine-mediated behaviours and dopamine release, regulates insulin release in pancreas

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

2.1. Regulation of locomotor activity and interaction with the dopamine system

The motor inhibition induced by CCK agonists (CCK-8s and caerulein) in mice manifested as a reduction both in the motility and frequency of rearing. The parenteral and intracerebroventricular administration of CCK-8s and caerulein, but not CCK-4, induced hypolocomotion and blocked amphetamine-induced hyperlocomotion in mice (Zetler, 1985; Moroji et al., 1987; Hagino, Moroji, 1989; Vasar et al., 1991; Hirosue et al., 1992). The pretreatment of mice with a selective CCK_1 receptor antagonist devazepide antagonised the motor depressant action of systemically and intracerebroventricularly administered CCK-8s and caerulein, reflecting the involvement of the CCK_1 receptors in the action of CCK agonists (Khosla, Crawley, 1988, O'Neill et al., 1991; Vasar et al., 1991). It is thought that the motor inhibition and suppression of dopaminergic activity induced by CCK agonists are of peripheral origin since they could be abolished by abdominal vagotomy in rats (Crawley, Kiss, 1985; Hamamura et al., 1989; Vasar et al., 1994a). However, not all authors have been able to reproduce the evidence that vagotomy can reverse the behavioural effects of CCK agonists in rodents. Moroji and Hagino (1987) have demonstrated that bilateral subdiaphragmatic vagotomy does not prevent the behavioural effects of subcutaneously injected caerulein in mice. The suppression of electrical self-stimulation by caerulein is also completely insensitive to vagotomy in rats (De Witte et al., 1986). Moreover, L–365,260, an antagonist of CCK_2 receptors, caused an opposite action to devazepide because the motor inhibition elicited by caerulein and CCK-8 became stronger under the influence of CCK antagonist (Vasar et al., 1991, 1994b). These data demonstrate the opposite effect of CCK in the regulation of locomotor activity, depending on the CCK receptor subtype involved. CCK-8 is shown to co-localise with dopamine in the mesencephalic dopaminergic neurones (Höckfelt et al., 1980). Co-localisation of dopamine and CCK in the ventral tegmental area and in the ascending mesolimbic pathway suggests that CCK could act as a neuromodulator of dopaminergic transmission (Hökfelt et al., 1980b; Vanderhaeghen et al., 1980). Locomotor activity is obviously dependent on the functional activity of the mesolimbic dopaminergic system (Bradbury et al., 1983; Costall et al., 1985). Dopaminergic drugs like apomorphine, an agonist of dopamine D_1 and D_2 receptors, and L–DOPA, a precursor molecule of dopamine, induce the biphasic effect on the locomotor activity in rats and mice (Boissier and Simon 1966; Ungerstedt and Ljungberg 1977). Low doses of apomorphine and L–DOPA cause hypomotility due the stimulation of the dopamine autoreceptors (Di Chiara et al., 1976), which belong to the dopamine

D_2 receptor family (Meltzer 1980; Sokoloff et al., 1992). Co-administration of caerulein with low doses of apomorphine induced an almost complete suppression of locomotor activity in mice (Vasar et al., 1986; 1991). On the other hand, the pre treatment of mice with devazepide significantly antagonized the motor suppression caused by apomorphine, whereas the CCK_2 receptor antagonist L–365,260 apparently potentiated the sedative effect of apomorphine (Vasar et al., 1991). It shows that similar neurochemical mechanisms are responsible for the motor suppressant action of caerulein and apomorphine in mice. CCK-8s also significantly potentiates hypolocomotion induced by dopamine if simultaneously injected into the ventral tegmental area of rat brain, suggesting that CCK-8s acts as a facilitatory modulator of dopamine at CCK receptors on the dopamine A10 cell bodies (Crawley 1989). High doses of apomorphine and L–DOPA induce a significant increase in locomotor activity in rodents. This hypermotility can also be elicited by the specific dopamine D_2 receptor agonists (bromocriptine, quinipirole) but not by dopamine D_1 agonists (Jackson et al., 1988; Koller and Herbster 1988). However, dopamine D_1 agonists seem to enhance the hypermotility elicited by the dopamine D_2 agonists (Jackson et al., 1988; Koller and Herbster 1988). Higher doses of caerulein not only inhibit locomotor activity but also amphetamine-induced hyperlocomotion showing that central mechanisms are probably involved in the action of peripherally administered CCK agonist. This effect of caerulein was also antagonized by devazepide demonstrating the involvement of CCK_1 receptors (Vasar et al., 1991). In rats the effect of centrally administered CCK-8s on amphetamine-induced hyperlocomotion is dependent on the brain site administered. In the anterolateral part of nucleus accumbens CCK-8s suppresses via CCK_2 receptors the amphetamine-induced hyperlocomotion, whereas in the posteromedial part CCK-8s potentiates via CCK_1 receptors the action of a dopamine agonist (Crawley, Corwin, 1994).

Repeated administration, but not acute treatment, of different dopamine antagonists (clozapine, chlorpromazine and haloperidol) evidently increases the amount of CCK-8s in the striatum and in mesolimbic structures (Frey et al., 1983). Chang et al. (1983) showed that the long-term treatment with haloperidol increases the density of CCK receptors in the cortical and limbic structures of mice and guinea pigs. By contrast, chronic administration of metamphetamine, an indirect dopamine agonist, decreases the number of CCK-8 receptors in the rat cerebral cortex (Suzuki and Moroji 1989). Moreover, only the repeated administration of dopamine antagonists (haloperidol, chlorpromazine, clozapine etc.) induced the CCK_1 receptor mediated depolarisation and the subsequent inactivation of the dopaminergic neurones in the rat midbrain (Chiodo and Bunney 1983; Bunney et al., 1985; Jiang et al., 1988; Zhang et al., 1991; Minabe et al., 1991).

Brain dopamine is also related to the motivational mechanisms and reward (Wise and Rompre 1989). Therefore, by influencing the dopamine system CCK has also a role in the regulation of motivated behaviour. As could be expected

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

2.2. Pain sensitivity and opioid–induced antinociception: interaction with the endopioidergic system

At the beginning of the eighties two studies described the ability of CCK-8s to antagonize opioid-induced analgesia (Faris et al., 1983; Itoh et al., 1985). These findings aroused considerable interest in exploring the possibility that CCK-8s may act as an endogenous antagonist of the endopioidergic system (Faris 1985a,b). In addition, the distribution of CCK peptides in CNS matches that of opioid peptides — enkephalin, β–endorphin, and dynorphin (Gall et al., 1987; Baber et al., 1989; Ghilardi et al., 1992; Skinner et al., 1997). It was shown that CCK_2 antagonists are able to prevent the development of opioid tolerance and dependence (Idanpää-Heikkila et al., 1997; Kaysler et al., 1998). There is also some evidence that CCK_1 receptors are responsible for the rewarding properties of morphine, whereas CCK_2 receptors modulate the analgesic activity of morphine (Singh et al., 1996). Interestingly, the increased number of CCK receptors in the supraoptic nucleus after the chronic morphine administration suggests that CCK could have a role in the development of tolerance to the analgesic effect of morphine (Munro et al., 1998). A clear anti-opioid action of CCK-8s is described on the level of the spinal cord (Suh and Tseng 1990). The supraspinal structures, particularly the periaqueductal grey matter, are also involved in the interaction between CCK-8s and endopioid peptides (Hendrie et al., 1989a; Dourish 1992). However, the cellular mechanisms of this antagonism are not clear. Electrophysiological studies demonstrated that CCK-8s diminished morphine-induced inhibition of dorsal horn neuronal firing in response to painful stimuli, whereas CCK antibodies and antagonists enhanced this inhibition (Suberg and Watkins 1987). This effect seems to

involve the intracellular calcium content because CCK-8s has been shown to increase the level of intracellular calcium in the presynaptic terminals by mobilization from the intracellular stores and, therefore, antagonising the suppression of cytosolic calcium levels induced by the opioid agonists (Wang et al., 1992).

2.3. Emotional behaviour, learning, and memory

CCK interacts in neural systems that form the networks of fear, anxiety, and panic behaviour (Harro et al., 1993b, Bradwejn, Vasar 1995; Crawley 1995). The electrophysiological experiments by Bradwejn and De Montegny (1984) demonstrated that the anxiolytic drugs belonging to benzodiazepines could block the excitatory effect of CCK in the hippocampus at clinically relevant doses. An anecdotal observation was made by Jens Rehfeld and Thue Schwartz, who self-injected CCK-4, the CCK_2 receptor agonist, and experienced severe anxiety and de-realization (Rehfeld 1992a). This study lead to a systematic assessment of CCK-4 in healthy volunteers and psychiatric patients, and subsequently confirmed the panic-like action of CCK-4 (De Montegny 1989; Koszycki et al., 1991). In animal experiments CCK–related peptides produce an anxiogenic-like effect in mice, rats, cats, and monkeys (Fekete et al., 1984; Csonka et al., 1988; Harro et al., 1990; Harro and Vasar 1991a; Singh et al., 1991; Palmour et al., 1992). Similarly to people, in animal experiments the dose response and behavioural patterns after CCK-4 challenge depend on the baseline anxiety of the animal. In monkeys the uptight animals — typically restless, submissive to threat, and excessively reactive to the environment, are affected by low doses of CCK-4 (Palmour et al., 1992). Rodents with anxious behaviour, as assessed in the elevated plus-maze, had a significantly higher density of CCK receptors in the cerebral cortex as compared to animals with the opposite behaviour (Harro et al., 1990). The non-selective CCK receptor antagonist proglumide and selective CCK_2 antagonists (L 365,260, CI–988, and LY 262691) show an anxiolytic effect in animals in several anxiety tests. The selective CCK_1 receptor antagonist lorglumide and devazepide show similar properties, but at doses probably active also at CCK_2 receptors (Woodruff and Hughes 1991; Harro and Vasar 1991a,b). However, in some cases the anxiolytic effect of CCK_2 antagonists as the only treatment has not been evident (Harro and Vasar 1991b; Crawley 1992). This can be explained in the light of the evidence that the peptidergic neurotransmission is activated by bursting or high-frequency neuronal activity; the peptide antagonists therefore should not necessarily show any effect under normal tonic activity (Hökfelt 1991). Thus, it is possible that the anxiolytic effect of peptide antagonist is observed only if the endogenous neurobiological mechanisms underlying anxiety are already activated. As stated above, a part of the anxiogenic-like action of CCK is mediated via dopaminergic

mechanisms in the posterior part of nucleus accumbens (Daugé et al., 1989; Derrien et al., 1993). Recent evidence suggests that the antagonistic interaction between CCK and endopioid peptides exists in the mechanisms regulating anxiety behaviour (Kõks et al., 1998; 1999). The concurrent administration of subeffective doses of CCK agonists and naloxone induces a significant anxiogenic-like action in rats (Kõks et al., 1998), and pretreatment of rats with CCK-4 completely antagonizes the anxiolytic-like action of morphine in the elevated plus-maze (Kõks et al., 1999).

There is an increasing amount of evidence from preclinical studies that CCK may be involved in memory processing. CCK availability is conspicuous in such brain regions as hippocampal formation, amygdaloid nuclei, and cerebral cortex, which are believed to underlie memory functions (Rehfeld et al., 1995). Parenteral administration of non-selective agonists of CCK receptors, such as CCK-8s and caerulein, prolongs extinction of already learned tasks (Kadar et al., 1981; Derrien et al., 1994), accelerates habituation to a novel environment (Crawley, 1984), and prevents experimental amnesia in rodents (Kadar et al., 1984; Katsuura et al., 1986a; Vasar et al., 1988; Itoh et al., 1990; Maurice et al., 1994). Recent evidence suggests that CCK_1 and CCK_2 receptors may have different roles in memory functions (Harro et al., 1993a). In particular, a balance has been proposed between CCK_1 receptor mediated facilitative effects and CCK_2 receptor mediated inhibitory effects on memory retention (Lemaire et al., 1992; 1994). However, the data describing the effects of CCK_2 receptor agonists on memory in laboratory animals are inconsistent. For instance, some researchers reported that administration of selective CCK_2 receptor agonists (CCK-4, BC–264) impairs memory functions in rodents (Katsuura et al., 1986a,b; Derrien et al., 1994; Lemaire et al., 1994) while others demonstrated memory enhancement (Gerhardt et al., 1994). Administration of BC264 increased vigilance in monkeys and behavioural arousal in rodents (Daugé et al., 1995), suggesting a role of CCK_2 receptors in attentional activation that may facilitate learning. Considering the role of CCK in anxiety, it cannot be excluded that much of the influence of CCK–related peptides on memory is due to their anxiogenic-like action.

3. Phenotype of CCK_2 receptor–deficient mice

Recently, mice with targeted disruption of CCK_2 receptor gene have been generated (Nagata et al. 1996). These mice are fertile and no obvious behavioural abnormalities have been observed up to the age of 24 months (Nagata et al., 1996; Kopin et al., 1999). The animals without CCK_2 receptors display atrophic development of gastric mucosa and altered learning abilities (Nagata et al., 1996; Sebret et al., 1999). The feeding behaviour in general

seems not to be disturbed in CCK_2 receptor-deficient mice compared to wild-type (+/+) littermates (Kopin et al., 1999). Nevertheless, the lack of CCK_2 receptors results in increased energy expenditure and a higher basal metabolic rate (Miyasaka et al., 2002). The activity of the dopaminergic system is also affected in mice by a corrupted function of CCK_2 receptors, and the sensitivity of dopamine D_2 receptors tended to be increased in mutant mice (Daugé et al., 2001a; Pommier et al., 2002). The performed studies also suggest that the function of the endopioidergic system is significantly altered in mice lacking CCK_2 receptors. Pommier et al. (2002) demonstrated that these mice displayed hyperalgesia and a reduced response to morphine-induced analgesia in the hotplate test. Moreover, the studies show that mutant mice exhibited hypersensitivity to morphine-induced locomotor activity and developed a more severe withdrawal syndrome after chronic morphine treatment (Pommier et al., 2002) leading the authors to the statement that the deletion of CCK_2 receptors has resulted in the upregulation of the endopioidergic system in these mice.

OBJECTIVES

The present work characterizes the behavioural and biochemical phenotype of mice lacking CCK_2 receptors. As described above, CCK is located in the same neurones with dopamine and endopioid peptides in the mammalian brain (Hökfelt et al., 1980a,b; Gall et al., 1987). This is an obvious reason why CCK closely interacts with the dopamine- and endopioidergic system in the regulation of various forms of behaviour (Shlik et al., 1997; Noble et al., 1999). Therefore, an attempt was made to study, using the behavioural, biochemical and pharmacological means, changes in the function of the dopamine- and endopioidergic system in mice without CCK_2 receptors.

The specific objectives of the present study are as follows:

1. To study the properties of dopamine D_2 and μ-opioid receptors in the forebrain structures of mice with the corrupted function of CCK_2 receptors.
2. To evaluate the changes in the locomotor activity and motor coordination of genetically modified mice.
3. To study the changes in the behavioural effects of apomorphine, an agonist of dopamine receptors, and amphetamine, increasing the release of dopamine from presynaptical terminals, in mice lacking CCK_2 receptors.
4. To measure the performance of genetically modified mice in the nociceptive tests.
5. To study the changes in the behavioural effects of morphine, an agonist of μ-opioid receptors, and naloxone, an antagonist of opioid receptors, in CCK_2 receptor-deficient mice, using the behavioural tests reflecting pain sensitivity, motivational responses, and locomotor activity.

MATERIALS AND METHODS

1. Animals

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

2. Drugs

All the injections were performed intraperitoneally, and the drugs were dissolved in sterile 0.9% NaCl (saline) solution. The injections were given in a volume 10 ml/kg.

The locomotor effects of amphetamine sulphate (3.0 and 6.0 mg/kg; Sigma), and apomorphine hydrochloride (0.1 mg/kg; Sigma) were studied in separate groups of animals. Both drugs were injected intraperitoneally 20 min and 15 min, respectively, before the beginning of an experiment.

Morphine sulphate (Boehringer-Ingelheim) in doses 1–10 mg/kg, was used in pain assessment experiments to evaluate the analgesic effect of this drug, and it was administered 15 min before the test.

In the plantar-analgesia test, 1 mg/kg of morphine sulphate was chosen, since lower doses (0.05–0.5 mg/kg i.p.) of the drug did not cause any statistically significant effect (data not shown). The effect of higher doses of morphine was impossible to study because of the increased locomotor activity

of mice and, therefore, the correct measurement of hind paw withdrawal latency was impossible. Injections were made after a 30 min adaptation period of mice at the enclosure assembly of the test equipment. After the injection, mice were released back to the enclosure assembly. The test was started after 15 min, the time being sufficient for mice to calm down again.

In the hotplate test the mice were taken out for injection and released back to the home cage 15 min before the test. Morphine sulphate (5–10 mg/kg) was used and saline injections served as controls in both the plantar-analgesia and hotplate tests.

In place preference tests the doses of naloxone hydrochloride (Sigma, 1 and 10 mg/kg) and morphine sulphate (3 and 9 mg/kg) were selected according to the data available in the literature. These doses are shown to induce a significant action in the place conditioning studies. In the locomotor activity studies the higher doses of morphine were applied since the pilot study revealed that only 10 mg/kg of morphine induced a significant increase in locomotor activity in mice with 129Sv/C57Bl/6 background.

3. Radioligand binding studies

Mice were decapitated, the brains were rapidly removed, quickly dissected on ice, and cooled down in the liquid nitrogen. The following brain areas were dissected from the sections according to the atlas by Franklin and Paxinos (1997): the cerebral cortex, both striata, mesolimbic structures (nucleus accumbens and tuberculum olfactorium), and the hippocampus. The dissected tissues were frozen in liquid nitrogen and stored at –80°C until sample preparation. The brain structures from six mice were pooled. The radioligand binding studies were performed according to the method of Kõks et al. (1997). The tissue was homogenized in 20 volumes of ice-cold 50 mM Tris–HCl (pH 7.4 at 4°C) using a Potter–S glass-teflon homogenizer (1000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48000 x g for 20 min) and resuspension. After the last centrifugation the crude brain cell membranes were suspended in the incubation buffer (50 mM Tris–HCl, pH 7.4 at 4°C) for the binding assay. The protein content was measured according to the method of Bradford (1976). The saturation curves of [^{3}H]–labelled ligand binding were analysed using GraphPad Prism (Version 3.00) for Windows software. The experiments were repeated four times.

3.1. Measurement of parameters of CCK receptors

The cerebral cortex and subcortical structures (involving the striatum, mesolimbic structures, and septum pellucidum) were used. [Propionyl–^{3}H]–propionylated–CCK-8–sulphated ([^{3}H]–pCCK-8s, with specific activity of 73 Ci/mmole, Amersham Radiochemicals) was used for the labelling of CCK receptors. The parameters of the receptors were determined in the presence of 0.05–2.4 nM of [^{3}H]–pCCK-8s at 23°C for 120 min. An antagonist of CCK_2 receptors L–365,260 (from Merck Sharp & Dohme, 1 μM) was added to determine the non-specific binding.

3.2. Measurement of parameters of dopamine and serotonin (5-HT) receptors

In one experiment the cerebral cortex and subcortical structures (involving the striatum, mesolimbic structures, and septum pellucidum) were dissected. In the other experiment the striata, and mesolimbic structures were separated. The striata, mesolimbic, and subcortical structures were used to study the parameters of dopamine D_2 receptors, whereas 5-HT_2 receptors were studied in the cerebral cortex. The parameters of dopamine and serotonin receptors were determined in the presence of 0.06–2 nM [^{3}H]–spiperone (specific activity 107 Ci/mmole, Amersham Radiochemicals) incubated at 37°C for 30 min. Raclopride (Astra, 1 μM), an antagonist of dopamine D_2 receptors, was added 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.

3.3. Measurement of parameters of opioid receptors

The cerebral cortex (including the frontal and parietal cortices), striata, mesolimbic structures, and the hippocampus were dissected. The parameters of opioid receptors were determined in the presence of 0.05–2.5 nM [^{3}H]–diprenorphine (specific activity 58.0 Ci/mmole, Amersham Radiochemicals) incubated at 23°C for 60 min. Naloxone hydrochloride (Sigma, 1 μM), an antagonist of opioid receptors, was added to determine the non-specific binding at opioid receptors.

4. Behavioural experiments

The animals were brought into the experimental room one hour before the experiment. All behavioural experiments were performed between 11:00 and 19:00. The locomotor activity and conditioned place preference tests were performed on separate groups of animals. Since some behavioural experiments lasted 6–8 h (place preference test; locomotor activity test), precautions were taken to control the possible daily fluctuations in the locomotor activity of animals. Therefore, these experiments were always performed in randomised order — wild-type (+/+) mice were always used in parallel with mutant animals. In the radioligand binding study we used animals that had not been exposed to behavioural testing. In some cases pilot studies were performed to establish the optimal experimental conditions.

4.1. Rotarod test

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

4.2. Locomotor activity

For the study of locomotor activity, an animal was placed singly into the photoelectric motility boxes (448×448×450 mm) connected to a computer (TSE Technical and Scientific Equipment GMBH, Germany). The illumination level of transparent test boxes was ~450 lux. Time in locomotion (s), distance of locomotion (m), the number of rearings and corner entries was registered during the 30–min observation period.

4.3. Nociceptive tests

4.3.1. Plantar-analgesia test

The plantar-analgesia test was performed according to the method of Hargreaves et al. (1988). The instrument for the measurement of plantar-analgesia (TSE Technical & Scientific Equipment Gmbh, Germany) consists of a

movable infrared generator, platform, mice enclosure assembly, and controller. In order to avoid the confounding effects of novelty and handling stress, prior handling of mice and habituation to the test equipment was performed. The animals were adapted to the equipment for three consecutive days, a 60–minute session each day. Before the experiment, mice were allowed a sufficient time to explore the equipment without a stimulus. The mice were also observed during the adaptation sessions to assess any unusual behaviour of mutant mice. The mice entered the adaptation session according to their cage mates and always in the same order to adapt also to the odours. After every session the enclosures were cleaned with a 5% alcohol solution. After the adaptation period the animals stayed quietly in a resting position, with occasional bouts of grooming, allowing to direct a beam of infrared light (intensity equal to 50°C) to the palmar surface of a hind paw. Latency for the mouse to withdraw the hind paw was measured automatically by the equipment. The stimulation of the hind paw was repeated up to four times, and the mean latency was calculated. There was at lest two-minute interval (usually longer) between two measurements. After the injections 15 minutes was sufficient for the animals to calm down to perform the measurements. The plantar-analgesia test was favoured instead of the tail-flick test, both reflecting the function of the spinal mechanisms of nociception, to avoid the stress from handling in mice.

4.3.2. Hotplate test

The animal was placed on a surface (30 x 50 cm) maintained at 52±0.3°C and a glass funnel (diameter 15 cm, height 12.5 cm) was used to restrict the movements of the animal. The time to the licking or to a clear shake of either of the hind paw was noted manually (using the stopwatch) as the pain threshold in seconds. The mice were taken out and released back to the home-cage after the experiment and observed for possible thermal damage. Another experiment was performed where the latency to jump was measured and a 2 minute cut-off time was used in this case. The two minute cut-off time was chosen according to our pilot studies, where we noticed that the majority of wild-type (+/+) and CCK_2 receptor-deficient mice responded with a jump during that period. After the experiment the mice, when released to the home-cage, did not show any behaviour to conclude that they had been damaged in any way by the temperature.

4.4. Place preference and aversion test

Place preference and aversion experiments were performed in three identical shuttle boxes (50×25×30). Each shuttle box was divided into two compartments of equal size by a sliding door having a partition. The door allows free movement of a test animal between the two compartments if opened or restricts the movement of a mouse to the other compartment if closed. These compartments were distinguished by wall and floor colour (dark green versus pale green). The illumination level in the shuttle boxes was ~250 lux. The place conditioning experiment was performed according to the method described by Bespalov et al. (1999). Each experiment consisted of pre-conditioning, conditioning, and post-conditioning periods. During the pre-conditioning period mice were placed three times into the apparatus for 15 minutes. The first and the second pre-conditioning tests were held on day 1. The third pre-conditioning test was held on day 2 of the experiment, and the time spent in the pale green compartment was measured. During the pre-conditioning and post-conditioning tests, animals were allowed to explore both compartments freely. Each pre- and post-conditioning session began with initial placement of an animal into the dark green compartment. The shuttle boxes were cleaned carefully after each animal using a 5% ethanol solution. The conditioning period consisted of a 30-min experimental session performed twice a day for four consecutive days (days 3–6). On each day animals received a saline injection before being placed into one compartment and were then injected with morphine (3 and 9 mg/kg) or naloxone (1 and 10 mg/kg) before placement into the opposite compartment. Injections were given immediately before the conditioning sessions. One half of the animals received saline as the first injection, whereas the other half were at first treated with morphine or naloxone. The morphine injections were paired with the placement of a mouse into the pale green compartment avoided in the pre-conditioning test (conditioning of place preference) and the naloxone injections were paired with the placement of an animal into the preferred compartment (dark green, conditioning of place avoidance). The control animals received the injection of saline in both compartments. Fifteen-minute post-conditioning tests were performed on day 7 of the experiments and the time spent in the pale green side of the shuttle box was measured. The behaviour of the animals was recorded on the videotape, and it was analysed by the experienced person unaware about the previous conditioning sessions and the genotype of mice. On day 8 we also studied the effect of morphine on place preference, but the response of the animals did not differ from that in the drug-free state. Therefore, these data were not included in the further analysis. This biased design has often been criticized because it may lead to false-positive results in the place conditioning test (Tzschentke, 1998). For example, the drugs with a strong anxiolytic action may overcome the initial aversion for the non-preferred compartment. Several steps were taken to minimize the problem linked to the biased design. The animals having a significantly different

response profile in the pre-conditioning session were excluded from further experiments. The effect of morphine was not compared with the pre-conditioning session but with a group of animals receiving saline injections in both compartments.

5. Data analysis

Age- and weight-matched mice were used. The results are expressed as mean values ± S.E.M. The results were analysed by using one- or/and two-way analysis of variance (ANOVA/MANOVA). *Post hoc* comparisons between the individual groups were performed by the Statistica software for Windows. The saturation curves of [^{3}H]–labelled ligand binding were analysed using GraphPad Prism (Version 3.00) software for Windows. The Student's t-test was applied for radioligand binding studies.

RESULTS

1. Radoligand binding studies (papers I, II, III, IV)

1.1. CCK receptors (papers I, II)

In wild-type mice (+/+) the density of CCK binding sites was significantly higher in the cerebral cortex compared to the subcortical structures. In heterozygous (+/–) mutant mice the density of CCK binding sites was significantly lower — about 30–50% of the corresponding value in wild-type (+/+) animals, but it was again higher in the cerebral cortex than in the sub-cortical structures. The specific binding of [^{3}H]–pCCK-8 was not detectable in the cortical and subcortical regions of homozygous (–/–) mutant mice as had been expected showing that the majority of CCK binding sites in these brain structures belong to the CCK_2 receptor subtype.

1.2. Dopamine D_2 and 5-HT_2 receptors (papers I, II)

The parameters of dopamine D_2 and 5-HT_2 receptors were evaluated by means of radio-labelled antipsychotic drug [^{3}H]–spiperone. The parameters of dopamine D_2 receptors were studied in the subcortical structures (including the striatum, tuberculum olfactorium, nucleus accumbens, and septum) in one study and in the striatum and mesolimbic structures (tuberculum olfactorium and nucleus accumbens) in other study. Therefore, the results were somewhat different. In the subcortical structures of homozygous (–/–) mice the affinity of [^{3}H]–spiperone binding sites was increased compared to wild-type (+/+) littermates reflecting an increased affinity of dopamine D_2 receptors for ligand. In the striatum of homozygous (–/–) animals the number of [^{3}H]–spiperone binding sites was significantly elevated compared to wild-type (+/+) mice. In heterozygous (+/–) mice the increase of [^{3}H]–spiperone binding was not statistically significant. We did not find any significant changes in the parameters of [^{3}H]–spiperone binding sites in the mesolimbic region. Nevertheless, the functional meaning of two studies is the same because there is an increase in the functional activity of dopamine receptors in the subcortical structures of CCK_2 receptor-deficient mice, and this change is located in the striatum. The parameters of [^{3}H]–spiperone binding were not affected in the cerebral cortex of the mice lacking CCK_2 receptors. Consequently, the targeted mutation of CCK_2 receptors in male mice induces changes in dopamine D_2 receptors but not in 5-HT_2 receptors.

1.3. Opioid receptors (papers III, IV)

The parameters of opioid receptors labelled by [^{3}H]–diprenorphine were evaluated in four brain regions, including the cerebral cortex, striatum, mesolimbic structures, and the hippocampus. Like in the case of [^{3}H]–spiperone binding the targeted genetic invalidation of CCK_2 receptors induced distinct changes in the parameters of opioid receptors in various brain regions. Again, as in the case of [^{3}H]–spiperone binding the biggest changes in the parameters of [^{3}H]–diprenorphine binding were found in the striatum. In homozygous (–/–) mice the density of [^{3}H]–diprenorphine binding sites was significantly elevated, but their affinity was reduced compared to wild-type (+/+) littermates. By contrast, the affinity of [^{3}H]–diprenorphine binding sites was increased in the cerebral cortex of homozygous (–/–) mice. In the mesolimbic structures and the hippocampus the parameters of [^{3}H]–diprenorphine binding of homozygous (–/–) mice did not differ from that in wild-type (+/+) animals. The changes established in [^{3}H]–diprenorphine binding of heterozygous (+/–) mice were not statistically significant. Altogether, the targeted genetic invalidation of CCK_2 receptors induced significant alterations in the parameters of opioid receptors, but these changes are restricted to certain brain structures, including the striatum and the cerebral cortex.

2. Locomotor activity and motor coordination (papers I, II, IV)

2.1. Motor coordination in the rotarod test (paper I)

In the first rotarod experiment at day 1, the performance of heterozygous (+/–) and homozygous (–/–) CCK_2 receptor-deficient mice was impaired relative to wild-type (+/+) littermates. This difference however was statistically significant only in the case of homozygous (–/–) mice. Significant difference remained between the mutant and wild-type mice in the second experiment on the second day. On the third day the behaviour of heterozygous (+/–) mice did not differ from that of wild-type (+/+) animals, whereas the ability of homozygous (–/–) mice to stay on the rotating wheel remained poor relative to wild-type (+/+) animals. This finding is in good agreement with the study by Daugé et al. (2001b), demonstrating an impaired motor coordination of CCK_2 receptor-deficient mice compared to their wild-type (+/+) littermates. Nevertheless, the established difficulties in staying on the rotating wheel in homozygous (–/–) mice seem to depend on the genetic background. It is noteworthy that in the rotarod test C57Bl/6 mice show much longer latencies to fall than 129Sv mice (Homanics et al., 1999). Moreover, our recent unpublished studies show that the backcrossing of mice to C57Bl/6 genetic background obviously diminished the

above-described motor coordination problems in CCK_2 receptor-deficient mice. It is interesting that the same phenomenon was established in the case of dopamine D_2 receptor deficient mice by Kelly et al. (1998). Therefore, the impairment of motor coordination induced by the genetic invalidation of CCK_2 receptors is present if the genes from the 129Sv strain dominate over the genes of the C57Bl/6 strain.

2.2. Locomotor activity in motility boxes (papers I, II, IV)

The studies performed for the evaluation of spontaneous locomotor activity of CCK_2 receptor-deficient mice show that the motor performance of mice depends on two major factors. First the genetic background and second, the adaptation of mice to the experimental environment. This statement is based on the data published in papers I, II, and IV. It should be noted that there is a difference between wild-type (+/+) mice in the first (paper II) and third study (paper IV) showing that moving out from the 129Sv genetic background obviously increases the motor activity in mice. In the beginning the locomotor activity (papers I, II) of mutant mice tended to be lower than in their wild-type (+/+) littermates, and in some experiments this difference reached a statistical significance. In the last experiment (paper IV), the locomotor activity of mutant mice, not habituated to the motility boxes, did not differ from that of wild-type (+/+) littermates. Only the number of rearing was higher in homozygous (–/–) animals. However, the situation changed when the animals were repeatedly exposed to the motility boxes. There was a significantly stronger inhibition of locomotor activity in the wild-type (+/+) mice compared to the homozygous (–/–) littermates. It has to be stressed that our data differ from that of Daugé et al. (2001a,b) and Pommier et al. (2002) because we were unable to find the increase of the horizontal component of locomotor activity in homozygous (–/–) mice relative to the wild-type (+/+) animals not habituated to the motility boxes. Only the frequency of rearing was higher in mice without CCK_2 receptors in the last experiment (paper IV), but not in the previous studies (papers I, II). The reason for the differences between our studies and Daugé et al. (2001a,b, Pommier 2002) is unclear but could be attributed to different research designs — the motility study by the French group was performed in dark box and to the distinct genetic background of mice. The repeated exposure of mice to the motility boxes (paper IV) caused a significant reduction of locomotor activity in the wild-type (+/+) mice. The described behavioural change was significantly weaker in animals lacking CCK_2 receptors. This difference can be explained in the light of the suggestion that CCK is an important factor in the adaptation of rodents to a novel environment (Wiertelak et al., 1992; Kõks et al., 1998; 1999). Thus, lack of CCK_2 receptors apparently attenuates normal adaptation of mice to a novel environment.

2.3. Changes in locomotor activity induced by dopamine- and endopioidergic drugs (papers I, II, IV)

2.3.1. Apomorphine-induced hypolocomotion (papers I, II)

The treatment with a low dose (0.1 mg/kg i.p.) of apomorphine, an unselective agonist of dopamine D_1 and D_2 receptors, reduced the locomotor activity in wild-type (+/+) mice. It should be noted that this dose of apomorphine did not cause any sign of stereotyped behaviour. The motor suppressant effect of apomorphine is believed to be caused by the stimulation of dopamine D_2 autoreceptors located on the dopamine neurones (Meltzer, 1980). Intrestingly, in two separate studies (papers I, II) the motor suppressant action of apomorphine was significantly stronger in mice lacking CCK_2 receptors and it is important to stress that the increased effect of apomorphine was already present in heterozygous (+/–) mice, having still 50% of CCK_2 receptors left in the brain (paper I). This finding is in agreement with our previous pharmacological studies, in which the CCK_2 receptor antagonist L365,260 potentiated the motor suppressant action of apomorphine (Vasar et al., 1991). Altogether, it is likely that the genetic invalidation of CCK_2 receptors increases the sensitivity of presynaptic dopamine receptors in the brain.

2.3.2. Amphetamine-induced hyperlocomotion (papers I, II)

The administration of amphetamine (3 and 6 mg/kg i.p.), increasing the release of dopamine from the pre-synaptic terminals (Kuczenski, 1983), induced a dose-dependent locomotor stimulation in wild-type (+/+) mice. However, as established in two separate studies (papers I, II), in mutant mice the effect of amphetamine was different. In heterozygous (+/–) mice, a lower dose of amphetamine (3 mg/kg) tended to increase the motor activity, but the higher dose had the same effect as in wild-type (+/+) animals. In homozygous (–/–) mice, a lower dose of amphetamine reduced the number of rearing, but did not affect the other parameters of locomotor activity. The treatment with a higher dose (6 mg/kg) of amphetamine induced in two separate studies (paper I, II) a significant increase in three parameters of locomotor activity: time in locomotion, travelled distance, and the number of corner entries. It should be noted that the stimulating effect of the higher dose (6 mg/kg) of amphetamine in homozygous (–/–) mice was significantly stronger compared with heterozygous (+/–) and wild-type (+/+) littermates. The increased action of amphetamine could be related to the enhanced sensitivity of the postsynaptic dopamine receptors in the forebrain regions. Indeed, the functional activity (see the data in the section 1.2.) of dopamine D_2 receptors was significantly higher in the subcortical structures (including the corpus striatum) of homozygous (–/–) mice relative to their wild-type (+/+) littermates. The increased motor stimulating

effect of amphetamine in CCK_2 receptor-deficient mice is consistent with previous studies where CCK antagonists were used. The administration of CCK into the anterior nucleus accumbens inhibits dopamine-induced hyperlocomotion, and this effect is mediated via CCK_2 receptors (Daugé et al., 1990; Crawley et al., 1992). On the other hand, the blockade of CCK_2 receptors in the nucleus accumbens increases amphetamine-induced dopamine release and hyperlocomotion (Altar, Boyar, 1989).

2.3.3. Action of morphine and naloxone on locomotor activity (paper IV)

In wild-type (+/+) mice, habituated to the motility boxes for three days, the administration of morphine (5 mg/kg i.p.) tended to increase the horizontal component of locomotor activity and significantly inhibited the frequency of rearing. A higher dose of morphine (10 mg/kg i.p.) induced significant hyperlocomotion in the wild-type (+/+) animals. By contrast, in the CCK_2 receptor-deficient mice this dose of morphine induced a significantly weaker motor activation. This finding contradicts the study of Pommier et al. (2002) demonstrating that in mutant mice the administration of morphine or inhibition of enkephalin metabolism induces a significantly stronger hyperlocomotion compared to wild-type (+/+) littermates. As mentioned above, the reason for these differences could be attributed to the different research design and distinct genetic background of mice used in these studies. Also differently from the study of Pommier et al. (2002) only a high dose (10 mg/kg i.p.) of naloxone reduced the horizontal component in mice not habituated to the motility boxes, and this effect was similar in the wild-type (+/+) and mutant mice. Nevertheless, a lower dose of naloxone (1 mg/kg i.p.) antagonized the increased frequency of rearing established in homozygous (–/–) mice showing that the elevation of this behavioural parameter is probably due to the increased function of the endopioidergic system in mice lacking CCK_2 receptors. Moreover, we established that naloxone caused a different effect in the wild-type (+/+) and homozygous (–/–) mice adapted to the motility boxes. In these mice a high dose of naloxone (10 mg/kg) induced a significant inhibition of locomotor activity in homozygous (–/–) but not in wild-type (+/+) mice. Therefore, it is likely that the impaired adaptation of CCK_2 receptor-deficient mice to the novel environment is due to the increased function of the endopioidergic system.

3. Assessment of nociceptive sensitivity (paper III)

3.1. Plantar-analgesia test (paper III)

The testing of heterozygous (+/–) and homozygous (–/–) mice in the plantar-analgesia test established a significantly increased hind paw withdrawal latency in these mice compared to wild-type (+/+) littermates. The latency was two to three times longer in both homozygous (–/–) and heterozygous (+/–) animals compared with wild-type (+/+) littermates. The pretreatment of mice with saline decreased the nociceptive threshold in all genotypes. However, the subsequent statistical analysis established a significant reduction only in mutant mice. The administration of morphine (1 mg/kg i.p.), a μ–opioid receptor agonist, induced a significant decrease in pain sensitivity in wild-type (+/+) mice compared to the injection of saline. However, the antinociceptive effect of morphine (1 mg/kg) was significantly stronger in homozygous (–/–) CCK_2 receptor-deficient mice compared to heterozygous (–/+) and wild-type (+/+) mice. Consequently, CCK_2 receptor-deficient mice display significantly reduced pain sensitivity in the plantar-analgesia test, and this effect is probably related to the increased sensitivity of μ-opioid receptors.

3.2. Hotplate test (paper III)

In the hotplate test homozygous (–/–) mice also displayed a delayed hind paw licking/shaking time compared to wild-type (+/+) littermates. The pretreatment of mice with saline similarly reversed this phenomenon in mutant mice if the measurement was performed 15 min after the injection. However, when we tested the mice 90 min after the treatment with saline, we did not find any differences between the saline-injected and untreated homozygous (–/–) mice. Also isolation of mice for 30 min attenuated the reduced nociception in homozygous (–/–) mice. The experiment performed 24 hours after the last saline injection (once daily for 10 days) demonstrated that this manipulation abolished the reduced nociception in homozygous (–/–) mice. Differently from the plantar test a significantly higher dose of morphine (7,5 mg/kg) was necessary to induce a significant antinociceptive effect. However, the antinociceptive effect of morphine (5–10 mg/kg) did not differ in mutant and wild-type (+/+) mice compared to saline treatment. If the jumping latency was used as a measure of nociception, then the results were totally opposite to those established by measuring the hind paw licking/shaking response. Namely, the jump latency was significantly longer in wild-type (+/+) mice compared to heterozygous (–/+) and homozygous (–/–) animals. Accordingly, in the hotplate test, the response of mutant mice was exactly related to the endpoint used to determine

the threshold of nociceptive behaviour. The reduced jump latency established in the present study is in good agreement with the study by Pommier et al. (2002).

4. Place conditioning experiments (paper IV)

In the pre-conditioning test the wild-type (+/+) and homozygous (–/–) mice preferred to stay in the dark green compartment of the shuttle box. The wild-type (+/+) animals spent only 115±15 sec in the pale green compartment, whereas the respective value for the homozygous (–/–) animals was 177±22 sec. The pairing of the dark and pale green boxes with saline injections did not change the preference of animals compared to the pre-conditioning session. The pairing of the dark green box with naloxone (1 and 10 mg/kg i.p.) induced a significant shift of exploratory activity from the dark to the pale green compartment. However, the effect of naloxone was stronger in the wild-type (+/+) animals compared with the homozygous (–/–) mice. Already 1 mg/kg of naloxone tended to shift the preference in the wild-type (+/+) mice, whereas in the homozygous (–/–) mice even the highest dose (10 mg/kg) did not cause any significant effect. The highest dose of naloxone (10 mg/kg) induced a significant place aversion in the wild-type (+/+) mice because they started to prefer the initially non-preferred side. These data are in favour of the assumption that the activity of the endopioidergic system in CCK_2 receptor-deficient mice increases in the neural circuits related to the development of place aversion. This could be an explanation why the effect of naloxone, an antagonist of opioid receptors, is weaker in mutant mice. The pairing of morphine (3 mg/kg) with the pale green compartment induced a significant shift of exploratory activity from the dark green to the pale green part both in the wild-type (+/+) and homozygous (–/–) mice. However, a higher dose of morphine (9 mg/kg) induced a significant effect only in homozygous (–/–) mice. Accordingly, the similar effect of morphine in mutant and wild-type (+/+) mice shows that the sensitivity of opioid receptors related to the reward system is probably not altered due to the targeted mutation of CCK_2 receptors in mice.

DISCUSSION

1. Changes in locomotor activity: evidence for increased sensitivity of dopamine D_2 receptors (Papers I and II)

1.1. Motor coordination and spontaneous locomotor activity

As expected, the mice lacking the CCK_2 receptor gene (–/–) did not have a measurable specific binding of [^{3}H]–pCCK-8 in the cerebral cortex and subcortical structures. In the heterozygous mutant (+/–) mice, the density of CCK receptors was about 30–50% of the corresponding value of the wild-type (+/+) animals. In heterozygous (+/–) mice, the affinity of CCK receptors was also somewhat reduced compared to wild-type (+/+) animals. This finding is different from the study by Nagata et al. (1996), who did not find any difference in the affinity of CCK receptors in wild-type (+/+) and heterozygous (+/–) animals. We used the saturation of CCK receptors by increasing the concentration of [^{3}H]–pCCK-8, whereas Nagata et al. (1996) applied the method of single concentration binding study with [^{125}I]–CCK. Several studies have shown that CCK_1 receptors have a wide distribution in the brain and they are not located only in the discrete brain nuclei (Hill at al., 1990; Honda et al., 1993). However, their density in most brain regions is very low and therefore not detectable by the method used in the present study. Mice lacking CCK_2 receptors displayed impaired motor coordination in the rotarod test. Motor impairment was stronger in homozygous (–/–) than in heterozygous (+/–) animals. On the third day of the study, the difference between wild-type (+/+) and heterozygous (+/–) mice disappeared, whereas the performance of homozygous (–/–) animals remained poor compared to their wild-type (+/+) littermates. The established differences between wild-type (+/+) and mutant mice may be related to some extent to the impaired learning abilities of homozygous (–/–) CCK_2 receptor-deficient mice (Sebret et al., 1999). However in our unpublished data the performance of heterozygous (+/–) mice in the memory tests did not differ from that of wild-type (+/+). In our first studies the locomotor activity of mutant mice was lower compared to wild-type (+/+) littermates. However, after the repeated back-crossings of mice into C57Bl/6 background the differences between the genotypes became less evident and finally the locomotor activity of mutant mice did not differ from that of their wild-type (+/+) littermates. Also the performance of mutant mice in the rotarod test improved considerably after back-crossings into C57Bl/6 background. It has been shown that the performance of C57Bl/6 mice is much better in the rotarod test compared to 129Sv mice (Homanics et al., 1999). Altogether it shows that the effect of targeted mutagenesis of CCK_2 receptors on locomotor activity is dependent on the genetic background of mice. Therefore, the impairment of motor coordination and reduction of locomotor activity, induced

by the genetic invalidation of CCK_2 receptors, is present if the genes from 129Sv strain dominate over the genes of the C57Bl/6 strain. Despite this significant improvement in locomotor activity of CCK_2 receptor-deficient mice we were unable to find that the horizontal component of locomotor activity of mutant mice, not habituated to the motility boxes, is increased compared to their wild-type (+/+) littermates. Only the frequency of rearing was higher in mice without CCK_2 receptors. A repeated exposure of mice to the motility boxes caused a significant reduction of locomotor activity in wild-type (+/+) mice. The latter behavioural change was significantly weaker in the animals lacking CCK_2 receptors. This difference can be explained in the light of the suggestion that CCK is an important factor in the adaptation of rodents to a novel environment. Namely, the administration of CCK antagonizes morphine-induced antinociception in a novel, but not in a familiar environment (Wiertelak et al., 1992). Therefore, lack of CCK_2 receptors apparently attenuates normal adaptation to a novel environment. As stated above, the learning ability of the mice without CCK_2 receptors is significantly affected in the T-maze and in the Morris water-maze tests (Sebret et al., 1999, our unpublished data). CCK_2 receptor-deficient mice display an impaired response in these learning tasks. However, it is unclear whether the affected adaptation to a novel environment and diminished learning abilities are linked to the same neurochemical changes in these mice.

1.2. Amphetamine-induced hyperlocomotion and apomorphine-induced hypolocomotion

The effect of apomorphine and amphetamine was studied twice. In the first experiment (paper II) we found that the response of CCK_2 receptor-deficient mice to the administration of dopamine agonists, amphetamine and apomorphine, was significantly altered. Treatment with a low dose (0.1 mg/kg) of apomorphine, an agonist of dopamine D_1/D_2 receptors, reduced the locomotor activity in wild-type (+/+) mice. This effect is linked to the stimulation of dopamine autoreceptors located on the dopamine neurones (Meltzer, 1980). However, the motor suppressant action of apomorphine was apparently stronger in mice, lacking CCK_2 receptors. This finding is in good agreement with our previous studies, where the pre-treatment with CCK_2 receptor antagonist L–365,260 potentiates the motor suppressant action of apomorphine in male mice (Vasar et al., 1991). Electrophysiological studies in rats demonstrated that the acute and long-term administration of LY262691 and related pyrazolidinone CCK_2 antagonists, decreased the number of spontaneously active dopamine cells in the midbrain structures, probably via action in the nucleus accumbens and prefrontal cortex (Rasmussen et al, 1993). The other dopamine agonist amphetamine increases the release of dopamine from the presynaptic terminals

(Kuczenski, 1983) and thus stimulates the pre- and postsynaptic dopamine receptors. The administration of amphetamine (3–6 mg/kg) induces dose-dependent locomotor stimulation in wild-type (+/+) mice. However, in mice lacking CCK_2 receptors a lower dose (3 mg/kg) of amphetamine reduces the number of rearing, whereas a higher dose (6 mg/kg) causes significantly stronger locomotor stimulation compared to wild-type (+/+) animals. The reduced effect of lower dose of amphetamine could be related to the increased sensitivity of dopamine autoreceptors in mutant mice established in the studies with apomorphine, whereas the increased effect of a higher dose of amphetamine seems to be related to the increased sensitivity of post-synaptic dopamine receptors. Indeed, the affinity of dopamine D_2 receptors labelled by [^{3}H]–spiperone is significantly increased in the forebrain subcortical regions of CCK_2 receptor-deficient mice. This finding is in accordance with the experiments by Ferraro et al. (1996) showing that the application of CCK-8s to the striatal membranes reduces the affinity of dopamine D_2 receptors, and this effect is counteracted by the CCK_2 receptor antagonist PD134308. By contrast, the affinity of 5-HT_2 receptors is not affected in male mice lacking CCK_2 receptors. In the second experiment (paper I) the treatment with a low dose (0.1 mg/kg) of apomorphine caused the same reduction of locomotor activity in wild-type (+/+) mice as in the first study. It should be noted that this dose of apomorphine did not cause any sign of stereotyped behaviour. As in the first study, the motor-depressant effect of apomorphine (0.1 mg/kg) was apparently stronger in heterozygous (+/–) and homozygous (–/–) CCK_2 receptor-deficient mice compared to their wild-type (+/+) littermates. The administration of amphetamine (3 and 6 mg/kg) again induced a dose-dependent motor stimulation in wild-type (+/+) mice as in the previous study. And again, the impact of amphetamine in the mutant mice differed from that seen in wild-type (+/+). In heterozygous (+/–) mice, a lower dose of amphetamine (3 mg/kg) tended to increase the motor activity, but the higher dose of this indirect dopamine agonist (6mg/kg) had the same effect as in wild-type (+/+) animals. In homozygous (–/–) mice the effect of amphetamine (3–6 mg/kg) on locomotor activity was exactly the same as in the previous study. A lower dose of amphetamine (3 mg/kg) reduced the number of rearing but did not affect the other parameters of locomotor activity. The treatment with a higher dose (6 mg/kg) of amphetamine induced a significant increase in two parameters of locomotor activity: the distance travelled and number of corner entries. It should be noted that the effect of a higher dose (6 mg/kg) of amphetamine in homozygous (–/–) animals on these parameters was significantly different from that seen in heterozygous (+/–) and wild-type (+/+) animals. Despite the behavioural changes we did not find any significant difference in the content of dopamine and serotonin, and their metabolites in three brain structures of wild-type (+/+) and homozygous (–/–) mice. Only the concentration of dopamine and its major metabolite homovanillic acid (HVA) tended to be somewhat lower in mutant mice (Kõks et al., 2001). Corwin et al. (1995) showed that the local administration of CI–988, an antagonist of CCK_2

receptors, increases the levels of dopamine in the nucleus accumbens without changing dopamine metabolites (DOPAC and HVA). However, this effect was apparent only at high concentrations of CI–988, lacking specificity for the CCK_2 receptors. Therefore, the increase in the action of amphetamine could be related to the increased sensitivity of the postsynaptic dopamine receptors in the forebrain structures. Indeed, the density of dopamine D_2 receptors was significantly increased in the striatum of homozygous (–/–) mice compared to their wild-type (+/+) littermates. In heterozygous (+/–) mice, the density of dopamine D_2 receptors was also somewhat elevated in the striatum. By contrast, the density of serotonin 5-HT_2 receptors in the cerebral cortex was not different in homozygous (–/–) and heterozygous (+/–) mice compared to wild-type (+/+) animals. The increased motor stimulating effect of amphetamine in CCK_2 receptor-deficient mice is consistent with the previous studies of CCK antagonists. The administration of CCK into the anterior nucleus accumbens inhibits dopamine-induced hyperlocomotion, and this effect is mediated via CCK_2 receptors (Daugé et al., 1990; Crawley 1992). On the other hand, the blockade of CCK_2 receptors in the nucleus accumbens increases amphetamine-induced dopamine release and hyperlocomotion (Altar and Boyar 1989).

Altogether, the results of two independent studies demonstrate that the targeted mutation of CCK_2 receptor gene induces significant changes in the motor performance of animals. The performance of CCK_2 receptor-deficient mice was impaired in the rotarod test and their general locomotor activity tended to be reduced. However, as noted above this motor impairment and the inhibition of locomotor activity in the CCK_2 receptor-deficient mice largely depends on the genetic background of mice. Moreover, the motor suppressant action of a small dose of apomorphine was stronger in mutant mice and homozygous (–/–) animals displayed an increased response to the administration of a higher dose of amphetamine. A clear gene-dose effect is evident from these studies since the rotarod performance is the most impaired in homozygous (–/–) mice, and an increased response to amphetamine-induced locomotor stimulation is also established in mice lacking CCK_2 receptors. Although we did not find any major differences in the metabolism of dopamine in three brain structures, these changes in the behaviour seems to be related to the above-established changes in the dopaminergic system. One possible reason for not finding any differences in the metabolite content could be the detection limits of post-mortem HPLC analysis of `crude` brain regions. Therefore, more sensitive techniques (in vivo microdialysis for example) should be used for further analysis. The reduced locomotor activity, impaired motor performance in the rotarod test, and the increased response to apomorphine-induced motor suppression are probably related to the increased sensitivity of the presynaptic dopamine receptors. The increased response to amphetamine in homozygous (–/–) animals is probably related to the increased sensitivity of the postsynaptic dopamine receptors as confirmed in radioligand binding studies. It is therefore obvious that targeted mutation of CCK_2 receptors leads to compensatory

changes in the activity of dopaminergic system, for the sensitivity of both pre- and postsynaptic dopamine receptors is increased.

2. Changes in pain sensitivity and morphine-induced antinociception: evidence for altered function of the endopioidergic system (Paper III)

A major finding of pain sensitivity studies is that the nociceptive response of mice lacking CCK_2 receptors depends on the paradigm used for the measurement of pain sensitivity. In the plantar-analgesia test, the pain sensitivity of heterozygous (+/–) and homozygous (–/–) mice was significantly lower compared to wild-type (–/–) littermates. In the hotplate test, the response of mutant mice was related to the point used to determine the threshold for painful stimuli. If the hind paw licking/shaking was used as the endpoint, homozygous (–/–) mice had a significantly delayed response to the noxious influence. The situation was completely different when we used jump latency as the threshold. The jump latency of wild-type mice (+/+) was significantly longer compared to heterozygous (+/–) and homozygous (–/–) mutant animals. It is important to note that heterozygous (+/–) animals, having 50% of CCK_2 receptors left, have a clear phenotype both in the plantar-analgesia and hotplate tests. In the plantar-analgesia test these mice responded similarly to homozygous (–/–) mice while in the hotplate test their response profile was closer to that in wild-type (+/+) littermates. In the jump latency experiment the heterozygous (+/–) mice reacted again like their homozygous (–/–) littermates. It is remarkable for heterozygous (+/–) animals, differently from homozygous (–/–) mice, do not have any disturbances in the development of gastric mucosa (Nagata et al., 1996), which may potentially interfere with the pain sensitivity in mice without CCK_2 receptors. The reduced jump latency is in good agreement with a recent study by Pommier et al., (2002), where wild-type (+/+) and homozygous (–/–) mice were compared. Nevertheless, it is unclear whether the jump latency is a true threshold to the pain stimuli. Wilson and Mogil (2001) believe that the best endpoint in the mouse is almost always hind paw licking or vigorous shaking, whichever occurs first. Hotplate-naive mice very rarely jump until long after they have responded in another way (Wilson, Mogil, 2001). The jump response is considered as a measure of pain tolerance, and it is heavily influenced by morphine (Frederickson et al., 1977; Bar-Or, Brown, 1989). This behavioural response is clearly an escape from the noxious stimuli and therefore involves more complex neural circuits (probably involving fear, anxiety, and central motor circuits) than hind paw licking/shaking. The reduced jump latency along with the increased pain thresholds in the plantar-analgesia and hotplate tests could be explained as a compensatory reaction to reduced pain sensitivity in

mice lacking CCK_2 receptors. Otherwise, these animals would inflict significant injuries due to the inadequate response to the noxious stimuli. Distinct behavioural responses in the models of pain sensitivity are also established in the animals lacking the other genes related to the regulation of nociceptive behaviour. The animals lacking pre-pro-enkephalin, tachykinin-1, and cannabinoid receptor CB1 genes were all reported to display altered sensitivity in the hotplate test but unchanged sensitivity in the tail-flick test (Konig et al., 1996; Zimmer et al., 1998, 1999).

Another interesting finding of the present study is that the reduced pain sensitivity in mice lacking CCK_2 receptors is affected by the administration of saline and isolation of mice for 30 min. The treatment with saline and isolation of mice attenuates the reduced pain sensitivity in mutant mice. It is worth noting that the effect was measured at 15 min, but not at 90 min, after the administration of saline in the hotplate test. Accordingly it has been shown that after saline injection there occur significant changes in the CCK system in the prefrontal cortex of experimental animals. Namely, the release of CCK is significantly diminished at 20 minutes after saline injection (Radu et al., 2001). Evidence suggests that CCK receptor antagonist proglumide antagonizes nocebo hyperalgesia induced by saline in humans (Benedetti et al., 1997). Moreover, Drago et al. (2001) found that the administration of saline increases the immobility of rats in the forced swimming test. It has been suggested that catecholamines and endorphins may play a role in the saline effect in the forced swimming test (Drago et al., 2001). Nevertheless, the background of saline-induced algesia remains unclear but could also be attributed to the previous findings that stressful factors affect the interaction between CCK and morphine in behavioural experiments. It has been shown that the administration of CCK antagonizes morphine-induced antinociception in a novel but not in a familiar environment (Wiertelak et al., 1992). The same is true about the potentiation of morphine-induced antinociception by the CCK_2 receptor antagonists (Lavigne et al., 1992). This could be linked to the fact that CCK is also involved in the regulation of anxiety (Harro et al., 1993b). Indeed, CCK agonists induce an anxiogenic-like action in an unfamiliar stressful but not in a safe environment (Kõks et al., 2000a). The combination of sub-threshold doses of CCK agonists and opioid antagonist (naloxone) induces a significant neophobia in rats in the elevated plus-maze (Kõks et al., 1998). Therefore, it is possible that CCK and endopioid peptides play an opposite role in the adaptation to a novel environment. CCK signals that the environment is unsafe, whereas the endopioidergic system mediates the information that there is no danger in the surrounding environment (Kõks et al., 1998; 2000b). This could explain why the baseline pain sensitivity of mice without CCK_2 receptors is reduced. Repeated treatments with saline increase alcohol consumption in low preference mice, and the effect of saline is blocked by the administration of CCK_2 receptor antagonist CAM1028 (Little et al., 1999). This is probably not linked to the anxiolytic action of CAM1028 because diazepam (1 mg/kg) was ineffective under these

circumstances. In agreement with this finding we found that repeated treatment with saline (once daily for 10 days) abolished the reduced nociception in mice lacking CCK_2 receptors. Accordingly, it is obvious that the repeated stressful manipulations reverse the reduced nociception in CCK_2 receptor-deficient mice.

The effect of morphine, μ-opioid receptor agonist, also depends on the approach used. In the plantar-analgesia test the antinociceptive effect of morphine (1 mg/kg) in homozygous (–/–) mice is significantly higher compared to wild-type (+/+) littermates. This is in good agreement with the previous studies where the administration of CCK_2 receptor antagonists or use of CCK_2 receptor antisense potentiates morphine-induced antinociception in rodents (Vanderah et al., 1994; 1996). In the hotplate test a significantly higher dose of morphine (7.5 mg/kg) is necessary to induce the antinociceptive effect, and there is no difference in the action of opioid agonist in wild-type (+/+) and mutant mice. Pommier et al. (2002) established the reduction of opioid receptors on the supraspinal level in CCK_2 receptor-deficient mice. We measured the density of opioid receptors by [^{3}H]–diprenorphine in four forebrain structures (cerebral cortex, hippocampus, striatum, and mesolimbic area) and at least in these structures no decline in the density of opioid receptors can be observed. Indeed, the number of opioid receptors is elevated in the striatum of mutant mice, whereas the other structures reveal no difference in the density of receptors between the homozygous (–/–) and wild-type (+/+) animals. On the other hand, the affinity of opioid receptors is decreased in the striatum. This could be in favour of the initial idea of Pommier et al. (2002) that the levels of endopioid peptides are increased in certain brain regions, and therefore the affinity of opioid receptors is decreased. In contrast to the striatum the affinity of opioid receptors tended to be increased in the cerebral cortex of mutant mice compared to wild-type (+/+) littermates. Accordingly, the targeted mutation of CCK_2 receptors affects differently the parameters of opioid receptors in the forebrain structures. The distinct changes in the sensitivity of opioid receptors in various brain structures may explain why the effect of morphine is increased in the plantar-analgesia test but remains unchanged in the hotplate test in CCK_2 receptor-deficient mice. Pommier et al. (2002) showed that mutant mice revealed not only a reduced latency to jump, but their response to the anti-jumping effect of morphine was also reduced, which in turn may reflect the increased function of some anti-opioid systems in the brain. Indeed, Pommier et al. (2002) demonstrated the role of NMDA receptors as they found that MK–801, an antagonist of NMDA receptors, effectively reversed the increased response of CCK_2 receptor-deficient mice.

Altogether, we established a reduced pain sensitivity of CCK_2 receptor-deficient mice in the plantar-analgesia and hotplate tests. However, CCK_2 receptor-deficient mice seem to have a reduced tolerance to pain stimuli as measured by the jump latency in the hotplate test. Moreover, a clear dissociation of morphine effects is present in mice lacking CCK_2 receptors. In the plantar-analgesia test the antinociceptive action of morphine is significantly stronger in

mutant mice, whereas in the hotplate test the effect of μ-opioid receptor agonist remains unchanged compared with wild-type (+/+) littermates. In the jump latency test, as we consider it the measure of pain tolerance, the effect of morphine is significantly reduced in CCK_2 receptor-deficient mice compared to wild-type (+/+) littermates (Pommier et al., 2002). The molecular background of these phenomena is unclear but could be explained in the light of the data that the targeted mutation of CCK_2 receptors induces distinct changes in the properties of opioid receptors in various brain structures.

3. Changes in behavioural effects of morphine and naloxone: further support for affected function of the endopioidergic system (Paper IV)

In wild-type (+/+) mice, habituated to the motility boxes, the administration of morphine (5 mg/kg) tended to increase the horizontal component of locomotor activity and significantly inhibited the frequency of rearing. A higher dose of morphine (10 mg/kg) induced a significant hyperlocomotion in wild-type (+/+) animals. By contrast, in the CCK_2 receptor-deficient mice this dose of morphine induced a significantly weaker motor activation. This finding contradicts the study by Pommier et al. (2002) demonstrating that the administration of morphine and the inhibition of enkephalin metabolism increases locomotor activity in mice without CCK_2 receptors. The reason for these differences is unclear but could be attributed to the different research design and distinct genetic background of mice used in different studies. Differently from the study by Pommier et al. (2002) only a high dose (10 mg/kg) of naloxone reduced the horizontal component of locomotor activity in mice, and the effect was similar in wild-type (+/+) and mutant mice. Nevertheless, a lower dose of naloxone (1 mg/kg) antagonized the increased frequency of rearing established in the homozygous (–/–) mice showing that the elevation of this behavioural parameter is probably due to the increased function of the endopioidergic system in mice lacking CCK_2 receptors. Moreover, we established that naloxone caused a different effect in the wild-type (+/+) and homozygous (–/–) mice adapted to the motility boxes. In these mice a high dose of naloxone (10 mg/kg) induced a significant inhibition of locomotor activity in the homozygous (–/–) but not in the wild-type (+/+) mice. It has been shown that naloxone at this high dose is lacking selectivity for the subtypes of opioid receptors (Tsuda et al. 1996). Therefore, it is likely that the impaired adaptation of CCK_2 receptor-deficient mice to a novel environment is due to the increased function of the endopioidergic system.

Morphine (3 mg/kg) induces a similar effect both in the wild-type (+/+) and homozygous (–/–) mice in the conditioned place preference test by increasing

the time spent in the drug-associated compartment during the post-conditioning phase. It should be noted that the pre-conditioning preference of animals for dark side of the compartment was very strong. This biased design has often been criticized because it may lead to false-positive results in the place conditioning test (Tzschentke et al., 1998). For example, the drugs with a strong anxiolytic action may overcome the initial aversion for the non-preferred compartment. Several steps were taken to minimize the problem linked to the biased design. The animals having a significantly different response profile in the pre-conditioning session were excluded from further experiments. The effect of morphine was not compared with the pre-conditioning session but with a group of animals receiving saline injections in both compartments. Indeed, the response of mice after saline treatments did no differ from that seen during the pre-conditioning session. During the conditioning session half of the animals received saline treatment as the first daily injection, whereas the other half were at first treated with morphine. Our previous studies had shown that morphine induced an anxiolytic-like action in rats (Kõks et al., 1998; 1999). However, morphine was effective in the elevated plus-maze and zero-maze when studied in rats that had not been handled and exposed repeatedly to the experimental room (Kõks et al., 2000b). Therefore, we tested the action of morphine (3 mg/kg) in the non-habituated wild-type (+/+) mice using the dark-light exploration paradigm, and no increase in exploratory activity was established (our unpublished data). Accordingly, the described shift in the behaviour of mice in the place preference test is not linked to the anxiolytic-like properties of morphine. The results of the present study differ from the data of the pharmacological experiments where the blockade of CCK_2 receptors induced a significant potentiation of morphine-induced place preference in rats (Higgins et al., 1992; Valverde et al., 1996). The compensatory changes in the other neurotransmitter systems due to the complete inactivation of CCK_2 receptors may explain this discrepancy.

Moreover, the present study established dissociation in the action of morphine in the motor activity and place preference tests. It is noteworthy that a significantly lower dose of morphine was effective in the place preference test compared to the locomotor activity study. It is possible that the distinct neural circuits are responsible for these two behavioural effects of morphine. The stronger effect of morphine on the locomotor activity in the wild-type (+/+) mice may be a reason why a higher dose of morphine causes a weaker effect in the place preference studies in the wild-type (+/+) mice compared to mutant animals. Increased motor stereotypy (psychosis-like behaviour) in the wild-type (+/+) mice under the influence of morphine may cause impaired perception of the surrounding environment and reduce the conditioning to the drug-paired environment in comparison with the mutant animals. Interestingly, Maldonado et al., (1997) described dissociation between the opioid-mediated motor and motivational responses in mice lacking dopamine D_2 receptors. They established that morphine-induced place preference was absent in these mice,

whereas morphine-induced hyperlocomotion remained unchanged compared with the wild-type (+/+) mice. This study clearly demonstrates the role of dopamine D_2 receptors of the nucleus accumbens in the mediation of the rewarding effects of morphine, whereas dopamine D_1 receptors and non-dopaminergic mechanisms are responsible for the opioid-induced enhancement of locomotion (Kalivas et al., 1983; Longoni et al., 1987; Daugé et al., 1989; Maldonado et al., 1997).

In the place conditioning experiments a lower dose of naloxone (1 mg/kg) tended to cause a conditioned place aversion in the wild-type (+/+) mice, whereas a high dose of opioid antagonist (10 mg/kg) caused significant place aversion. By contrast, naloxone-induced place aversion was weaker in mice without CCK_2 receptors because a high dose of naloxone (10 mg/kg) only tended to shift the behaviour of mutant mice from the non-preferred to the preferred side. Consequently, there is also dissociation in the behavioural effects of naloxone in the CCK_2 receptor-deficient mice. These data may also reflect an increased tone of the endopioidergic system in the neural circuits responsible for the development of place aversion in mutant mice. It was shown that naloxone (10 mg/kg) failed to produce conditioned place aversion in μ-opioid receptor-deficient mice, whereas the effect of κ-opioid agonist U50,488H remained unchanged (Skoubis et al., 2001). This finding obviously supports the involvement of μ-opioid receptors in the mediation of naloxone-induced place aversion. This behavioural phenomenon can be induced by the local administration of naloxone into the ventral tegmental area and nucleus accumbens but not into the striatum and medial prefrontal cortex (Shippenberg, Bals-Kubik, 1995). Recent evidence suggests that naloxone-induced action is not linked only to the mesolimbic dopaminergic system because the blockade of μ-opioid receptors in the dorsal periaqueductal grey matter causes the conditioned place aversion (Sante et al., 2000). We did not find any differences in the density of opioid and dopamine D_2 receptors in the mesolimbic area, but we established, as stated above, a significant reduction in pain sensitivity in the CCK_2 receptor-deficient mice. The role of periaqueductal grey matter in the regulation of pain sensitivity is well established (Mason, 1999). Therefore, it is tempting to speculate that the increased tone of the endopioidergic system in this brain region could be linked to the reduced effect of naloxone in homozygous (–/–) mice. To some extent this study tends to support the finding of pharmacological experiments showing that CCK_2 receptor antagonists attenuate naloxone-induced place aversion in rats (Valverde, Roques, 1998).

Once again we were unable to repeat the results of radioligand binding studies performed by Pommier et al. (2002). First, they found that the number of opioid receptors was decreased if measured in the whole brain of CCK_2 receptor-deficient mice in *in vivo* conditions. Pommier et al. (2002) suggested that this effect was due to the increased levels of endopioid peptides in the brain of homozygous (–/–) mice. Second, in *in vitro* studies they did not found any changes in the parameters of opioid receptors if the cell membranes were

prepared from the whole brain (Pommier et al., 2002). We measured the density of opioid receptors by [^{3}H]–diprenorphine in four distinct forebrain structures (cerebral cortex, striatum, mesolimbic area, and hippocampus), and at least in these structures no decline in the density of opioid receptors was observed. Indeed, the number of opioid receptors was elevated in the striatum of mutant mice, whereas the other structures show no difference in the density of receptors between the homozygous (–/–) and wild-type (+/+) animals. On the other hand, the affinity of opioid receptors was reduced in the striatum. The reduced affinity of opioid receptors in the striatum could support the initial idea of Pommier et al. (2002) that the levels of endopioid peptides are increased in certain brain regions, and therefore the affinity of opioid receptors is decreased. A micro-array study was performed in the striatum in order to reveal the differences between the wild-type (+/+) and homozygous (–/–) mice in the gene expression (our unpublished data). Accordingly the expression of the μ–opioid receptor gene was increased, whereas the expression of pre-proenkephalin, a precursor molecule of enkephalins, and nociceptin genes was reduced. In contrast with the striatum the affinity of opioid receptors was increased in the cerebral cortex of mutant mice compared to wild-type (+/+) littermates. Accordingly, the targeted mutation of CCK_2 receptors affects differently the parameters of opioid receptors in the forebrain structures. These distinct changes in the sensitivity of opioid receptors in various brain structures may explain the dissociation of behavioural effects of morphine and naloxone described in animals without CCK_2 receptors. The mesolimbic structures, and especially the nucleus accumbens, are targets for the rewarding effect of morphine (Maldonado et al., 1997). However, we did not find any differences in the parameters of opioid receptors in the mesolimbic area, and no change in morphine-induced place preference was established when the wild-type (+/+) and mutant mice were compared. Maldonado et al. (1997) demonstrated the role of dopamine D_2 receptors in the nucleus accumbens for the motivational effects of morphine. As stated above, we did not find any significant differences in the density of dopamine D_2 receptors in the mesolimbic area of wild-type (+/+) and CCK_2 receptor-deficient mice. Accordingly, the reduction of morphine-induced motor stimulation in mutant mice has to be explained by other mechanisms than opioid and dopamine D_2 receptors in the mesolimbic structures. It could be associated with changes in the parameters of opioid receptors in the striatum where the increased number and reduced affinity was found. This may reflect an increased tone of the endogenous opioid system in the striatum and could explain the reduced morphine-induced motor stimulation in mice without CCK_2 receptors. Altogether, the distinct changes of opioid receptors in the striatum and mesolimbic area are most likely linked to the dissociation of behavioural effects of morphine in CCK_2 receptor-deficient mice.

In conclusion, the results of our study support to some extent the initial idea of Pommier et al. (2002) that the activity of the endopioidergic system is increased due to the targeted invalidation of CCK_2 receptor gene in mice.

However, in these mice a clear dissociation of the effects of both morphine and naloxone occurs if different beheviournal paradigmas are employed. The molecular background of this phenomenon is unclear but could be explained in the light of the data that the targeted mutation of CCK_2 receptors induces distinct changes in the properties of opioid receptors in various brain structures.

4. Concluding remarks and suggestions for further studies

Finally, it is time to summarise the major findings of the present thesis (Table 2). Indeed, the present study clearly shows that the targeted mutation of the CCK_2 receptor gene induces substantial alterations in the function of the dopamine- and endopioidergic system. Actually this is not surprising because CCK is co-localised with dopamine and endopioid peptides within the same neurones and brain regions (Hökfelt et al., 1980a,b; Gall et al., 1987). Despite some discrepancies the results of the present study are in accordance with the experiments conducted by Daugé and colleagues (Daugé et al., 2001a,b; Pommier et al., 2002). However, it should always be kept in mind that two mouse lines 129Sv and C57Bl/6, differing markedly by their behavioural and neurochemical parameters, are used for the generation of genetically modified animals. Therefore, the background of genetically modified mice has to be considered if their biochemical and behavioural phenotype is analysed. It does mean that the behavioural and biochemical phenotype should be studied in the different stages of moving from 129Sv to C57Bl/6 genetic background and, of course, the experiments have to be repeated several times in order to avoid false-positive results. Indeed, we found significant changes in the motor coordination and locomotor activity of mice at the beginning of our studies with the dominating 129Sv genotype. It is well documented that the performance of 129Sv mice in the motor coordination and locomotor activity test is significantly inhibited compared to C57Bl/6 mice (Homanics et al., 1999; Võikar et al., 2001). The back-crossings to the C57Bl/6 genetic background apparently attenuated disturbances in the motor performance of mice lacking CCK_2 receptors. In this respect our findings are in good agreement with a number of previous studies showing that the genetic background plays a major role in the behavioural and biochemical phenotype of genetically modified mice (Tang et al., 2001; Võikar et al., 2001; Gerlai 2002a,b,c; Vyssotsky et al., 2002). As a matter of fact, only a part, but not all, of the effects induced by the targeted mutation of CCK_2 receptors are dependent on the genes of 129Sv mice.

As mentioned above, the behavioural and biochemical analysis revealed significant alterations in the activity of the dopamine- and endopioidergic system in CCK_2 receptor-deficient mice. It is important to stress that changes occurring in these two neurotransmitter systems are not similar throughout the

brain because these established alterations are localized in discrete brain regions. The radioligand binding studies detected significant changes in the parameters of dopamine D_2 receptors in the striatum but not in the mesolimbic area. Moreover, the pharmacological studies demonstrated that the sensitivity of both pre- and postsynaptic dopamine receptors is increased in mice lacking CCK_2 receptors. The increased affinity of presynaptic dopamine receptors explains not only the increased hypolocomotion induced by apomorphine, an agonist of dopamine receptors, but it could explain why a low dose of amphetamine tended to reduce the exploratory activity in mutant mice. It is noteworthy that the levels of dopamine and its major metabolite homovanillic acid are somewhat lower in the striatum of CCK_2 receptor-deficient mice (Kõks et al., 2001), and this change can also be attributed to the increased function of presynaptic dopamine receptors. The significantly stronger amphetamine-induced hyperlocomotion together with the increased binding of [^{3}H]–spiperone in the striatum are in favour of the increased sensitivity of postsynaptic dopamine D_2 receptors in mutant mice. On the other hand, the membrane potential of dopamine-containing neurones and release of dopamine from presynaptic terminals are not affected directly by morphine, the agonist of μ-opioid receptors. The increased release of dopamine, induced by morphine is caused via the inhibition of afferent GABA–containing neurones in the midbrain and reduced release of GABA from presynaptic terminals (Johnson, North, 1992). Recent evidence suggests that the activity of the GABAergic system is increased in the brain of mice lacking CCK_2 receptors (Raud et al., 2003), and this may be a reason for the reduced effectiveness of morphine in inducing hyperlocomotion in mutant mice.

The changes in pain sensitivity and dissociation of behavioural effects of morphine in mice lacking CCK_2 receptors are in favour of distinct changes in the endopioidergic system in the brain. This statement is confirmed by the radioligand binding studies showing that the parameters of opioid receptors are affected in the striatum and cerebral cortex but not in the other structures. Nociceptive response of homozygous (–/–) mice is reduced in the plantar-analgesia and hotplate test, but their response is significantly augmented if the jumping response in the hotplate test is used as the endpoint of nociceptive behaviour. Therefore, it is likely that CCK_2 receptor-deficient mice have decreased pain sensitivity but reduced pain tolerance. Moreover, the anti-nociceptive effect of morphine is significantly increased in the plantar-analgesia test, not changed in the hotplate test, and apparently reduced in the jumping test (Pommier et al., 2002). In the experiments where the adaptation of mice to the motility boxes, was studied, the altered function of the endopioidergic system was confirmed. CCK_2 receptor-deficient mice demonstrated decreased adaptation to the motility boxes and this effect was antagonized by the administration of naloxone, an antagonist of opioid receptors. The dissociation of behavioural effects of morphine in mice lacking CCK_2 receptors was also evident in other behavioural tests. The place conditioning induced by

morphine was unaffected, whereas morphine-induced hyperlocomotion was significantly lower in the genetically reduced mice. These findings are in good agreement with the study by Maldonado et al. (1997) showing that the described behavioural effects of morphine are mediated via distinct dopaminergic mechanisms. Indeed, we found changes in opioid and dopamine D_2 receptors in the striatum but not in the mesolimbic structures. This may be a likely reason for the dissociation of behavioural effects of morphine in CCK_2 receptor-deficient mice. There is also a difference in positively and aversively motivated behaviour in these mice. Differently from the place conditioning effect of morphine, naloxone-induced place aversion is apparently reduced in homozygous (–/–) mice compared to their wild-type (+/+) littermates. One possible explanation is that the effects of morphine and naloxone are mediated via different neural circuits displaying different changes in the activity of the endopioidergic system. On the other hand, according to our recent study, anxiety of CCK_2 receptor-deficient mice is reduced compared to their wild-type (+/+) littermates (Raud et al., 2003). Kõks et al. (1998) have shown that the administration of CCK-4, a selective agonist of CCK_2 receptors, and naloxone at subeffective doses induces a significant anxiogenic-like action in rats. Therefore, the reduced anxiety could be a possible reason for the decreased effectiveness of naloxone in inducing a place aversion in mutant mice.

One might ask whether CCK_2 receptor-deficient mice can be used for the pre-clinical screening of new drugs. Concerning the distinct changes in the activity of the dopamine- and endopioidergic system at least three different groups of drugs, including antipsychotic, analgetic, and anti-addictive compounds, can be evaluated using these genetically modified mice. Indeed, further research has to be planned and performed in this direction. For example, the behavioural and biochemical effects of distinct groups of antipsychotic drugs (including haloperidol, raclopride, and clozapine) should be explored in these mice. It is interesting that patients suffering from schizophrenia have an increased sensitivity of the dopamine receptors and an enhanced response to the psychotomimetic action of amphetamine (Gainedtinov et al., 2001). This is exactly the same what we have established in mice lacking CCK_2 receptors. Taking into account the distinct changes in the effectiveness of morphine in the models of nociception and place preference, it is of a great interest to explore the effects of drugs affecting pain sensitivity and motivational responses in mice without CCK_2 receptors. The drugs interacting with the CB1 cannabinoid receptors are of particular interest. Moreover, there is some evidence that CB1 cannabinoid receptors are expressed predominantly by axons of CCK–containing inter-neurones in the hippocampus, amygdala, and neocortex, and that reduction of GABA and CCK release occures when there receptores are activated (Freund, 2003). In conclusion, we believe that this model can be of great interest for the pre-clinical screening of new drugs interacting with the neurotransmitter systems mediating nociception, motivational responses, and psychotic behaviour.

Table 2. Established changes in the function of the dopamine- and endopioidergic system due to genetic invalidation of CCK_2 receptors

Established change in the function	Characteristic behavioural and biochemical features
1. Increase in the activity of the dopaminergic system	1. Elevated number of dopamine receptors in the striatum 2. Enhanced apomorphine-induced hypolocomotion (increased sensitivity of presynaptic dopamine receptors) 3. Enhanced amphetamine-induced hyperlocomotion (increased sensitivity of postsynaptic dopamine receptors)
2. Partial increase in the activity of the endopioidergic system	1. Increased number of μ-opioid receptors in the striatum 2. Increased affinity of μ-opioid receptors in the cerebral cortex 3. Reduced pain sensitivity and increased effect of morphine in the plantar-analgesia test (increased sensitivity of opioid receptors) 4. Reduced adaptation to the novel environment antagonised by naloxone, an antagonist of opioid receptors 5. Reduced effectiveness of naloxone in place aversion test
3. Partial reduction in the activity of the endopioidergic system	1. Reduced tolerance to pain and decreased effect of morphine against the jumping behaviour in the hotplate test (decreased sensitivity of opioid receptors) 2. Reduced morphine-induced hyperlocomotion (increased activity of the GABAergic system in the midbrain?)

CONCLUSIONS

1. The moving from 129Sv to C57Bl/6 genetic background affects several, but not all, behavioural effects induced by the genetic invalidation of CCK_2 receptors. The back-crossings of animals into the C57Bl/6 genetic background apparently attenuate the disturbances of motor coordination and diminished locomotor activity established at the beginning of studies with the dominating 129Sv genetic background.
2. The targeted mutation of CCK_2 receptors induces changes in the activity of the dopaminergic system. According to the radioligand binding studies, the number of dopamine D_2 receptors is significantly increased in the striatum but not in the mesolimbic area. The enhanced apomorphine-hypolocomotion is in favour of the increased sensitivity of presynaptic dopamine receptors, whereas enhanced amphetamine-induced hyperlocomotion reflects an increased sensitivity of postsynaptic dopamine receptors.
3. The genetic invalidation of CCK_2 receptors causes changes in the affinity and number of μ-opioid receptors. The affinity of μ-opioid receptors is reduced in the striatum but increased in the cerebral cortex, and the number of μ-opioid receptors is increased in the striatum.
4. CCK_2 receptor-deficient mice show decreased pain sensitivity and reduced pain tolerance. Nociceptive sensitivity of mice lacking CCK_2 receptors is significantly decreased in the plantar-analgesia and the hotplate tests. By contrast, the jumping response of genetically modified mice is apparently augmented in the hotplate test compared to their wild-type littermates.
5. There is a dissociation of behavioural effects of morphine, an agonist of μ-opioid receptors, in various behavioural tests. The effect of morphine is increased in the plantar-analgesia test, unchanged in the place conditioning and the hotplate test, and reduced in the jumping behaviour and locomotor activity tests. These behavioural changes reflect distinct changes in the parameters of opioid receptors in the forebrain structures.
6. CCK_2 receptor-deficient mice show reduced adaptation in a novel environment, and this effect is reversed by naloxone, an opioid receptor antagonist, demonstrating an increased activity of the endopioidergic system. Moreover, naloxone-induced place aversion is decreased in mutant mice showing that the targeted mutation of CCK_2 receptors also inhibits negatively motivated learning.

REFERENCES

Altar CA, Boyar WC (1989) Brain CCK–B receptors mediate the suppression of dopamine release by cholecystokinin. Brain Res 483:321–326

Baber NS, Dourish CT, Hill DR (1989) The role of CCK caerulein, and CCK antagonists in nociception. Pain 39:307–328

Bar-Or A, Brown GM (1989) Pineal involvement in the diurnal rhythm of nociception in the rat. Life Sci 44:1067–1075

Barrett RW, Steffey ME, Wolfram CA (1989) Type-A cholecystokinin binding sites in cow brain: characterization using (–)–[3H]L364718 membrane binding assays. Mol Pharmacology 36:285–290

Beinfeld MC, Meyer DK, Eskay RL, Jensen RT, Brownstein MJ (1981) The distribution of cholecystokinin immunoreactivity in the central nervous system of the rat as determined by radioimmunoassay. Brain Res 212:51–57

Beinfeld MC (1983) Cholecystokinin in the central nervous system: a minireview. Neuropeptides 3:411–427

Benedetti F, Amanzio M, Casadio C, Oliaro A, Maggi G (1997) Blockade of nocebo hyperalgesia by the cholecystokinin antagonist proglumide. Pain 71:135–140

Bespalov AY, Tokarz ME, Bowen SE, Balster RL, Beardsley PM (1999) Effects of test conditions on the outcome of place conditioning with morphine and naltrexone in mice. Psychopharmacology 141:118–122.

Blandizzi C, Song I, Yamada T (1994) Molecular cloning and structural analysis of the rabbit gastrin/CCKB receptor gene. Biochem Biophys Res Commun 202:947–953

Boden PR, Hill RG (1988) Effects of cholecystokinin and pentagastrin on rat hippocampal neurones maintained in vitro. Neuropeptides 12:95–103

Boden PR, Hill RG (1988) Effects of cholecystokinin and related peptides on neuronal activity in the ventromedial nucleus of the rat hypothalamus. Br J Pharmacology 94:246–252

Boissier JR, Simon P (1966) On the potentiation of DOPA effects by monoamine oxidase inhibitors. Psychopharmacologia 8:428–436

Bradbury AJ, Costall B, Naylor RJ, Neumeyer JL (1983) Motor inhibition induced by apomorphine derivatives in the mouse. J Pharm Pharmacol 35:494–499.

Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem 72:248–254.

Bradwejn J, Vasar E (1995) Cholecystokinin and anxiety: from neuron to behavior. Austin: Springer Verlag–R.G.Landes Company

Bunney BS, Chiodo LA, Freeman AS (1985) Further studies on the specificity of proglumide as a selective cholecystokinin antagonist in the central nervous system. Ann N Y Acad Sci 448:345–351

Chang RS, Lotti VJ, Martin GE, Chen TB (1983) Increase in brain 125I–cholecystokinin (CCK) receptor binding following chronic haloperidol treatment, intracisternal 6-hydroxydopamine or ventral tegmental lesions. Life Sci 32:871–878

Chen D, Zhao CM, Al-Haider W, Hakanson R, Rehfeld JF, Kopin AS (2002) Differentiation of gastric ECL cells is altered in CCK(2) receptor-deficient mice. Gastroenterology 123:577–585

Corwin RL, Jorn A, Hardy M, Crawley JN (1995) The CCK–B antagonist CI–988 increases dopamine levels in microdialysate from the rat nucleus accumbens via a tetrodotoxin- and calcium-independent mechanism. J Neurochem 65:208–217

Costall B, Domeney AM, Naylor RJ (1985) The continuity of dopamine receptors antagonism can dictate the long-term consequences of a mesolimbic infusion of dopamine. Neuropharmacology 24:193–198.

Costall B, Domeney AM, Hughes J, Kelly ME, Naylor RJ, Woodruff GN (1991) Anxiolytic effects of CCK–B antagonists. Neuropeptides 19 Suppl:65–73

Crawley JN (1984) Cholecystokinin accelerates the rate of habituation to a novel environment. Pharmacol Biochem Behav 20:23–27.

Crawley JN, Kiss JZ (1985) Paraventricular nucleus lesions abolish the inhibition of feeding induced by systemic cholecystokinin. Peptides 6:927–935

Crawley JN (1989) Microinjections of cholecystokinin into the rat ventral tegmental area potentiates dopamine-induced hypolocomotion. Synapse 3:346–355

Crawley JN (1991) Cholecystokinin-dopamine interactions. Trends Pharmacol Sci 12:232–236

Crawley JN (1992). Subtype-selective cholecystokinin receptor antagonists block cholecystokinin modulation of dopamine-mediated behaviours in the rat mesolimbic pathway. J. Neurosci. 12:3380–3391.

Crawley JN, Corwin RL (1994). Biological actions of cholecystokinin. Peptides 15:741–755.

Crawley JN (1995) Interactions between cholecystokinin and other neurotransmitter systems. In: Bradwejn J, Vasar E, editors. Cholecystokinin and anxiety: from neuron to behavior. Austin: Springer Verlag–R.G.Landes Company, pp 101–126

Crawley JN (2000) What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. John Wiley and Sons, New York.

Csonka E, Fekete M, Nagy G, Szanto-Fekete M, Feledyg G, Penke B, and Kovacs K (1988) Anxiogenic effect of cholecystokinin in rats. In: Penke B, Török A (eds) Peptides, Walter de Gryter & Co, New York, pp 249–252

Daugé V, Rossignol P, Roques BP (1989) Blockade of dopamine receptors reverses the behavioural effects of endogenous enkephalins in the nucleus caudatus but not in the nucleus accumbens: differential involvement of delta- and mu-opioid receptors. Psychopharmacology 99:168–175.

Daugé V, Böhme GA, Crawley JN, Durieux C, Stutzmann JM, Feger J, Blanchard JC, Roques BP (1990) Investigation of behavioural and electrophysiological responses induced by selective stimulation of CCK–B receptors using a new highly potent CCK analog, BC 264. Synapse 6:73–80.

Daugé V, Roques BP (1995) Opioid and CCK systems in anxiety and reward. In: Bradwejn J, Vasar E, editors. Cholecystokinin and anxiety: from neuron to behavior. Austin: Springer Verlag-R.G.Landes Company, pp 151–171

Daugé V, Beslot F, Matsui T, Roques BP (2001a) Mutant mice lacking the cholecystokinin2 receptor show a dopamine-dependent hyperactivity and a behavioural sensitization to morphine. Neurosci Lett 306:41–44.

Daugé V, Sebret A, Beslot F, Matsui T, Roques BP. (2001b) Behavioral Profile of CCK2 Receptor deficient Mice Neuropsychopharmacology 25:690–698

De Montigny C (1989) Cholecystokinin tetrapeptide induces panic-like attacks in healthy volunteers. Preliminary findings. Arch Gen Psychiatry 46:511–517

De Witte P, Goldman S, Gewiss M, Poels JF, Van Boxez P, Van Der Veken E, and Vanderhaghen JJ (1986) Similar effects of caerulein on intracranial self-stimulation in vagotomised and non-vagotomised rats. Neurochem Int 8:339–343

Derrien M, Durieux C, Dauge V, Roques BP (1993) Involvement of D2 dopaminergic receptors in the emotional and motivational responses induced by injection of CCK-8 in the posterior part of the rat nucleus accumbens. Brain Res 617:181–188

Derrien M, Dauge V, Blommaert A, Roques BP (1994) The selective CCK–B agonist, BC 264, impairs socially reinforced memory in the three-panel runway test in rats. Behav Brain Res 65:139–146

Deschenes RJ, Lorenz LJ, Haun RS, Roos BA, Collier KJ, Dixon JE (1984) Cloning and sequence analysis of a cDNA encoding rat preprocholecystokinin. Proc Natl Acad Sci USA 81:726–730

Di Chiara G, Porceddu ML, Vargiu L, Argiolas A, Gessa GL (1976) Evidence for dopamine receptors mediating sedation in the mouse brain. Nature 264:564–567

Dockray GJ, Taylor IL (1976) Heptadecapeptide gastrin: measurement in blood by specific radioimmunoassay. Gastroenterology 71:971–977

Dockray (1992) CCK neurons and receptors in the CNS: introduction. In: Dourish CT, Cooper SJ, Iversen SD, Iversen LL (eds) Multiple Cholecystokinin receptors in the CNS. Oxford University Press, New-York, pp 3–7

Dodd J, Kelly JS (1979) Excitation of CA1 pyramidal neurones of the hippocampus by the tetra- and octapeptide C-terminal fragments of cholecystokinin [proceedings] J Physiol 295:61P–62P

Dodd J, Kelly JS (1981) The actions of cholecystokinin and related peptides on pyramidal neurones of the mammalian hippocampus. Brain Res 205:337–350

Dourish CT (1992) The role of CCK_A and CCK_B receptors in mediating the inhibitory effect of CCK on opioid analgesia. In: Dourish CT, Cooper SJ, Iversen SD, Iversen LL (eds) Multiple Cholecystokinin receptors in the CNS. Oxford University Press, New-York, pp 456–479

Drago F, Nicolosi A, Micale V, Lo Menzo G (2001) Placebo affects the performance of rats in models of depression: is it a good control for behavioural experiments. Eur Neuropsychopharmacol 11:209–213

Emson PC, Lee CM, Rehfeld JF (1980) Cholecystokinin octapeptide: vesicular localization and calcium dependent release from rat brain in vitro. Life Sci 26:2157–2163

Faris PL, Komisaruk BR, Watkins LR, Mayer DJ (1983) Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. Science 219:310–312.

Faris PL (1985a) Role of cholecystokinin in the control of nociception and food intake. Prog Clin Biol Res 192:159–166

Faris PL (1985b) Opiate antagonistic function of cholecystokinin in analgesia and energy balance systems. Ann NY Acad Sci 448: 437–447

Fekete M, Lengyel A, Hegedus B, Penke B, Zarandy M, Toth G, Telegdy G (1984) Further analysis of the effects of cholecystokinin octapeptides on avoidance behaviour in rats. Eur J Pharmaco l98:79–91

Ferraro L, O´Connor WT, Li XM, Rimondini R, Beani L, Ungerstedt U, Fuxe K, Tanganelli S (1996) Evidence for a differential cholecystokinin–B and –A receptor regulation of GABA release in the rat nucleus accumbens mediated via dopaminergic and cholinergic mechanisms. Neuroscience 73:941–950.

Franklin KBJ, Paxinos G (1997) The mouse brain in stereotaxic coordinates. Academic Press, San Diego
Frederickson RCA, Burgis V, Edwards JD (1977) Hyperalgesia induced by naloxone follows a diurnal rhythm in responsitivity to painful stimuli. Science 198:756–758
Freund TF (2003) Interneuron diversity series: rhythm and mood in perisomatic inhibition. Trends in Neurosciences (in press)
Frey P (1983) Cholecystokinin octapeptide levels in rat brain are changed after subchronic neuroleptic treatment. Eur J Pharmacol 95:87–92
Gall C, Lauterborn J, Burks D, Seroogy K (1987) Co-localization of enkephalin and cholecystokinin in discrete areas of rat brain. Brain Res 403:403–408.
Gerhardt P, Voits M, Fink H, Huston JP (1994) Evidence for mnemotropic action of cholecystokinin fragments Boc–CCK-4 and CCK-8S. Peptides 15:689–697
Gerlai R (2002a) Afraid of complications in gene targeting: Anxiety plays a role! Trends in Neurosciences 25:136
Gerlai R (2002b) Mutants at large: ethology meets transgenic technology! Trends in Neurosciences 25:344
Gerlai R (2002c) Phenomics: fiction or the future? Trends In Neurosciences 25:506–509
Ghilardi JR, Allen CJ, Vigna SR, McVey DC, Mantyh, PW (1992) Trigeminal and dorsal root ganglion neurons express CCK receptor binding sites in the rat, rabbit, and monkey: possible site of opiate-CCK analgesic interactions. J Neurosci 12:4854–4866.
Goltermann NR, Rehfeld JF, Roigaard-Petersen H (1980) In vivo biosynthesis of cholecystokinin in rat cerebral cortex. J Biol Chem 255:6181–6185
Hagino Y, Moroji T, Iizuka R (1989) A behavioural pharmacological study on intracerebroventricularly administered CCK-8 related peptides in mice. Neuropeptides 13:107–113
Hamamura T, Kazahaya Y, Otsuki S (1989) Ceruletide suppresses endogenous dopamine release via vagal afferent system, studied by in vivo intracerebral dialysis. Brain Res 483:78–83
Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77–88
Harro J, Kiivet RA, Lang A, Vasar E (1990) Rats with anxious or non-anxious type of exploratory behaviour differ in their brain CCK-8 and benzodiazepine receptor characteristics. Behav Brain Res 39:63–71
Harro J, Vasar E (1991a) Cholecystokinin-induced anxiety: how is it reflected in studies on exploratory behaviour? Neurosci Biobehav Rev 15:473–477
Harro J, Vasar E (1991b) Evidence that CCKB receptors mediate the regulation of exploratory behaviour in the rat. Eur J Pharmacol 193:379–381
Harro J, Oreland L (1993a) Cholecystokinin receptors and memory: a radial maze study. Pharmacol Biochem Behav 44:509–517.
Harro J, Vasar E, Bradwejn J (1993b) Cholecystokinin in animal and human research of anxiety. Trends Pharmacol Sci 14:244–249
Hays SE, Beinfeld MC, Jensen RT, Goodwin FK, Paul SM (1980) Demonstration of a putative receptor site for cholecystokinin in rat brain. Neuropeptides 1:53–62.
Hendrie CA, Shepherd JK, Rodgers RJ (1989) Differential effects of the CCK antagonist, MK–329, on analgesia induced by morphine, social conflict (opioid) and defeat experience (non-opioid) in male mice. Neuropharmacology 28:1025–1032

Hill DR, Shaw TM, Graham W, Woodruff GN (1990) Autoradiographical detection of cholecystokinin–A receptors in primate brain using 125I–Bolton Hunter CCK-8 and 3H–MK–329. J Neurosci 10:1070–1081

Hirosue Y, Inui A, Miura M, Nakajima M, Okita M, Himori N, Baba S, Kasuga M (1992) Effects of CCK antagonists on CCK-induced suppression of locomotor activity in mice. Peptides 13:155–157

Homanics GE, Quinlan JJ, Firestone LL (1999) Pharmacological and behavioural responses of inbred C57BL/6J and strain 129/SvJ mouse lines. Pharmacology Biochemistry and Behavior 63:21–26.

Honda T, Wada E, Battery JF, Wank SA (1993) Differential gene expression of CCKA and CCKB receptors in the rat brain. Mol Cell Neurosci 4:143–154

Hökfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M, Markey K (1980a) Evidence for coexistence of dopamine and CCK in meso-limbic neurones. Nature 285:476–478

Hökfelt T, Skirboll L, Rehfeld JF, Goldstein M, Markey K, Dann O (1980b) A subpopulation of mesencephalic dopamine neurons projecting to limbic areas contains a cholecystokinin-like peptide: evidence from immunochemistry combined with retrograde tracing. Neuroscience 5:2093–2110

Hökfelt T (1991) Neuropeptides in perspective: The last ten years. Neuron 7:867–879

Idanpään-Heikkilä JJ, Guilbaud G, Kayser V (1997) Prevention of tolerance to the antinociceptive effects of systemic morphine by a selective cholecystokinin–B receptor antagonist in a rat model of peripheral neuropathy. J Pharmacol Exp Ther 282:1366–1372

Ingram SM, Krause RG, Baldino F, Skeen LC, Lewis ME (1989) Neuronal localization of cholecystokinin mRNA in the rat brain by using in situ hybridization histochemistry. J Comp Neurol 287:260–272

Innis RB, Snyder SH (1980) Cholecystokinin receptor binding in brain and pancreas: regulation of pancreatic binding by cyclic and acyclic guanine nucleotides. Eur J Pharmacol 65:123–124

Ishibashi S, Oomura Y, Okajima T, Shibata S (1979) Cholecystokinin, motilin and secretin effects on the central nervous system. Physiol Behav 23:401–403

Itoh S, Katsuura G, Yoshikawa K, Rehfeld JF (1985) Potentiation of beta-endorphin effects by cholecystokinin antiserum in rats. Can J Physiol Pharmacol 63:1 81–83

Itoh S, Lal H (1990) Influences of cholecystokinin and analogues on memory processes. Drug Dev Res 21: 257–276

Jackson DM, Ross SB, Hashizume M (1988) Dopamine-mediated behaviours produced in naive mice by bromocriptine plus SKF 38393. J Pharm Pharmacol 40:3 221–223

Jagerschmidt A, Popovici T, O'Donohue M, Roques BP (1994) Identification and characterization of various cholecystokinin B receptor mRNA forms in rat brain tissue and partial determination of the cholecystokinin B receptor gene structure. J Neurochem 63:4 1199–1206

Jensen RT, Wank SA, Rowley WH, Sato S, Gardner JD (1989) Interaction of CCK with pancreatic acinar cells. Trends Pharmacol Sci 10:418–423

Jiang LH, Kasser RJ, Wang RY (1988) Cholecystokinin antagonist lorglumide reverses chronic haloperidol-induced effects on dopamine neurons. Brain Res 473:165–168

Johnson SW, North RA (1992) Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neuroscience 12: 493–488.

Kadar T, Fekete M, Telegdy G (1981) Modulation of passive avoidance behaviour of rats by intracerebroventricular administration of cholecystokinin octapeptide sulphate ester and no sulphated cholecystokinin octapeptide. Acta Physiol Acad Sci Hung 58:269–274

Kadar T, Pesti A, Penke B, Telegdy G (1984) Inhibition of seizures induced by picrotoxin and electroshock by cholecystokinin octapeptides and their fragments in rats after intracerebroventricular administration. Neuropharmacology 23:955–961

Kalivas PW, Widerlow E, Stanley D, Breese G, Prange AJ (1983) Enkephalin action on the mesolimbic system: a dopamine-dependent and a dopamine-independent increase in locomotor activity. J Pharmacol Exp Ther 227: 229–237.

Katsuura G, Itoh S (1986a) Preventive effect of cholecystokinin octapeptide on experimental amnesia in rats. Peptides 7:105–110

Katsuura G, Itoh S (1986b) Passive avoidance deficit following intracerebroventricular administration of cholecystokinin tetrapeptide amide in rats. Peptides 7:809–814

Kayser V, Idanpään-Hekkilä JJ, Christensen D, Guilbaud G (1998) The selective cholecystokininB receptor antagonist L–365,260 diminishes the expression of naloxone-induced morphine withdrawal symptoms in normal and neuropathic rats. Life Sci 62:947–952

Kelly MA, Rubinstein M, Phillips TJ, Lessov CN, Burkhart-Kasch S, Zhang G, Bunzow JR, Fang Y, Gerhardt GA, Grandy DK, Low MJ (1998) Locomotor activity in D2 dopamine receptor-deficient mice is determined by gene dosage, genetic background, and developmental adaptations. J Neurosci 18:3470–3479

Khosla S, Crawley JN (1988) Potency of L–364,718 as an antagonist of the behavioral effects of peripherally administered cholecystokinin. Life Sci 42:153–159

Koller WC, Herbster G (1988) D1 and D2 dopamine receptor mechanisms in dopaminergic behaviors. Clin Neuropharmacol 11:221–231

Konig M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ, Zimmer A (1996) Pain responses, anxiety and aggression in mice deficient in pre-pro-enkephalin. Nature 383:535–538

Kopin AS, Mathes WF, McBride EW, Nguyen M, Al Haider W, Schmitz F, Bonner-Weir S, Kanarek R, Beinborn M (1999) The cholecystokinin–A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. J Clin Invest 103:383–391

Koszycki D, Bradwejn J, Bourin M (1991) Comparison of the effects of cholecystokinin-tetrapeptide and carbon dioxide in health volunteers. Eur Neuropsychopharmacol 1:137–141

Kõks S, Vasar E, Soosaar A, Lang A, Volke V, Võikar V, Bourin M, Männistö PT (1997) Relation of exploratory behavior of rats in elevated plus-maze to brain receptor binding properties and serum growth hormone levels. Eur Neuropsychopharmacol 7:289–294

Kõks S, Soosaar A, Võikar V, Volke V, Ustav M, Männistö PT, Bourin M, Vasar E (1998) Opioid antagonist naloxone potentiates anxiogenic-like action of cholecystokinin antagonists in elevated plus-maze. Neuropeptides 32:235–240

Kõks S, Soosaar A, Võikar V, Bourin M, Vasar E (1999) BOC–CCK-4, CCK_B receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides 33:63–69.

Kõks S, Mannisto PT, Bourin M, Shlik J, Vasar V, Vasar E (2000a) Cholecystokinin-induced anxiety in rats: relevance of pre-experimental stress and seasonal variations. J Psychiatry Neurosci 25:33–42

Kõks S, Bourin M, Vasar E (2000b) Adaptation of rats to the experimental conditions modifies the behavioral effects of naloxone and morphine in the elevated zero-maze. Eur Neuropsychopharmacol 10: Suppl. 2, S66.

Kuczenski, R., (1983). Biochemical actions of amphetamine and other stimulants. In: Creese, I. (Ed.), Stimulants: Neurochemical, Behavioral and Clinical Perspectives. Raven Press, New York, pp 31–61.

Lavigne GJ, Millington WR, Mueller GP (1992) The CCK–A and CCK–B receptor antagonists, devazepide and L–365,260, enhance morphine antinociception only in non-acclimated rats exposed to a novel environment. Neuropeptides 21:119–129

Lee YM, Beinborn M, McBride EW, Lu M, Kolakowski LF, Kopin AS (1993) The human brain cholecystokinin–B/gastrin receptor. Cloning and characterization. J Biol Chem 268:8164–8169

Lemaire M, Piot O, Roques BP, Bohme GA, Blanchard JC (1992) Evidence for an endogenous cholecystokininergic balance in social memory. Neuroreport 3:929–932

Lemaire M, Bohme GA, Piot O, Roques BP, Blanchard JC (1994) CCK–A and CCK–B selective receptor agonists and antagonists modulate olfactory recognition in male rats. Psychopharmacology (Berl) 115:435–440

Lindefors N, Linden A, Brene S, Sedvall G, Persson H (1993) CCK peptides and mRNA in the human brain. Prog Neurobiol 40:671–690

Little HJ, O'Callaghan MJ, Butterworth AR, Wilson J, Cole J, Watson WP (1999) Low alcohol preference among the "high alcohol preference" C57 strain of mice: preference increased by saline injections. Psychopharmacology 147:182–189

Longoni R, Spina L, Di Chiara G (1987) Dopaminergic D1 receptors: essential role in morphine-induced hypermotility. Psychopharmacology 93:401–402.

Lopes da Silva FH, Witter MP, Boejinga PH, Lehman AH (1990) Anatomic organization and physiology of the limbic cortex. Physiol Rev 70:453–511

MacVicar BA, Kerrin JP, Davison JS (1987) Inhibition of synaptic transmission in the hippocampus by cholecystokinin (CCK) and its antagonism by a CCK analog (CCK27–33). Brain Res 406:130–135

Maldonado R, Saiardi A, Valverde O, Ṣama TA, Roques BP, Borrelli E (1997) Absence of opiate rewarding effects in mice lacking dopamine D_2 receptors. Nature 388: 586–589.

Mason P (1999) Central mechanisms of pain modulation. Curr Opin Neurobiol 9: 436–441.

Maurice T, Hiramatsu M, Kameyama T, Hasegawa T, Nabeshima T (1994) Cholecystokinin-related peptides, after systemic or central administration, prevent carbon monoxide-induced amnesia in mice. J Pharmacol Exp Ther 269:665–673

Meltzer HY (1980) Relevance of dopamine autoreceptors for psychiatry. Preclinical and clinical studies. Schizophrenia Bull 3:456–467

Migaud M, Durieux C, Roques BP (1993) Evidence for cholecystokinin octapeptide (CCK8) uptake in rat cortex synaptosomal fractions. J Neurochem 61:S83B

Minabe Y, Ashby CR, Wang RY (1991) The CCK–A receptor antagonist devazepide but not the CCK–B receptor antagonist L–365,260 reverses the effects of chronic clozapine and haloperidol on midbrain dopamine neurons. Brain Res 549:151–154

Miyake A (1995) A truncated isoform of human CCK–B/gastrin receptor generated by alternative usage of a novel exon. Biochem Biophys Res Commun 208:230–237

Miyasaka K, Ichikawa M, Ohta M, Kanai S, Yoshida Y, Masuda M, Nagata A, Matsui T, Noda T, Takiguchi S, Takata Y, Kawanami T, Funakoshi A (2002) Energy metabolism and turnover are increased in mice lacking the cholecystokinin–B receptor. The Journal of Nutrition 132:739–741

Moran TH, Robinson PH, Goldrich MS, McHugh PR (1986) Two brain cholecystokinin receptors: implications for behavioral actions. Brain Res 362:175–179

Moroji T, Hagino Y (1987) Bilateral subdiaphragmatic vagotomy does not prevent the behavioral effects of systematically administered ceruletide in mice. Neuropeptides 9:217–224

Munro G, Pumford KM, Russell JA (1998) Altered cholecystokinin binding site density in the supraoptic nucleus of morphine-tolerant and -dependent rats. Brain Res 780:190–198.

Nagata A, Ito M, Iwata N, Kuno J, Takano H, Minowa O, Chihara K, Matsui T, Noda T (1996) G protein-coupled cholecystokinin–B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proc Natl Acad Sci USA 93:11825–11830.

Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, Roques BP (1999) International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev 51:745–781

O'Neill MF, Dourish CT, Iversen SD (1991) Hypolocomotion induced by peripheral or central injection of CCK in the mouse is blocked by the CCKA receptor antagonist devazepide but not by the CCKB receptor antagonist L–365,260. Eur J Pharmacol 193:203–208

Palmour RM, Bradwejn J, Ervin FR (1992) The anxiogenic effect of CCK-4 in monkeys are reduced by CCK–B antagonists, benzodiazepines or adenosine A2 agonists. Eur Neuropsychopharmacology 2:193–195

Pélaprat D, Broer Y, Studler JM, Peschanski M, Tassin JP, Glowinski J, Rostenes W, Roques BP (1987) Autoradiography of CCK receptors in the rat brain using (3H)–Boc(Nle28,31)CCK27–33 and (125I) Bolton-Hunter CCK8. Functional significance of subregional distributions. Neurochem Int 10:495–508

Peters A, Miller M, Kimerer LM (1983) Cholecystokinin-like immunoreactive neurons in rat cerebral cortex. Neuroscience 8:431–448

Pettit HO, Mueller K (1989) Infusions of cholecystokinin octapeptide into the ventral tegmental area potentiate amphetamine conditioned place preferences. Psychopharmacology (Berl) 99:423–426

Pinget M, Straus E, Yalow RS (1978) Localization of cholecystokinin-like immunoreactivity in isolated nerve terminals. Proc Natl Acad Sci USA 75:6324–6326

Pinget M, Straus E, Yalow RS (1979) Release of cholecystokinin peptides from a synaptosome-enriched fraction of rat cerebral cortex. Life Sci 25:339–342

Pommier B, Da Nascimento S, Dumont S, Bellier B, Million E, Garbay C, Roques BP, Noble F (1999) The cholecystokininB receptor is coupled to two effector pathways through pertussis toxin-sensitive and -insensitive G proteins. J Neurochem 73:281–288

Pommier B, Beslot F, Simon A, Pophillat M, Matsui T, Daugé V, Roques B P, Noble F (2002) Deletion of CCK_2 receptor in mice results in an upregulation of the endogenous opioid system. J Neurosci 22:5 2005–2011

Radu D, Brodin E, Weber G, Lindefors N (2001) Delayed stress-induced increase in tissue level of cholecystokinin in rat prefrontal cortex: modulation by microdialysis probe implantation and systemic ketamine. Brain Res 908:197–203

Rasmussen K, Czachura JF, Stockton ME, Howbert JJ (1993) Electrophysiological effects of diphenylpyrazolidinone cholecystokinin–B and cholecystokinin–A antagonists on midbrain dopamine neurons. J Pharmacol Exp Ther 264:480–488

Raud S, Rünkorg K,Veraksitš A, Reimets A, Nelovkov A, Abramov U, Matsui T, Bourin M, Volke V, Kõks S, Vasar E (2003) Targeted mutation of CCK2 receptor gene modifies the behavioural effects of diazepam in female mice. Psychopharmacology (Berl) 168:417–425.

Rehfeld JF (1985) Neuronal cholecystokinin: one or multiple transmitters? J Neurochem 44:1–10

Rehfeld JF, Hansen HF (1986) Characterization of preprocholecystokinin products in the porcine cerebral cortex. Evidence of different processing pathways. J Biol Chem 261:5832–5840

Rehfeld JF (1992a) CCK and anxiety: introduction. In: Dourish CT, Cooper SJ, Iversen SD, Iversen LL (eds) Multiple Cholecystokinin receptors in the CNS. Oxford University Press, New-York, pp 117–120

Rehfeld JF, Mogensen NW, Bardram L, Hilsted L, Monstein HJ (1992b) Expression, but failing maturation of preprocholecystokinin in cerebellum. Brain Res 576:111–9

Rehfeld JF, Nielsen FC (1995) Molecular forms and regional distribution of cholecystokinin in the central nervous system. In Bradwejn J, Vasar E (eds). Cholecystokinin and Anxiety: From Neuron to Behavior. Austin R.G. Landers Company pp 33–56

Roche S, Bali JP, and Magous R (1990) Involvement of a pertussis toxin-sensitive G-protein in the action of gastrin on gastric parietal cells. Biochim Biophys Acta 1055:287–294.

Roques BP, Noble F (1998) Cholecystokinin receptors. In: Girdlestone D (ed) The IUPHAR receptor compendium of receptor characterization and classification. IUPHAR Media, London, pp 128–133

Saito A, Sankaran H, Goldfine ID, Williams JA (1980) Cholecystokinin receptors in the brain: characterization and distribution. Science 208:1155–1156.

Sankaran H, Goldfine ID, Deveney CW, Wong KY, Williams JA (1980) Binding of cholecystokinin to high affinity receptors on isolated rat pancreatic acini. J Biol Chem 255:1849–1853

Sante AB, Nobre MJ, Brandao ML (2000) Place aversion induced by blockade of mu or activation of kappa opioid receptors in the dorsal periaqueductal grey matter. Behav Pharmacol 11:583–589.

Savasta M, Palacios JM, Mengod G (1988) Regional localization of the mRNA coding for the neuropeptide cholecystokinin in the rat brain studied by *in situ* hybridization. Neurosci Lett 93:132–138

Savasta M, Palacios JM, Mengod G (1990) Regional distribution of the messenger RNA coding for the neuropeptide cholecystokinin in the human brain examined by *in situ* hybridization. Brain Res Mol Brain Res 7:91–104

Schalling M, Friberg K, Bird E, Goldstein M, Schiffmann S, Mailleux P, Vanderhaeghen JJ, Hokfelt T (1989) Presence of cholecystokinin mRNA in dopamine cells in the ventral mesencephalon of a human with schizophrenia. Acta Physiol Scand 137:467–468

Sebret A, Lena I, Crete D, Matsui T, Roques BP, Daugé V (1999) Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin–B receptor stimulation. J Neurosci 19:7230–7237.

Shippenberg TS, Bals-Kubik R (1995) Involvement of the mesolimbic dopamine system in mediating aversive effects of opioid antagonists in the rat. Behav Pharmacol 6:99–106.

Shlik J, Vasar E, Bradwejn J (1997) Cholecystokinin and Psychiatric disorders. Role in Aetiology and Potential of Receptor Antagonists in Therapy. CNS Drugs 8:134–152

Singh L, Field MJ, Hughes J, Menzies R, Oles RJ, Vass CA, Woodruff GN (1991) The behavioural properties of CI–988, a selective cholecystokininB receptor antagonist. Br J Pharmacol 104:239–245

Singh L, Oles RJ, Field MJ, Atwal P, Woodruff GN, Hunter JC (1996) Effect of CCK receptor antagonists on the antinociceptive, reinforcing and gut motility properties of morphine. Br J Pharmacol 18:1317–1325

Skinner K, Basbaum AI, Fields HL (1997) Cholecystokinin and enkephalin in brain stem pain modulating circuits. Neuroreport 8:2995–2998

Skoubis PD, Matthes HW, Walwyn WM, Kieffer BL, Maidment NT (2001) Naloxone fails to produce conditioned place aversion in μ-opioid receptor knock-out mice. Neuroscience 106:757–763.

Sokoloff P, Andrieux M, Besancon R, Pilon C, Martres MP, Giros B, Schwartz JC (1992) Pharmacology of human dopamine D3 receptor expressed in a mammalian cell line: comparison with D2 receptor. Eur J Pharmacol 225:331–337

Song I, Brown DR, Wiltshire RN, Gantz I, Trent JM, and Yamada T (1993) The human gastrin/cholecystokinin type B receptor gene: alternative splice donor site in exon 4 generates two variant mRNAs. Proc Natl Acad Sci USA 90:9085–9089.

Suberg SN, Watkins LR (1987) Interaction of cholecystokinin and opioids in pain modulation. Pain Headache 9:247–265

Suh HH, Tseng LF (1990) Differential effects of sulphated cholecystokinin octapeptide and proglumide injected intrathecally on antinociception induced by beta-endorphin and morphine administered intracerebroventricularly in mice. Eur J Pharmacol 179:329–338

Suzuki T, Moroji T (1989) Cholecystokinin binding sites in the rat forebrain: effects of acute and chronic methamphetamine administration. J Neural Transm 77:181–195

Swerdlow NR, van der Kooy D, Koob GF, Wenger JR (1983) Cholecystokinin produces conditioned place-aversions, not place-preferences, in food-deprived rats: evidence against involvement in satiety. Life Sci 32:2087–2093

Takahashi Y, Kato K, Hayashizaki Y, Wakabayashi T, Ohtsuka E, Matsuki S, Ikehara M, Matsubara K (1985) Molecular cloning of the human cholecystokinin gene by use of a synthetic probe containing deoxyinosine. Proc Natl Acad Sci USA 82:1931–1935

Tang Y, Shimizu E, and Tsien JZ (2001) Do 'smart' mice feel more pain, or are they just better learners? Nature Neuroscience 4:453

Taniguchi T, Matsui T, Ito M, Murayama T, Tsukamoto T, Katakami Y, Chiba T, Chihara K (1994) Cholecystokinin–B/gastrin receptor signalling pathway involves tyrosine phosphorylations of p125FAK and p42MAP. Oncogene 9:861–867

Tsuda M, Suzuki T, Misawa M, Nagase H (1996) Involvement of the opioid system in the anxiolytic effect of diazepam in mice. Eur J Pharmacol 307:7–14.

Tzschentke TM (1998) Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Progress in Neurobiology 56:613–672.

Ulrich CD, Ferber I, Holicky E, Hadac E, Buell G, Miller LJ (1993) Molecular cloning and functional expression of the human gallbladder cholecystokinin A receptor. Biochem Biophys Res Commun 193:204–211

Ungerstedt U, Ljungberg T (1977) Behavioral patterns related to dopamine neurotransmission: effect of acute and chronic antipsychotic drugs. Adv Biochem Psychopharmacology 16:193–199

Vaccarino FJ, Rankin J (1989) Nucleus accumbens cholecystokinin (CCK) can either attenuate or potentiate amphetamine-induced locomotor activity: evidence for rostral-caudal differences in accumbens CCK function. Behav Neurosci 103:831–6

Valverde O, Fournie-Zaluski MC, Roques BP, Maldonado R (1996) The CCKB antagonist PD–134,308 facilitates rewarding effects of endogenous enkephalins but does not induce place preference in rats.Psychopharmacology 123:119–126.

Vanderah TW, Lai J, Yamamura HI, Porreca F (1994) Antisense oligonucleotides to the CCKB receptor produces naltrindole- and [Leu5]enkephalin antiserum-sensitive enhancement of morphine antinociception. Neuroreport 5:2601–2605.

Vanderah TW, Bernstein RN, Yamamura HI, Hruby VJ, Porreca F (1996) Enhancement of morphine antinociception by a CCKB antagonist in mice is mediated via opioid delta receptors. J Pharmacol Exp Ther 278:212–219.

Vanderhaeghen JJ, Lotstra F, De Mey J, Gilles C (1980) Immunohistochemical localization of cholecystokinin- and gastrin-like peptides in the brain and hypophysis of the rat. Proc Natl Acad Sci USA 77:1190–1194

Vanderhaeghen JJ, Schiffmann SN (1992) Distribution of brain neuronal CCK. An in situ hybridization study. In: Dourish CT, Cooper SJ, Iversen SD, Iversen LL (eds) Multiple Cholecystokinin receptors in the CNS. Oxford University Press, New-York, pp 38–56

Vanderhaeghen JJ, Signeau JC, Gepts W (1975) New peptide in the vertebrate CNS reacting with antigastrin antibodies. Nature 257:604–605

Vasar E, Maimets M, Nurk A, Soosaar A, Allikmets L (1986) Comparison of motor depressant effects of caerulein and N-propylnorapomorphine in mice. Pharmacol Biochem Behav 24:469–478

Vasar EE, Allikmets LH, Soosaar AH (1988) Antagonism of caerulein, a CCK-8 receptor agonist, to the behavioral effects of ketamine in mice and rats. Bull Exp Biol Med 105:43–45

Vasar E, Harro J, Lang A, Põld A, Soosaar A (1991) Differential involvement of CCK–A and CCK–B receptors in the regulation of locomotor activity in the mouse. Psychopharmacology 105:393–399

Vasar E, Lang A, Harro J, Kõks S, Volke V, Sihver S, Bourin M, Bradwejn J, Männistö PT (1994a) Subdiaphragmatic vagotomy does not prevent the anti-exploratory effect of caerulein in the elevated plus-maze. Neuropeptides 26:39–45

Vasar E, Lang A, Harro J, Bourin M, Bradwejn J (1994b) Evidence for potentiation by CCK antagonists of the effect of cholecystokinin octapeptide in the elevated plus-maze. Neuropharmacology 33:729–735

Vasar E, Kõks S, Beljajev S, Abramov U, Koovit I, Matsui T (2000) CCK_B receptor knockout mice: gender related behavioural differences. Eur Neuropsychopharmacol 10:Suppl. 2, S69.

Verhage M, Ghijsen WE, Nicholls DG, Wiegant VM (1991) Characterization of the release of cholecystokinin–8 from isolated nerve terminals and comparison with exocytosis of classical transmitters. J Neurochem 56:1394–1400

Vickroy TW, Bianchi BR, Kerwin JF, Kopecka H, Nazdan AM (1988) Evidence that type A CCK receptors facilitate dopamine efflux in rat brain. Eur J Pharmacol 152:371–372

Vickroy TW, Bianchi BR (1989) Pharmacological and mechanistic studies of cholecystokinin-facilitated [^{3}H]dopamine efflux from rat nucleus accumbens. Neuropeptides 13:43–50

Võikar V, Kõks S, Vasar E, Rauvala H (2001) Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies Physiology & Behavior 72:271–281

Wank SA, Harkins R, Jensen RT, Shapira H, de Weerth A, Slattery T (1992) Purification, molecular cloning, and functional expression of the cholecystokinin receptor from rat pancreas. Proc Natl Acad Sci USA 89:3125–3129

Wank SA (1995) Cholecystokinin receptors. Am J Physiol 269:G628–646

Wiertelak EP, Maier SF, Watkins LR (1992) Cholecystokinin anti-analgesia: safety cues abolish morphine analgesia. Science 256:830–833.

Wilson SG, Mogil JS (2001) Measuring pain in the (knockout) mouse: big challenges in a small mammal. Behav Brain Res 125:65–73.

Wise RA, Rompre PP (1989) Brain dopamine and reward. Annu Rev Psychol 40:191–225

Woodruff GN, Hughes J (1991) Cholecystokinin antagonists. Annu Rev Pharmacol Toxicol 31:469–501

Zetler G (1985) Caerulein and its analogues: neuropharmacological properties. Peptides 6 Suppl 3:33–46

Zhang J, Chiodo LA, Freeman AS (1991) Effects of the CCK–A receptor antagonist CR 1409 on the activity of rat midbrain dopamine neurons. Peptides 12:339–343

Zimmer A, Zimmer AM, Baffi J, Usdin T, Reynold K, Konig M, Palkovits M, Mezey E (1998) Hypoalagesia in mice with a targeted deletion of the tachykinin 1 gene. Proc Natl Acad Sci USA 95:2630–2635.

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc Natl Acad Sci USA 96:5780–5785.

SUMMARY IN ESTONIA

Koletsüstokiniini teist tüüpi retseptori puudulikkusega hiirte käitumuslik ja biokeemiline iseloomustus: muutused dopamiin- ja endopioidergilise süsteemi talitluses

Sissejuhatus

Esimest korda kirjeldasid koletsüstokiniini (CCK) imetaja kesknärvisüsteemis 1975.a. Vanderhaeghen ja tema kolleegid. CCK on peptiid, mis esineb nii seedetraktis kui kesknärvisüsteemis (KNS-is). Peptiid esineb mitme alavormina ja KNS-is on esindatuim kaheksast aminohappest koosnev, positsioonis 7 (alates –COOH terminaalsest otsast) sulfakteeritud vorm CCK–8s. Koletsüstokiniinil on suur sarnasus teise olulise seedetrakti peptiidi — gastriinga. Mõlemal peptiidil on identne järjestus viie aminohappe ulatuses alates –COOH terminaalsest otsast ning nii CCK kui gastriin on endogeensed ligandid ülekaalukalt KNS-is esinevale CCK_2 retseptorile. CCK toimet organismi talitlusele ja käitumisele on laialdaselt uuritud. On leitud, et anatoomiliselt kattub CCK-ergiline süsteem KNS-is nii dopamiinergilise kui opioidergilise süsteemiga. Funktsionaalselt pärsib CCK oma teist tüüpi reteptori — CCK_2 vahendusel nimetatud süsteemide aktiivsust

Uuringute eesmärk

Käesoleva töö eesmärgiks oli iseloomustada koletsüstokiniini teist tüüpi retseptori (CCK_2) puudulikkusega hiirte fenotüüpi — võimalikke käitumuslikke iseärasusi ning muutusi neurokeemilistes protsessides. Põhiline tähelepanu on suunatud KNS-i dopamiin- ja endopioidergiliste süsteemide uurimisele, sest nende virgatsainete esinemine kesknärvisüsteemis kattub suuresti CCK-ergilise süsteemiga.

Töö konkreetsemateks eesmärkideks oli uurida CCK_2 retseptori puudulikkusega hiirtel:

1. dopamiini teist tüüpi (D_2) retseptorite ning opioid retseptorite afiinsust ja tihedust erinevates KNS-i struktuurides nagu ajukoores, juttkehas, meso-limbilistes struktuurides ja hipokampuses;
2. võimalikke muutusi liikumisaktiivsuses ja motoorses koordinatsioonis;
3. dopamiinergiliste ühendite apomorfiini, mitteselektiivse dopamiini D_1 ja D_2 retseptorite agonisti, ja amfetamiini, põhjustab KNS-is dopamiini vabanemise, toimet katseloomade liikumisaktiivsusele;
4. muutusi valutundlikkuses, kasutades selleks erinevaid uurimismeetodeid;

5. morfiini, μ-opioid retseptorite agonisti, analgeetilist toimet ning morfiini ja naloksooni, mitteselektiivse opioidi retseptorite antagonisti, mõju katseloomade liikumisaktiivsusele ning koha-eelistusele.

Meetodid

CCK_2 retseptori puudulikkusega hiired saadi professor Toshimitsu Matsui laboratooriumist (Nagata et al., 1996) ning neid paljundati ja kasvatati TÜ Biomeedikumi vivaariumis. Radioligandi sidumiskatsetes mõõdeti CCK_2, dopamiini D_2 ja μ-opioid retseptori omadusi kesknärvisüsteemi erinevates struktuurides: ajukoores, juttkehas, mesolimbilistes struktuurides ja hipokampuses. CCK_2 retseptorite märgistamiseks kasutati [^{3}H]–pCCK-8 ja mittespetsiifilise sidumise määramiseks CCK_2 retseptorite antagonisti L–365,260. Dopamiini D_2 retseptorite märgistamiseks kasutati [^{3}H]–spiperooni ning mittespetsiifilist sidumist määrati selle retseptorite antagonisti, raklopriidi abil. Opioidi retseptorite märgistamiseks rakendati [^{3}H]–diprenorfiini ja mittespetsiifilist sidumist määrati naloksooni abil. Motoorset aktiivsust hinnati aparatuuriga, mis koosnes fotosensoritega varustatud pleksiklaasist kastis ja arvutisüsteemist. Selle katse puhul hinnati hiirte üldist motoorset aktiivsust ja uudistamisaktiivsust — tõusu tagakäppadele. Samuti hinnati kohanemise mõju motoorsele aktiivsusele ning uuriti amfetamiini, naloksooni ja morfiini toimet selles katsekeskkonnas adaptatsiooni foonil. Motoorse koordinatsiooni hindamiseks kasutati rotarodi testi, kus uuriti katseloomade võimet püsida pöörleval silindril. Valutundlikkuse spinaalsete mehhanismide hindamiseks kasutati Hargreaves'i testi, mille puhul katselooma tagumise käpa alla juhiti kindla intensiivsusega infrapunaste kiirte kimp (intensiivsus vastas temperatuurile 50°C) ning automaatselt registreeriti katselooma reaktsiooni aeg ärritusele — tagumise käpa äratõmbamine. Valutundlikkuse supraspinaalsete mehhanismide uurimiseks kasutati kuumplaaditesti, mis kujutab endast katselooma reaktsiooni aja mõõtmist ja käitumise iseloomustamist kindla temperatuuriga (52°C) metallplaadil. Katselooma liikumine metallplaadil piirati klaasist koonuse abil. Kuumplaaditestis määrati kaks parameetrit: esmane valureaktsioon ehk valulävi ja võime taluda valu. Esmaseks valureaktsiooniks võeti hetk, mil katseloom kas lakkus või raputas tugevalt tagakäppa. Valu taluvusena mõõdeti aeg hetkeni millal hiir püüdis hüppamisega katsesituatsioonist põgeneda. Nii Hargreaves´i kui ka kuumplaaditesti hinnati morfiini antinotsitseptiivset toimet. Koha-eelistuse katses kasutati puuri, mis oli jagatud luugiga vaheseina abil heledaks ja tumedaks osaks. Selles katses hinnati morfiini ja naloksooni toimet katseloomade valikule puuri heleda ja tumeda osa vahel, eeldades, et hiirtel on loomulik eelistus puuri tumedama osa vastu.

Tulemused

Ühe vanemliku hiireliini — 129Sv hiireliini geneetiline taust mõjutab mitmeid käitumuslikke avaldusi CCK_2 retseptori puudulikkusega hiirtel. Korduv tagasiristamine C57Bl/6 hiireliiniga parandas oluliselt katseloomade motoorset koordinatsiooni ja langetas liikumisaktiivsust CCK_2 retseptor puudulikkusega hiirtel. CCK_2 retseptori geeni väljalülitamine tekitab olulisi muutusi dopamiinergilise süsteemi talitluses. Radioligandi sidumiskatsed näitasid, et dopamiini D_2 retseptorite arv on suurenenud juttkehas, kuid mitte mesolimbilistes struktuurides. Apomorfiini hüpolokomotoorse toime oluline võimendumine CCK_2 retseptori puudulikkusega hiirtel viitab presünaptiliste dopamiini retseptorite aktiivsuse tõusule. Samas amfetamiini hüperlokomotoorse toime suurenemine mutageensetel hiirtel näitab postsünaptiliste dopamiini retseptorite aktiivsuse tõusu. CCK_2 retseptorite geeni väljalülitamine põhjustab muutusi ka μ-opiodi retseptorite biokeemilistes parameetrites. Juttkehas on nende retseptorite arv suurenenud, nende afiinsus on aga langenud. Samas on ajukoores μ-opioidi retseptorite afiinsus tõusnud. Valutundlikkus on CCK_2 retseptori puudulikkusega hiirtel oluliselt muutunud. Valulävi on antud hiirtel oluliselt tõusnud nii spinaalset kui ka supraspinaalset valu impulsi regulatsiooni kajastavates mudelites. Samas on CCK_2 retseptori puudulikkusega hiirtel oluliselt vähenenud valu taluvuse võime. Seega on neil hiirtel kõrgem valulävi, kuid madalam valutaluvus. Morfiini, μ-opioidi retseptori agonisti, toimes esineb CCK_2 retseptori puudulikkusega hiirtel olulisi lahknevusi erinevates käitumiskatsetes. Morfiini toime on oluliselt suurenenud Hargreaves´i testis, kuid pole muutunud kuumplaaditestis, kui mõõta esmast valureaktsiooni, ega kohaeelistus katses. Samas ei avalda morfiin olulist aktiveerivat toimet nende hiirte liikumisaktiivsusele ega suurenda nende valu taluvuse võimet (Pommier et. al. 2002). Sellised vastuolulised muutused käitumises peegeldavad hästi kirjeldatud nihkeid μ-opioidi retseptorite afiinsuses juttkehas ja ajukoores. Liikumisaktiivsuse mõõtmise tulemused näitavad, et CCK_2 retseptor puudulikel hiirtel on langenud kohanemisvõime uudses keskkonnas, kuna võrreldes kontroll hiirtega püsis nende aktiivsus üksteisele järgnevate katsete käigus kõrge. Ilmselt on viimane seotud endopioidergilise süsteemi aktivatsiooniga, sest naloksoon, opioidi retseptorite antagonist, kõrvaldab selle muutuse. Lisaks eeltoodule ei kutsu naloksoon CCK_2 retseptori puudulikkusega hiirtel esile olulist kohavältimise käitumist, mis on ilmselt seotud asjaoluga, et nimetatud hiirtel on väiksem ärevus võrreldes CCK_2 retseptoriga liigikaaslastega. Tulenevalt märkimisväärsetes muutustes dopamiin- ja edopioidergilise süsteemi talitluses peame CCK_2 retseptori puudulikkusega hiirt heaks mudeliks uute psühhofarmakoloogiliste ravimite uurimisel. Selle mudeli abil oleks võimalik sedastada ja uurida uusi antipsühhootilisi aineid, medikamente ravimsõltuvuse ärahoidmiseks ning valu leevendamiseks.

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PUBLICATIONS

I

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ORIGINAL INVESTIGATION

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Cholecystokinin$_2$ receptor-deficient mice display altered function of brain dopaminergic system

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Abstract *Rationale:* Cholecystokinin (CCK) has been shown to coexist and interact with dopamine in the regulation of behaviour. Two different CCK receptors (CCK_1 and CCK_2) have an opposite influence on the activity of dopamine neurons. Stimulation of CCK_2 receptors decreases the release of dopamine and that receptor could mediate the neuroleptic-like effect of CCK. *Objective:* To investigate the activity of the dopaminergic system in pharmacological experiments on CCK_2 receptor (CCK2R)-deficient mice. *Methods:* We used age- and sex-matched littermates in all our experiments. To evaluate the behavioural differences, we performed the rotarod test and measured the locomotor activity of animals using computer-connected photoelectric motility boxes. Amphetamine and apomorphine, two dopaminergic drugs with different pharmacodynamic properties, were used to influence the activity of the dopaminergic system in the brain. Neurochemical differences related to the different genotype were analysed by means of high-performance liquid chromatography and radioligand binding studies. *Results:* Motor co-ordination was significantly impaired in the rotarod test of CCK2R receptor-deficient mice. Moreover, the locomotor activity of heterozygous (+/–) and homozygous (–/–) CCK2R receptor-deficient mice was somewhat reduced. A low dose of apomorphine (0.1 mg/kg), an unselective agonist of dopamine receptors, suppressed locomotor activity significantly more in homozygous (–/–) and heterozygous (+/–) mutant mice than in their wild-type (+/+) littermates. Amphetamine (3–6 mg/kg), increasing release of dopamine from the presynaptic terminals, caused a dose-dependent motor stimulation in wild-type (+/+) mice. In heterozygous (+/–) and homozygous (–/–) mice, a lower dose of amphetamine (3 mg/kg) did not alter the locomotor activity, whereas the higher dose of (6 mg/kg) induced a significantly stronger increase in locomotor activity in homozygous (–/–) mice than in their heterozygous (+/–) and wild-type (+/+) littermates. Despite the changes in the action of apomorphine and amphetamine in homozygous (–/–) mice, we did not find any significant differences in the concentration of dopamine and their metabolites in the striatum or cortex. However, the density of dopamine D_2 receptors was significantly increased in the striatum of homozygous (–/–) animals compared with wild-type (+/+) mice. *Conclusions:* The targeted mutation of the CCK_2 receptor gene induced gene dose-dependent changes in the activity of the dopaminergic system. The sensitivity of presynaptic dopamine receptors was increased in heterozygous (+/–) and homozygous (–/–) animals, whereas the increase in sensitivity of postsynaptic dopamine receptors was apparent only in homozygous (–/–) mice.

Keywords Targeted mutagenesis · Wild-type · Heterozygous · Homozygous · Dopamine · Serotonin · Receptor · Cholecystokinin · Cholecystokinin$_2$ receptor · Motor co-ordination · Rotarod test · Motility boxes · Locomotor activity

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Introduction

Cholecystokinin (CCK) coexists with dopamine (DA) in some mesencephalic neurones, innervating mesolimbic and cortical regions, and interacts with DA in the regulation of behaviour (Hökfelt et al. 1980). These interactions are complex and depend on the route of administra-

tion of CCK agonists and the subtype of CCK receptor involved (Altar and Boyar 1989; Crawley and Corwin 1994; Ladurelle et al. 1997). There are two distinct subtypes of CCK receptors, namely CCK_1 ("alimentary" subtype, CCK1R, former CCK-A) and CCK_2 ("brain" subtype, CCK2R, former CCK-B) receptors (Noble et al. 1999). Both subtypes are found in the brain structures linked to the mesolimbic DA system: the ventral tegmental area and nucleus accumbens (Hökfelt et al. 1980; Hill et al. 1990; Honda et al. 1993). The behavioural and biochemical studies have shown that CCK1R and CCK2R receptors mediate the opposite effects of CCK on the activity of DA neurones in mice and rats (Vasar et al. 1991; Noble et al. 1999). CCK increases the release of DA in the medial posterior nucleus accumbens by stimulating the CCK1R receptors, whereas the stimulation of CCK2R receptors in the anterior nucleus accumbens does the opposite (Vickroy et al. 1988; Altar and Boyar 1989; Marshall et al. 1991). Both subtypes of CCK receptors have been cloned, and targeted disruption of CCK1R and CCK2R receptor genes has been achieved in mice (Nagata et al. 1996; Kopin et al. 1999). The animals lacking CCK1R receptors did not respond to the hypophagic effect of CCK, but otherwise the feeding behaviour of these mice was not impaired relative to their wild-type littermates (Kopin et al. 1999). The animals without CCK2R receptors display disturbances in the development of gastric mucosa (Nagata et al. 1996) and in learning (Sebret et al. 1999).

Taking into account a role of CCK2R receptors in the regulation of activity of DA neurones (Studler et al. 1986; Crawley and Corwin 1994), an attempt has now been made to explore alterations in the DA system due to the genetic mutation of the CCK2R receptor gene in mice. It is known that mesolimbic DA plays a crucial role in the regulation of locomotor activity in rodents (Bradbury et al. 1983; Costall et al. 1985). Therefore, the motor co-ordination of mice in the rotarod test and their locomotor activity in the motility boxes were studied. Moreover, the action of two drugs, apomorphine (unselective D1 and D2 receptor agonist) and amphetamine (increases release of DA), was studied in CCK2R receptor-deficient mice. In a separate study, the concentrations of DA, serotonin (5-hydroxytryptamine; 5-HT) and their major metabolites were measured in the two brain structures of wild-type (+/+) and homozygous (–/–) mice. Also, the receptor-binding parameters of CCK, serotonin $5\text{-}HT_2$ and DA D_2 receptors were measured in the CCK2R receptor-deficient and wild-type mice.

Materials and methods

Animals

Nagata et al. (1996) generated CCK2 receptor-deficient mice by replacement of a part of exon 2 and exons 3, 4 and 5. Breeding and genotype analysis were performed in the Department of Physiology, University of Tartu. Genotyping was carried out by means of polymerase chain reaction (PCR) using two pairs of primers – HE2F (TGG AGT TGA CCA TTC GAA TCA C) and LacZrev (GTG CTG CAA GGC GAT TAA GTT G) were designed to detect the mutant allele, and HE3F (TAT CAG TGA GTG TGT CCA CTC T) and HE3R (ACA TTT GTT GGA CAC GTT CAC) were designed for the wild-type allele. For PCR, we used the following protocol: 96°C for 10 min (initial denaturation); 96°C for 50 s, 60°C for 50 s and 72°C for 2 min (25 cycles); and 72°C for 10 min (final amplification). PCR products were stored at 4°C until electrophoresis. Male homozygous (–/–) CCK_2 receptor-deficient, male heterozygous mutant (+/–) and male wild-type (+/+) (3-months old) mice were used. Mutant mice were crossed back three times to the C57/BL6 background to minimise the possible genetic effects from the 129 sv strain. The mice were kept in the animal house at 20±2°C under a 12-h/12-h light/dark cycle (lights on at 0700 hours). Tap water and food pellets were available ad libitum. All animal procedures were approved by the University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

Behavioural testing

The animals were brought into the experimental room 1 h before the beginning of the experiments which were performed between 1300 hours and 1900 hours.

Rotarod test

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

Motility test

For the study of locomotor activity, an animal was placed singly into the photoelectric motility boxes (448×448×450 mm) connected to a computer (TSE Technical and Scientific Equipment GMBH, Germany). The illumination level of transparent test boxes was ~750 lux. Time in locomotion (s), distance of locomotion (m), number of rearing and number of corner entries were registered during the 30-min observation period.

The locomotor effects of amphetamine (3 mg/kg and 6 mg/kg) – increasing a release of catecholamines – and apomorphine (0.1 mg/kg) – an unselective agonist of DA D_1 and D_2 receptors – were studied in the separate groups of animals. Amphetamine (Sigma) and apomorphine (Sigma) were injected intraperitoneally 20 min and 15 min, respectively, before the beginning of an experiment.

Analysis of DA, 5-HT and their metabolites in the mouse brain samples

Mice were decapitated, the brains were rapidly removed and cooled in liquid nitrogen. The following brain areas were dissected from the sections according to the atlas of Franklin and Paxinos (1997): (i) the cerebral cortex, (ii) both striata, and (iii) hypothalamus. The dissected tissues were frozen in liquid nitrogen and stored at –80°C until sample preparation.

The brain samples were homogenised in 10–50 volumes of 0.1 N $HClO_4$, depending on the tissues. The homogenates were centrifuged at 16,500 rpm (15 min +4°C, Heraeus Sepatech, Biofuge 17RS). The supernatant was removed, separated into two separate Eppendorf tubes and stored at –80°C until analysed.

Two different high-performance liquid chromatography (HPLC) runs determined DA, 5-HT and their metabolites: one assay for DA and its metabolite dihydroxyphenylacetic acid (DOPAC), and another assay for measuring homovanillic acid (HVA), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA).

Both chromatographic systems consisted of an isocratic Waters 510 pump (Waters, Milford, Mass.), a Waters 717 plus autosampler with cooler (Waters), a reverse-phase C18 column (Ultrasphere ODS, 4.6×250 mm, 5-µm particle size, Beckmann) and Hewlett Packard HP-3396A integrators. In HPLC system 1, the detector (ESA Coulochem II, ESA, Chelmsford, Mass.) was equipped with a 5021 (ESA) conditioning cell and a two-channel (Det 1 and Det 2) 5011 (ESA) analytical cell. The potentials applied were +0.10 V (conditioning cell), +0.375 V (Det 1) and –0.350 V (Det 2). The sensitivities were set at 100 nA (Det 1) and 5 nA (Det 2). In HPLC system 2, the detector (ESA Coulochem 5100A, ESA) was equipped with a 5021 conditioning cell (+0.10 V) and a 5014A analytical cell (+0.35 V, Det 1). The sensitivity was set at 15×100. The detection limits were 18 fmol for DA and DOPAC in HPLC 1 and 100 fmol for 5HT, 5HIAA and HVA in HPLC 2.

The mobile phase consisted of sodium acetate buffer (0.1 M), citric acid (0.1 M), methanol (7.5%) and sodium octyl sulphate 45 mg/l for the first HPLC method and 90 mg/l for the second. The pH of the mobile phase was adjusted to 3.6 with phosphoric acid, and the flow rate was 1.1 ml/min.

Radioligand binding studies

After decapitation, the brains were quickly dissected on ice. The cerebral cortex, both striata and mesolimbic structures were dissected (Franklin and Paxinos 1997). The brain structures from six mice were pooled, and the experiment was repeated three times. CCK receptor binding was performed in the cerebral cortex and in the striatum. The striatum and mesolimbic structures were used for studying the parameters of DA D_2 receptors, whereas the cerebral cortex was used for 5-HT_2 receptor studies. The radioligand binding studies were performed as described by Kõks et al. (1997). For labelling of DA D_2 and 5-HT_2 receptors, [^{3}H]-spiperone (specific activity 107 Ci/mmol, Amersham Radiochemicals) was used. The parameters of DA and 5-HT 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 DA D_2 receptors, was added to determine the non-specific binding at DA D_2 receptors. Ritanserin (RBI, 1 µM), an antagonist of 5-HT_2 receptors, was used to detect the non-specific binding at 5-HT_2 receptors. [Propionyl-^{3}H]-propionylated-CCK-8-sulphated ([^{3}H]-pCCK-8) (specific activity 73 Ci/mmol, Amersham Radiochemicals) was used for labelling of CCK receptors. The parameters of CCK receptors were determined in the presence of 0.05–2.4 nM [^{3}H]-pCCK-8 at 23°C for 120 min. L-365,260 (Merck Sharp & Dohme, 1 µM), an antagonist of CCK2R receptors, was added to determine the non-specific binding. The brain tissue was homogenised in 20 volumes of ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) using a Potter-S glass-teflon homogeniser (1000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48000×*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 (Kõks et al. 1997). The protein content was measured according to the method of Bradford (1976). The saturation curves of [^{3}H]-pCCK-8 and [^{3}H]spiperone binding were analysed using GraphPad Prism (Version 3.00) for Windows software.

Statistics

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

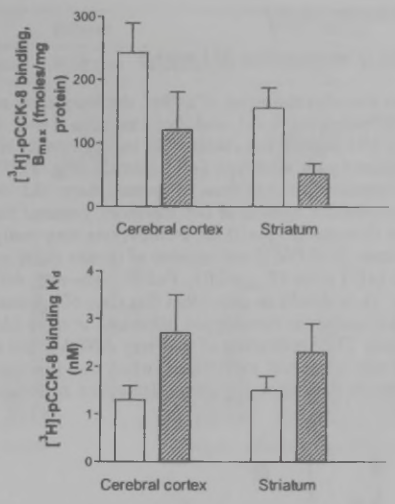

Fig. 1 The parameters of [^{3}H]-pCCK binding in the brain structures of cholecystokinin (CCK)2R receptor-deficient mice. The number of animals in each group was 18, brains of 6 mice were pooled, and the mean is a result of three experiments. *White bars* wild-type, *striped bars* heterozygous, K_d apparent dissociation constant, B_{max} apparent number of binding sites

Results

[^{3}H]-pCCK-8 binding in the brain

In wild-type mice (+/+), the density of CCK receptors was significantly higher in the cerebral cortex than the striatum (Fig. 1). In heterozygous (+/–) mutant mice, the density of CCK binding sites was lower and the affinity of these binding sites was decreased relative to their wild-type (+/+) littermates. The specific binding of [^{3}H]-pCCK-8 was not detectable in the cortex and striatum of homozygous (–/–) mutant mice.

Rotarod test

In the first rotarod experiment, the performance of heterozygous (+/–) and homozygous (–/–) CCK2R receptor-deficient mice was impaired relative to wild-type (+/+) animals ($F_{2,27}$=5.53, P<0.01, Fig. 2). However, only in the case of homozygous (–/–) mutant mice was this difference statistically significant. There was a statistically significant difference between the mutant and wild-type mice in the second experiment ($F_{2,27}$=4.15, P<0.05). On the third day, the behaviour of heterozygous (+/–) mice did not differ from that of wild-type animals, whereas the ability of homozygous (–/–) mice to stay on the rotating wheel remained poor relative to wild-type (+/+) animals ($F_{2,27}$=5.56, P<0.01).

Locomotor activity

Effect of apomorphine (0.1 mg/kg)

After the administration of saline, the locomotor activity of homozygous (–/–) and heterozygous (+/–) mutant mice was slightly but statistically insignificantly reduced compared with wild-type (+/+) animals (Fig. 3). The administration of a low dose of apomorphine (0.1 mg/kg), an unselective agonist of DA receptors, reduced the travelled distance [$F_{5,42}$=10.88, P<0.01, one-way analysis of variance (ANOVA)] and number of corner visits in wild-type (+/+) mice ($F_{5,42}$=7.63, P<0.01, one-way ANOVA) (Fig. 3). It should be noted that this dose of apomorphine did not cause the stereotyped behaviour in mice (data not shown). The application of two-way ANOVA did not reveal any statistical differences when the genotype and treatment data were compared. However, in mutant animals, the motor suppressant effect of apomorphine was stronger than in wild-type mice, since the DA agonist reduced not only the distance travelled and corner entries but also the time of locomotion ($F_{5,42}$=10.83, P<0.01, one-way ANOVA) and number of rearing ($F_{5,42}$=5.87, P<0.01, one-way ANOVA).

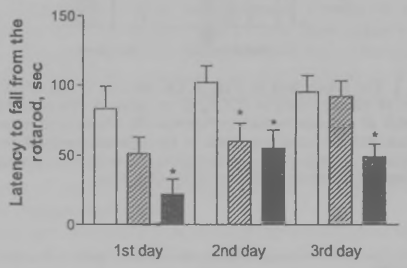

Fig. 2 The performance of cholecystokinin (CCK)2R receptor-deficient mice in the motor co-ordination test. The number of animals in each group was ten. The study was repeated on three consecutive days. *White bars* wild-type, *striped bars* heterozygous, *black bars* homozygous. *P<0.05 (compared with wild-type animals; Tukey HSD test after significant one-way analysis of variance)

Effect of amphetamine

The applying of two-way ANOVA (genotype; treatment) established the statistically significant differences when the effect of amphetamine (3 mg/kg and 6 mg/kg) was compared between wild-type (+/+) and mutant mice (time of locomotion $F_{4,117}$=5.51, P<0.01; distance travelled $F_{4,117}$=5.85, P<0.01; $F_{4,117}$=2.90, P<0.05; number of corner visits $F_{4,117}$=5.11, P<0.01). In this study, even after the treatment with saline, the locomotor activity of heterozygous (+/–) and homozygous (–/–) mutant mice was significantly lower than in wild-type (+/+) animals (Fig. 4). In heterozygous (+/–) mice, the time of locomotion and number of rearing were significantly decreased compared with wild-type (+/+) littermates; whereas, in homozygous (–/–) mice, number of rearing was reduced. The administration of amphetamine (3 mg/kg and 6 mg/kg) to wild-type mice induced a dose-dependent increase of the time of locomotion, distance travelled and number of corner visits but reduced the number of rearing. A lower dose of amphetamine (3 mg/kg) slightly, but insignificantly, increased the time of locomotion and distance travelled in heterozygous (+/–) mice, but not in homozygous (–/–) animals. The higher dose of amphetamine (6 mg/kg) caused a significant increase of the locomotor activity in mutant mice. In (+/–) heterozygous mice, the effect of amphetamine (6 mg/kg) did not differ from that in wild-type (+/+) littermates; whereas, in homozygous (–/–) mice, the higher dose of amphetamine

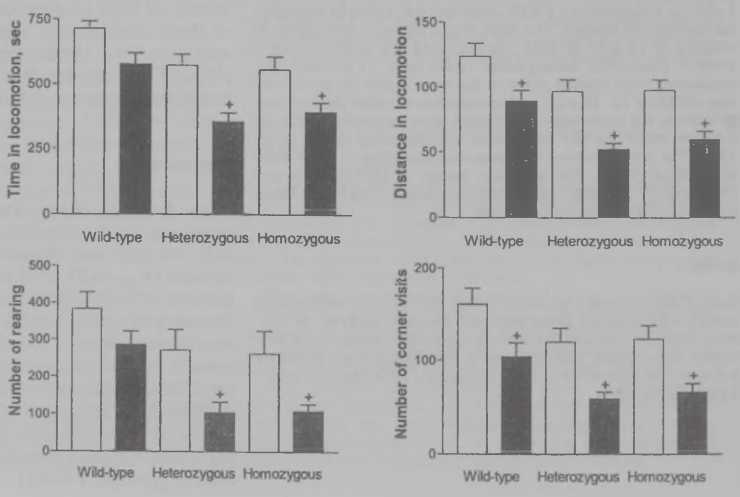

Fig. 3 Apomorphine (0.1 mg/kg) induced motor suppression in cholecystokinin (CCK)2R receptor-deficient mice. The number of animals in each group was 8. *White bars* saline, *black bars* apomorphine. +P<0.05 (compared with the respective saline-treated group; Tukey HSD test after significant one-way analysis of variance)

Table 1 The content of dopamine, serotonin and their metabolites in the brain structures of wild-type and cholecystokinin (CCK)2 receptor-deficient mice (pmol/mg wet weight tissue). The number of mice in each group was three. *HVA* homovanillic acid, *DOPAC* dihydroxyphenylacetic acid, *5-HIAA* 5-hydroxyindoleacetic acid

	Cerebral cortex		Striatum		Hypothalamus	
	Wild type	Homozygous	Wild type	Homozygous	Wild type	Homozygous
Dopamine	0.32±0.12	0.27±0.11	65.5±6.0	53.2±3.5	1.3±0.3	1.2±0.2
HVA	0.32±0.06	0.35±0.11	7.1±0.7	6.1±1.3	1.5±0.2	1.4±0.1
DOPAC	0.42±0.06	0.30±0.10	4.7±0.2	5.4±0.8	0.40±0.03	0.43±0.01
Serotonin	5.1±0.7	5.7±0.3	3.5±0.5	4.0±0.5	8.7±0.7	8.8±0.7
5-HIAA	1.5±0.1	1.6±0.1	2.8±0.2	2.8±0.3	4.5±0.3	4.4±0.2

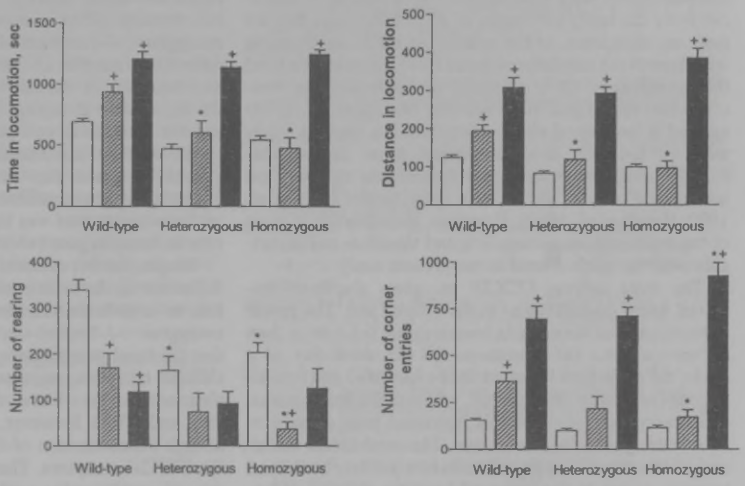

Fig. 4 Amphetamine (3–6 mg/kg) induced motor stimulation in cholecystokinin (CCK)2R receptor-deficient mice. The number of animals in each group was 14. *White bars* saline, *striped bars* amphetamine 3 mg/kg, *black bars* amphetamine 6 mg/kg. $^{+}P<0.05$ (compared with the respective saline-treated group; Tukey HSD test after two-way analysis of variance), $^{*}P<0.05$ (compared with the respective wild-type group)

caused the significantly stronger increase of distance travelled and number of corner visits.

Content of DA, 5-HT and their metabolites in the brain structures

The assay of DA, 5-HT and their metabolite levels in the cortex and hypothalamus did not reveal significant differences between wild-type (+/+) and homozygous (–/–) mice. Only in the striatum were the levels of DA and its metabolite HVA slightly lower in homozygous (–/–) animals, but this difference was not statistically significant (Table 1).

[^{3}H]-spiperone binding in the brain

The density of [^{3}H]-spiperone binding sites was significantly higher in the striatum of homozygous (–/–) mice than their wild-type (+/+) littermates (Fig. 5). Also in the mesolimbic structures, the number of [^{3}H]-spiperone binding sites was somewhat increased (not statistically significant) in the homozygous (–/–) mice. A similar trend of increased [^{3}H]-spiperone binding was apparent in the striatum of heterozygous (+/–) animals. In the cerebral cortex, we did not find any differences between the wild-type (+/+) and mutant mice.

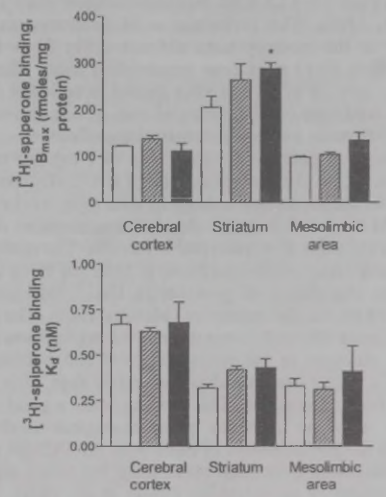

Fig. 5 The parameters of [^{3}H]-spiperone binding in the brain structures of cholecystokinin (CCK)2R receptor-deficient mice. The number of animals in each group was 18, brains of 6 mice were pooled, and mean is a result of three experiments. *White bars* wild-type, *striped bars* heterozygous, *black bars* homozygous. $^{*}P<0.05$ (compared with wild-type group, student's *t*-test)

Discussion

As expected, the mice lacking the CCK2R receptor gene (–/–) did not have a measurable specific binding of [^{3}H]-pCCK-8 in the cerebral cortex and striatum. In heterozygous mutant (+/–) mice, the density of CCK receptors was about 30–50% of the corresponding value of the wild-type (+/+) animals. In heterozygous (+/–) mice, the affinity of CCK receptors was also somewhat reduced compared with wild-type animals. This finding is different from the study of Nagata et al. (1996), who did not find any difference in the affinity of CCK receptors in wild-type (+/+) and heterozygous (+/–) animals. We used the saturation of CCK receptors by increasing the concentration of [^{3}H]pCCK-8, whereas Nagata et al. (1996) applied a method of single concentration binding study with [^{125}I]-CCK. Several studies have shown that CCK1R receptors have a wide distribution and they are not located only in the discrete brain nuclei (Hill at al. 1990; Honda et al. 1993). However, their density in most of the brain regions is very low and therefore not detectable with the method used in the present study.

The mice lacking CCK2R receptors displayed impaired motor co-ordination in the rotarod test. The motor impairment was stronger in homozygous (–/–) mice than in heterozygous (+/–) animals. On the third day of a study, the difference between wild-type (+/+) and heterozygous (+/–) mice disappeared, whereas the performance of homozygous (–/–) animals remained poor relative to their wild-type (+/+) littermates. The established differences between wild-type and mutant mice may be related to some extent to the impaired learning abilities of homozygous (–/–) CCK2R receptor-deficient mice (Sebret et al. 1999). The performance of heterozygous (+/–) mice in the memory tests did not differ from that of wild-type (+/+) mice (our unpublished data). The locomotor activity of mutant mice tended to be lower than in their wild-type (+/+) littermates and, in one experiment, this difference reached a statistical significance.

The treatment with a low dose (0.1 mg/kg) of apomorphine, an unselective agonist of DA D_1/D_2 receptors, reduces the locomotor activity in wild-type (+/+) mice. It should be noted that this dose of apomorphine did not cause any sign of stereotyped behaviour. The motor suppressant effect of apomorphine is believed to be caused by the stimulation of presynaptic DA "auto-receptors" located on the DA neurones (Meltzer 1980). The motor depressant effect of apomorphine (0.1 mg/kg) was apparently stronger in heterozygous (+/–) and homozygous (–/–) CCK2R receptor-deficient mice than their wild-type (+/+) littermates. This finding is in a good agreement with our previous pharmacological studies, in which the pre-treatment of mice with the CCK2R receptor antagonist L-365,260 potentiated the motor suppressant action of apomorphine (Vasar et al. 1991). Moreover, electrophysiological studies in rats demonstrated that the acute and long-term administration of LY262691 and related pyrazolidinone CCK2R receptor antagonists decreased the number of spontaneously active DA cells in the midbrain structures, probably via action in the nucleus accumbens and prefrontal cortex (Rasmussen et al. 1993).

The administration of amphetamine (3 mg/kg and 6 mg/kg), increasing the release of DA and noradrenaline from the presynaptic terminals (Kuczenski 1983), induced a dose-dependent motor stimulation in wild-type (+/+) mice. However, in the mutant mice, the effect of amphetamine was different. In heterozygous (+/–) mice, a lower dose of amphetamine (3 mg/kg) tended to increase the motor activity, but the higher dose (6 mg/kg) had the same effect as in wild-type (+/+) animals. In homozygous (–/–) animals, the lower dose of amphetamine reduced the number of rearing, but did not affect the other parameters of locomotor activity. The treatment with the higher dose (6 mg/kg) of amphetamine induced a significant increase in two parameters of locomotor activity: the travelled distance and number of corner entries. It should be noted that the effect of the higher dose (6 mg/kg) of amphetamine in homozygous animals (–/–) on these parameters was significantly different from that seen in heterozygous (+/–) and wild-type (+/+) animals.

Despite the behavioural changes, we did not find any difference in the content of DA, 5-HT and their metabolites in three brain structures of wild-type (+/+) and homozygous (–/–) mice. Corwin et al. (1995) have shown that the local administration of CI-988, an antagonist of CCK2R receptors, increases the levels of DA in the nucleus accumbens without changing DA metabolites (DOPAC and HVA). However, this effect was apparent only at high concentrations of CI-988, lacking specificity for the CCK2R receptors. Therefore, the increase in the action of amphetamine could be related to the increased sensitivity of the postsynaptic DA receptors in the forebrain structures. Indeed, the density of DA D_2 receptors was significantly increased in the striatum of homozygous (–/–) mice relative to their wild-type (+/+) littermates, and in the mesolimbic area a similar trend was seen. In heterozygous (+/–) mice, the density of DA D_2 receptors was somewhat elevated in the striatum, but not at all in the mesolimbic area. By contrast, the density of serotonin 5-HT_2 receptors in the cerebral cortex was not different in homozygous (–/–) and heterozygous (+/–) mice compared with wild-type (+/+) animals. The increased motor-stimulating effect of amphetamine in CCK2R receptor-deficient mice is consistent with the previous studies with CCK antagonists. The administration of CCK into the anterior nucleus accumbens inhibits DA-induced hyperlocomotion, and this effect is mediated via CCK2R receptors (Daugé et al. 1990; Crawley 1992). However, the blockade of CCK2R receptors in the nucleus accumbens increases amphetamine-induced DA release and hyperlocomotion (Altar and Boyar 1989).

In conclusion, the targeted mutation of the CCK2R receptor gene induces significant changes in the locomotor behaviour of animals. The performance of CCK2R receptor-deficient mice was impaired in the rotarod test, and their general locomotor activity tended to be reduced. Moreover, the motor suppressant action of a small dose of apomorphine was stronger in the mutant

mice, and homozygous (–/–) animals displayed the increased response to the administration of higher dose of amphetamine. A clear gene–dose effect is evident from these studies since the rotarod performance is impaired most in homozygous (–/–) mice, and the increased response to amphetamine-induced locomotor stimulation is also established in mice with the null mutation. Although we did not find the major differences in the metabolism of DA in three brain structures, the alteration in the function of the dopaminergic system seems to be related to these behavioural changes. One possible reason for negative findings could be the detection limits of postmortem HPLC analysis of "crude" brain regions and more sensitive techniques (in vivo microdialysis) for further analysis should be used. The reduced locomotor activity, impaired performance in the rotarod test and increased response to apomorphine-induced motor suppression are probably related to the increased sensitivity of the presynaptic DA receptors. The increased response to amphetamine in homozygous (–/–) animals is probably related to the increased sensitivity of the postsynaptic DA receptors, as is confirmed in the radioligand binding studies. It is therefore likely that the targeted mutation of CCK2R receptors leads to compensatory changes in the activity of the dopaminergic system.

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References

Altar CA, Boyar WC (1989) Brain CCK-B receptors mediate the suppression of dopamine release by cholecystokinin. Brain Res 483:321–326

Bradbury AJ, Costall B, Naylor RJ, Neumeyer JL (1983) Motor inhibition induced by apomorphine derivatives in the mouse. J Pharm Pharmacol 35:494–499

Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Corwin RL, Jörn A, Hardy M, Crawley JN (1995) The CCK-B antagonist Cl-988 increases dopamine levels in microdialysate from the rat nucleus accumbens via a tetrodoxin- and calcium-independent mechanisms. J Neurochem 65:208–217

Costall B, Domeney AM, Naylor RJ (1985) The continuity of dopamine receptors antagonism can dictate the long-term consequences of a mesolimbic infusion of dopamine. Neuropharmacology 24:193–198

Crawley JN (1992) Subtype-selective cholecystokinin receptor antagonists block cholecystokinin modulation of dopamine-mediated behaviors in the rat mesolimbic pathway. J Neurosci 12:3380–3391

Crawley JN, Corwin RL (1994) Biological actions of cholecystokinin. Peptides 15:741–755

Daugé V, Böhme GA, Crawley JN, Durieux C, Stutzmann JM, Feger J, Blanchard JC, Roques BP (1990) Investigation of behavioral and electrophysiological responses induced by selective stimulation of CCK-B receptors using a new highly potent CCK analog, BC 264. Synapse 6:73–80

Franklin KBJ, Paxinos G (1997) The mouse brain in stereotaxic coordinates. Academic Press, San Diego

Hill DR, Shaw TM, Graham W, Woodruff GN (1990) Autoradiographical detection of cholecystokinin-A receptors in primate brain using 125I-Bolton Hunter CCK-8 and 3H-MK-329. J Neurosci 10:1070–1081

Honda T, Wada E, Battery JF, Wank SA (1993) Differential gene expression of CCKA and CCKB receptors in the rat brain. Mol Cell Neurosci 4:143–154

Hökfelt T, Skirboll L, Rehfeld JF, Goldstein M, Markey K, Dann O (1980) A subpopulation of mesencephalic dopamine neurons projecting to limbic areas contains a cholecystokinin-like peptide: evidence from immunochemistry combined with retrograde tracing. Neuroscience 5:2093–2110

Kõks S, Vasar E, Soosaar A, Lang A, Volke V, Võikar V, Bourin M, Männistö PT (1997) Relation of exploratory behavior of rats in elevated plus-maze to brain receptor binding properties and serum growth hormone levels. Eur Neuropsychopharmacol 7:289–294

Kopin AS, Mathes WF, McBride EW, Nguyen M, Al-Haider W, Schmitz F, Bonner-Weir S, Kanarek R, Beinborn M (1999) The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. J Clin Invest 103:383–391

Kuczenski R (1983) Biochemical actions of amphetamine and other stimulants. In: Creese I (ed) Stimulants: neurochemical, behavioral and clinical perspectives. Raven Press, New York, pp 31–61

Ladurelle N, Geller G, Blommaert A, Roques BP, Daugé V (1997) The CCK-B agonist, BC 264, increases dopamine in the nucleus and facilitates motivation and attention after intraperitoneal injection in rats. Eur J Neurosci 9:1804–1814

Marshall FH, Barnes S, Hughes J, Woodruff GN, Hunter JC (1991) Cholecystokinin modulates the release of dopamine from the anterior and posterios nucleus accumbens by two different mechanisms. J Neurochem 56:917–922

Meltzer HY (1980) Relevance of dopamine autoreceptors for psychiatry. Preclinical and clinical studies. Schizophrenia Bull 3: 456–467

Nagata A, Ito M, Iwata N, Kuno J, Takano H, Minowa O, Chihara K, Matsui T, Noda T (1996) G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proc Natl Acad Sci USA 93:11825–11830

Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, Roques BP (1999) International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev 51:745–781

Rasmussen K, Czachura JF, Stockton ME, Howbert JJ (1993) Electrophysiological effects of diphenylpyrazolidinone cholecystokinin-B and cholecystokinin-A antagonists on midbrain dopamine neurons. J Pharmacol Exp Ther 264:480–488

Sebret A, Lena I, Crete D, Matsui T, Roques BP, Daugé V (1999) Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. J Neurosci 19:7230–7237

Studler JM, Reibaud M, Herve D, Blanc G, Glowinski J, Tassin JP (1986) Opposite effects of sulfated cholecystokinin on DA-sensitive adenylate cyclase in two areas of the rat nucleus accumbens. Eur J Pharmacol 126:125–128

Vasar E, Harro J, Lang A, Põld A, Soosaar A (1991) Differential involvement of CCK-A and CCK-B receptors in the regulation of locomotor activity in the mouse. Psychopharmacology 105:393–399

Vickroy TW, Bianchi BR, Kerwin JF, Kopecka H, Nazdan AM (1988) Evidence that type A CCK receptors facilitate dopamine efflux in rat brain. Eur J Pharmacol 152:371–372

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Sulev Kõks, Urho Abramov, **Alar Veraksitš**,
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CCK_2 receptor-deficient mice have increased sensitivity of dopamine D_2 receptors

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Abstract

The present study supports a role of CCK_2 receptors in the regulation of dopamine neurones. In pharmacological studies conducted on male CCK_2 receptor-deficient mice the changes in the activity of dopamine system were established. A low dose of dopamine agonist apomorphine (0.1 mg/kg), stimulating the pre-synaptic dopamine receptors, induced significantly stronger suppression of locomotor activity in mutant mice (−/−) compared to their wild-type littermates (+/+). The administration of amphetamine (3–6 mg/kg), a drug increasing dopamine release, caused a dose-dependent stimulation of locomotor activity in wild-type mice. In mice lacking CCK_2 receptors, a lower dose of amphetamine (3 mg/kg) tended to suppress the motor activity, whereas the higher dose (6 mg/kg) induced the significantly stronger motor stimulation in mutant mice. Moreover, in the CCK_2 receptor-deficient mice the affinity of dopamine D_2 receptors, but not 5-HT_2 receptors, was increased. Altogether, the targeted genetic suppression of CCK_2 receptors increased the sensitivity of pre- and post-synaptic dopamine D_2 receptors.

Keywords: CCK_2 receptor-deficient mice; Locomotor activity; 5-HT_2 receptors; Dopamine D_2 receptors; Apomorphine; Amphetamine

1. Introduction

Cholecystokinin (CCK) is a neuropeptide widely distributed in the central nervous system. CCK coexists with dopamine in some mesencephalic neurones, innervating mesolimbic and cortical region (Hökfelt et al., 1980). The interactions between dopamine and CCK are complex and depend on the route of administration of CCK, and the subtype of CCK receptor involved (Crawley and Corwin, 1994; Ladurelle et al., 1997). CCK modulates the activity of dopamine system via two distinct subtypes of CCK receptors, namely CCK_1 ("alimentary" subtype) and CCK_2 ("brain" subtype) receptors (Noble et al., 1999). Both subtypes of CCK receptors are found in the brain structures linked to the mesolimbic dopamine system: the ventral tegmental area and nucleus accumbens (Crawley and Corwin, 1994; Noble et al., 1999). The behavioural and biochemical studies have shown that CCK_1 and CCK_2 receptors mediate the opposite effects of CCK on the activity of dopamine neurones in mice and rats (Noble et al., 1999; Vasar et al., 1991). CCK increases the release of dopamine in the medial posterior nucleus accumbens by stimulating the CCK_1 receptors, whereas the stimulation of CCK_2 receptors in the anterior nucleus accumbens induces an opposite action (Crawley and Corwin, 1994). Both the subtypes of CCK receptors are cloned and targeted disruption of CCK_1 and CCK_2 receptor genes has been achieved in mice (Nagata et al., 1996; Kopin et al., 1999). The animals lacking CCK_1 receptors do not respond to the hypophagic effect of CCK, but otherwise the feeding behaviour of mice is not impaired compared to wild-type littermates (Kopin et al., 1999). The animals without CCK_2 receptors display the disturbances in the development of gastric mucosa (Nagata et al., 1996) and in the learning abilities (Sebret et al., 1999).

Taking into account a role of CCK_2 receptors in the regulation of activity of dopamine neurones (Crawley and Corwin, 1994), in the present study an attempt was done for the establishing of alterations in the dopamine

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system of CCK_2 receptor-deficient mice. It is known that the mesolimbic dopamine plays a crucial role in the regulation of locomotor activity in rodents (Bradbury et al., 1983; Costall et al., 1985). Therefore, in the present study the action of two drugs, apomorphine and amphetamine, affecting the dopamine transmission, was studied on the locomotor activity of CCK_2 receptor-deficient mice. Also, in a separate study the parameters of CCK, 5-HT_2 and dopamine D_2 receptors were measured in CCK_2 receptor-deficient and wild-type mice.

2. Materials and methods

2.1. Animals

CCK_2 receptor-deficient mice were provided from the original background 129sv/C57BL6 mice (Nagata et al., 1996). Breeding and genotype analysis have been done in the Department of Physiology, University of Tartu. Altogether, 62 male homozygous (−/−) CCK_2 receptor-deficient, 9 male heterozygous mutant (+/−) and 62 male wild-type mice (+/+) of 129sv/C57BL6 genetic background (3 months old) were used. The mice were kept in the animal house at 20 ± 2 °C in 12-h light/dark cycle (lights on at 07:00). Tap water and food pellets were available ad libitum. All animal procedures were approved by University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

2.2. Behavioural testing

The animals were brought into the experimental room 1 h before the beginning of experiment. According to the preliminary studies the behaviour of heterozygous (+/−) CCK_2 receptor-deficient mice did not differ from that in wild-type animals (+/+) (Vasar et al., 2000). Therefore, for the following studies only wild-type animals and homozygous (−/−) CCK_2 receptor-deficient mice were chosen. The behavioural effects of amphetamine (3 and 6 mg/kg), increasing release of dopamine, and apomorphine, stimulating dopamine receptors, were studied in the separate groups of animals. (±)-amphetamine (Sigma) was injected intraperitoneally 20 min and (±)-apomorphine subcutaneously (Sigma) 15 min before the beginning of an experiment. All behavioural experiments were performed between 13:00 and 19:00. In the radioligand binding studies the animals, not exposed to the behavioural testing, were used.

2.3. Motility test

For the study of locomotor activity an animal was placed singly into the photoelectric motility boxes (448 × 448 × 450 mm) connected to a computer (TSE Technical and Scientific Equipment GMBH, Germany). The illumination level of transparent test boxes was ~750 lux. Time in locomotion (in seconds), distance of locomotion (in metres), number and time of rearing (in seconds), and number of corner entries were registered during the 15-min observation period.

2.4. Radioligand binding studies

For the radioligand binding studies we used 18 male homozygous (−/−) CCK_2 receptor-deficient mice, 9 male heterozygous (+/−) mutant and 18 male wild-type (+/+) animals. After decapitation the brains were quickly dissected on ice. The cerebral cortex and sub-cortical structures (involving the striatum, mesolimbic structures and septum pellucidum) were dissected. The brain structures from three mice were pooled and the experiment was repeated three times. CCK receptor binding study was performed in the cerebral cortex and sub-cortical structures. The sub-cortical structures were used for studying of parameters of dopamine D_2 receptors, whereas in the cerebral cortex 5-hydroxytryptamine 5-HT_2 receptors were studied. The radioligand binding studies were performed according to Kõks et al. (1997). For labeling of dopamine D_2 and 5-HT_2 receptors [^{3}H]spiperone (specific activity 107 Ci/mmol, Amersham Radiochemicals) was used. The parameters of dopamine and 5-hydroxytryptamine 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 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. [Propionyl-^{3}H]propionylated-CCK-8-sulphated-([^{3}H]pCCK-8) (specific activity 73 Ci/mmol, Amersham Radiochemicals) was used for labelling of CCK receptors. The parameters of CCK receptors were determined in the presence of 0.05–2.4 nM of [^{3}H]pCCK-8 at 23EC for 120 min. L-365,260 (Merck Sharp and Dohme, 1 μM), an antagonist of CCK_2 receptors, was added to determine the non-specific binding. The brain tissue was homogenized in 20 volumes of ice-cold 50 mM Tris–HCl (pH 7.4 at 4 °C) using a Potter-S glass-Teflon homogenizer (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]pCCK-8 and [^{3}H]spiperone binding were analysed using GraphPad Prism (Version 3.00) for Windows software.

2.5. Statistics

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

3. Results

3.1. The increased affinity of D_2 receptors in CCK_2 receptor-deficient mice

In wild-type mice (+/+) the density of CCK receptors was significantly higher in the cerebral cortex (B_{max} 242 ± 47 fmol/mg protein, K_d 1.3 ± 0.3 nM) compared to the sub-cortical structures (B_{max} 125 ± 40 fmol/mg protein, K_d 1.5 ± 0.3 nM). In heterozygous (+/−) mutant mice the density of CCK binding sites was significantly lower, but again it was higher in the cerebral cortex (B_{max} 120 ± 60 fmol/mg protein, K_d 2.7 ± 0.8 nM) than in the sub-cortical structures (B_{max} 37 ± 24 fmol/mg protein, K_d 2.3 ± 1.3 nM). The specific binding of [^{3}H]pCCK-8 was not detectable in the cortical and sub-cortical regions of homozygous (−/−) mutant mice. The experiments with [^{3}H]spiperone also revealed the differences between mutant (−/−) and wild-type (+/+) animals (Table 1). Namely, in the sub-cortical structures, but not in the cerebral cortex, the affinity of [^{3}H]spiperone binding sites was increased (Student's *t* test $p < 0.01$) in mutant (−/−) mice.

3.2. Apomorphine suppresses motor activity more in CCK_2 receptor-deficient mice

The administration of low dose of apomorphine (0.1 mg/kg), an unselective agonist of dopamine receptors, induced the suppression of locomotor activity in wild-type (+/+) mice ($F_{3,36} = 63.5$, $p < 0.01$ [time in locomotion]; $F_{3,36} = 67.4$ $p < 0.01$ [distance in locomotion]; $F_{3,36} = 51.0$, $p < 0.01$ [number of rearing]; $F_{3,36} = 37.7$, $p < 0.01$ [time of rearing]; $F_{3,36} = 63.5$, $p < 0.01$ [number of corner entries]) (Fig. 1). The similar, but obviously stronger, inhibition of locomotor activity was established in mice lacking CCK_2 receptors (−/−). The application of two-way ANOVA (genotype × treatment) established the difference in the action of apomorphine in wild-type (+/+) and mutant mice (−/−) (Fig. 1), if the number of rearing ($F_{1,36} = 5.57$, $p < 0.05$) and time of rearing ($F_{1,36} = 7.59$, $p < 0.01$) were analysed.

3.3. The action of amphetamine is different in wild-type and CCK_2 receptor-deficient mice

The administration of amphetamine (3 and 6 mg/kg), increasing the release of dopamine, induced a dose-dependent increase in the motor activity of wild-type mice (+/+) ($F_{5,78} = 62.7$, $p < 0.01$ [time in locomotion]; $F_{5,78} = 92.0$, $p < 0.01$ [distance in locomotion]; $F_{5,78} = 9.7$, $p < 0.01$ [number of rearing]; $F_{5,78} = 11.1$, $p < 0.01$ [time of rearing]; $F_{5,78} = 70.3$, $p < 0.01$ [number of corner entries]) (Fig. 2). The application of two-way ANOVA (genotype × treatment) established that the action of amphetamine was different in wild-type (+/+) and mutant mice (−/−) ($F_{2,78} = 19.0$, $p < 0.01$ [time in locomotion]; $F_{2,78} = 31.4$, $p > 0.01$ [distance of locomotion]; $F_{2,78} = 19.0$, $p < 0.01$ [number of corner entries]). Three mg/kg of amphetamine induced a significant increase in the motor activity in wild-type mice (+/+), whereas in mutant animals (−/−) it reduced the number of rearing (Fig. 2). By contrast, the higher dose of (±)-amphetamine (6 mg/kg) caused the stronger motor stimulation in mice, lacking CCK_2 receptors, compared to their wild-type littermates.

4. Discussion

As it was expected, the animals, lacking CCK_2 receptor gene (−/−), did not have a specific binding of [^{3}H]pCCK-8 in the cerebral cortex and sub-cortical structures. In heterozygous mutant (+/−) mice the density of CCK receptors was about 30–50% of the corresponding value in wild-type (+/+) animals. Since we have not been able to establish the measurable differences in the behavioural reactions in heterozygous (+/−) and wild-type (+/+) mice (Vasar et al., 2000), the animals, lacking one CCK_2 receptor allele, are not included to the present study. According to the performed behavioural studies a response of CCK_2 receptor-defi-

Table 1
[^{3}H]Spiperone binding in the sub-cortical structures and cerebral cortex of CCK_2 receptor-deficient mice

	Sub-cortical structures (D_2)		Cerebral cortex (5-HT_2)	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Wild-type mice	0.16 ± 0.02	243 ± 15	0.33 ± 0.08	159 ± 15
CCK_2 receptor-deficient mice	0.08 ± 0.01*	235 ± 16	0.28 ± 0.06	145 ± 13

*$p < 0.01$ (compared to wild-type mice, Student's *t* test).

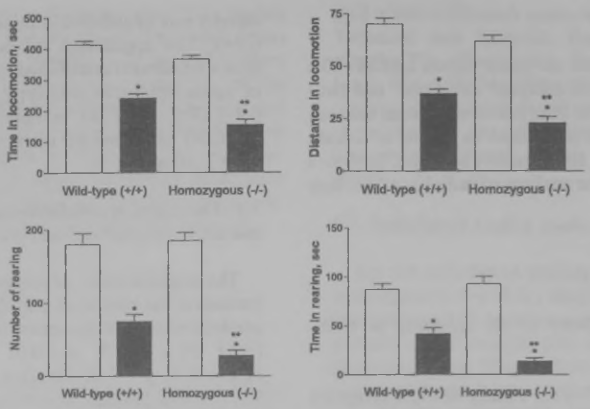

Fig. 1. The effect of apomorphine (0.1 mg/kg) on the locomotor activity of wild-type and CCK_2 receptor-deficient mice. The number of animals in each group was 10. White bars – saline treatment; black bars – apomorphine treatment. $*p < 0.05$ (compared to the corresponding saline-treated group, Scheffe test after the significant one-way ANOVA); $**p < 0.05$ (compared to apomorphine-treated wild-type mice).

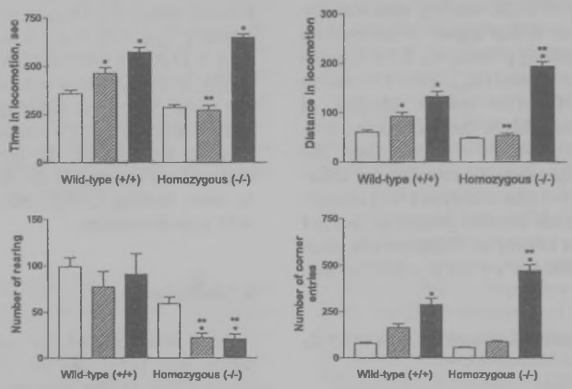

Fig. 2. The effect of amphetamine (3 and 6 mg/kg) on the locomotor activity of wild-type and CCK_2 receptor-deficient mice. The number of animals in each group was 14. White bars – saline treatment; striped bars – amphetamine (3 mg/kg); black bars – amphetamine (6 mg/kg). $*p < 0.05$ (compared to the corresponding saline-treated group, Scheffe test after the significant one-way ANOVA); $**p < 0.05$ (compared to amphetamine-treated wild-type group).

cient mice to the administration of dopamine agonists, amphetamine and apomorphine, is significantly altered. Treatment with a low dose (0.1 mg/kg) of apomorphine, an unselective agonist of dopamine receptors, reduces the locomotor activity in wild-type mice. This effect is linked to the stimulation of dopamine "auto-receptors" located on the dopamine neurones (Meltzer, 1980). However, the motor suppressant action of apomorphine is apparently stronger in mice lacking CCK_2 receptors. This finding is in a good agreement with our previous studies, where the pre-treatment with CCK_2 receptor antagonist L-365,260 potentiates the motor suppressant action of apomorphine in male mice (Vasar et al., 1991). Electrophysiological studies in rats demonstrated that the acute and long-term administration of LY262691 and related pyrazolidinone CCK_2 antagonists decreased the number of spontaneously active dopamine cells in the midbrain structures, probably via action in the nucleus accumbens and prefrontal cortex (Rasmussen et al., 1993). The other dopamine agonist amphetamine increases the release of dopamine from the pre-synaptic terminals (Kuczenski, 1983), and due to that stimulates the pre- and post-synaptic dopamine receptors. The administration of amphetamine (3–6 mg/kg) induces a dose-dependent locomotor stimulation in wild-type mice. However, in mice lacking CCK_2 receptors a lower

dose (3 mg/kg) of amphetamine reduces the number of rearing, whereas the higher dose (6 mg/kg) causes significantly stronger locomotor stimulation compared to wild-type animals. The reduced effect of lower dose of amphetamine could be related to the increased sensitivity of dopamine "auto-receptors" in mutant (–/–) mice established in the studies with apomorphine, whereas the increased effect of higher dose of amphetamine seems to be related to the increased sensitivity of post-synaptic dopamine receptors. Indeed, the affinity of dopamine D_2 receptors labelled by [^{3}H]spiperone is significantly increased in the forebrain sub-cortical regions of CCK_2 receptor-deficient mice. This finding is in good accordance with the experiments of Ferraro et al. (1996) showing that the application of CCK-8 to the striatal membranes reduces the affinity of dopamine D_2 receptors and this effect is counteracted by the CCK_2 receptor antagonist PD134308. By contrast, the affinity of 5-HT_2 receptors is not affected in male mice lacking CCK_2 receptors. The increased motor stimulating effect of amphetamine in the CCK_2 receptor-deficient mice is consistent with the previous studies. The administration of CCK into the anterior nucleus accumbens inhibits dopamine-induced hyper-locomotion and this effect is mediated via CCK_2 receptors (Daugé et al., 1990; Crawley, 1992). On the other hand, the blockade of CCK_2 receptors in the nucleus accumbens increases amphetamine-induced dopamine release and hyper-locomotion.

In conclusion, the targeted mutation of CCK_2 receptor gene induces the significant changes in the activity of dopamine system. CCK_2 receptor-deficient mice have the increased sensitivity of pre- and post-synaptic dopamine D_2 receptors. However, the molecular background of this change is unclear and remains to be established.

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References

Bradbury, A.J., Costall, B., Naylor, R.J., Neumeyer, J.L., 1983. Motor inhibition induced by apomorphine derivatives in the mouse. J. Pharm. Pharmacol. 35, 494–499.

Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Costall, B., Domeney, A.M., Naylor, R.J., 1985. The continuity of dopamine receptors antagonism can dictate the long-term consequences of a mesolimbic infusion of dopamine. Neuropharmacology 24, 193–198.

Crawley, J.N., 1992. Subtype-selective cholecystokinin receptor antagonists block cholecystokinin modulation of dopamine-mediated behaviors in the rat mesolimbic pathway. J. Neurosci. 12, 3380–3391.

Crawley, J.N., Corwin, R.L., 1994. Biological actions of cholecystokinin. Peptides 15, 741–755.

Daugé, V., Böhme, G.A., Crawley, J.N., Durieux, C., Stutzmann, J.M., Feger, J., Blanchard, J.C., Roques, B.P., 1990. Investigation of behavioral and electrophysiological responses induced by selective stimulation of CCK-B receptors using a new highly potent CCK analog, BC 264. Synapse 6, 73–80.

Ferraro, L., O'Connor, W.T., Li, X.M., Rimondini, R., Beani, L., Ungerstedt, U., Fuxe, K., Tanganelli, S., 1996. Evidence for a differential cholecystokinin-B and -A receptor regulation of GABA release in the rat nucleus accumbens mediated via dopaminergic and cholinergic mechanisms. Neuroscience 73, 941–950.

Hökfelt, T., Skirboll, L., Rehfeld, J.F., Goldstein, M., Markey, K., Dann, O., 1980. A subpopulation of mesencephalic dopamine neurons projecting to limbic areas contains a cholecystokinin-like peptide: evidence from immunochemistry combined with retrograde tracing. Neuroscience 5, 2093–2110.

Kopin, A.S., Mathes, W.F., McBride, E.W., Nguyen, M., Al-Haider, W., Schmitz, F., Bonner-Weir, S., Kanarek, R., Beinborn, M., 1999. The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. J. Clin. Invest. 103, 383–391.

Kuczenski, R., 1983. Biochemical actions of amphetamine and other stimulants. In: Creese, I. (Ed.), Stimulants: Neurochemical, Behavioral and Clinical Perspectives. Raven Press, New York, pp. 31–61.

Kõks, S., Vasar, E., Soosaar, A., Lang, A., Volke, V., Võikar, V., Bourin, M., Mönnistö, P.T., 1997. Relation of exploratory behavior of rats in elevated plus-maze to brain receptor binding properties and serum growth hormone levels. Eur. Neuropsychopharmacol. 7, 289–294.

Ladurelle, N., Geller, G., Blommaert, A., Roques, B.P., Daugé, V., 1997. The CCK-B agonist, BC 264, increases dopamine in the nucleus and facilitates motivation and attention after intraperitoneal injection in rats. Eur. J. Neurosci. 9, 1804–1814.

Meltzer, H.Y., 1980. Relevance of dopamine autoreceptors for psychiatry. Preclinical and clinical studies. Schizophrenia Bull. 3, 456–467.

Nagata, A., Ito, M., Iwata, N., Kuno, J., Takano, H., Minowa, O., Chihara, K., Matsui, T., Noda, T., 1996. G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proc. Natl. Acad. Sci. 93, 11825–11830.

Noble, F., Wank, S.A., Crawley, J.N., Bradwejn, J., Seroogy, K.B., Hamon, M., Roques, B.P., 1999. International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol. Rev. 51, 745–781.

Rasmussen, K., Czachura, J.F., Stockton, M.E., Howbert, J.J., 1993. Electrophysiological effects of diphenylpyrazolidinone cholecystokinin-B and cholecystokinin-A antagonists on midbrain dopamine neurons. J. Pharmacol. Exp. Ther. 264, 480–488.

Sebret, A., Lena, I., Crete, D., Matsui, T., Roques, B.P., Daugé, V., 1999. Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. J. Neurosci. 19, 7230–7237.

Vasar, E., Harro, J., Lang, A., Põld, A., Soosaar, A., 1991. Differential involvement of CCK-A and CCK-B receptors in the regulation of locomotor activity in the mouse. Psychopharmacology 105, 393–399.

Vasar, E., Kõks, S., Beljajev, S., Abramov, U., Koovit, I., Matsui, T., 2000. CCK_B receptor knockout mice: gender related behavioural differences. Eur. Neuropsychopharmacol. 10 (Suppl. 2), S69.

III

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Altered pain sensitivity and morphine-induced anti-nociception in mice lacking CCK_2 receptors

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Abstract *Rationale:* Cholecystokinin (CCK) interacts with the endopioid system in the regulation of various physiological functions, including the control of pain sensitivity, motor activity and emotional behaviour. *Objective:* The aim of the present work was to study the pain sensitivity, morphine-induced antinociception and density of opioid receptors in mice lacking CCK_2 receptors. *Methods:* Plantar analgesia and hotplate tests were used to evaluate pain sensitivity and morphine-induced antinociception. The parameters of opioid receptors were analysed by using [^{3}H]-diprenorphine binding. *Results:* In the plantar analgesia test the latency of hind paw withdrawal was significantly increased in CCK_2 receptor deficient mice compared to wild-type (+/+) littermates. The treatment with saline reversed the reduced pain sensitivity in heterozygous (+/–) and homozygous (–/–) mice. The administration of morphine (1 mg/kg) induced a significantly stronger antinociceptive effect in homozygous (–/–) mice compared with wild-type (+/+) animals. In the hotplate test, only homozygous (–/–) mutant mice displayed the delayed latency of hind paw licking/shaking in comparison with wild-type (+/+) mice. The injection of saline and isolation of mice for 30 min reversed the delayed response in homozygous (–/–) mice. However, in this test, the anti-nociceptive action of morphine (5–10 mg/kg) in mutant mice did not differ from that in wild-type (+/+) littermates. By contrast, the jump latency was decreased in both homozygous (–/–) and heterozygous (+/–) mice in the hotplate test. The increased density of opioid receptors was established in the striatum of homozygous (–/–) mice. *Conclusion:* It is apparent that the targeted mutagenesis of the CCK_2 receptor gene has different effects on the sensitivity of opioid receptors in various brain structures. This is a probable reason for the altered pain sensitivity and morphine-induced antinociception in mutant mice compared to wild-type (+/+) littermates.

Keywords Targeted mutagenesis · CCK · receptor · Wild-type · Heterozygous · Homozygous · μ-Opioid receptors · Pain sensitivity · Plantar analgesia test · Hotplate test · Mouse

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Introduction

Cholecystokinin (CCK) is generally believed to be the most widespread and abundant neuropeptide in the brain (Noble et al. 1999). High levels of CCK and preproCCK mRNA have been identified throughout the brain, including the cerebral cortex, olfactory bulb, olfactory tubercle, hippocampus, basal ganglia, hypothalamus and periaquaductal gray (Savasta et al. 1988; Vanderhaeghen and Schiffmann 1992; Shlik et al. 1997). The effects of CCK are mediated by two distinct receptor subtypes (CCK_1 and CCK_2) that have been cloned and functionally expressed (Noble et al. 1999). Binding and autoradiography studies have detected CCK_1 receptors in high concentration throughout the gastrointestinal tract and in a few discrete brain regions such as the area postrema, nucleus of the solitary tract, interpeduncular nucleus, dorsal raphe, nucleus accumbens, substantia nigra and ventral tegmental area (Roques and Noble 1998). CCK_2 receptors are mainly distributed in the brain, with the highest concentrations in the striatum, cerebral cortex and limbic system, but they can also be found in the stomach (Noble et al. 1999). Various studies support the antagonistic interaction between two neuropeptide systems,

CCK and endopioid peptides, in the regulation of physiological functions, including the control of pain sensitivity, motor activity and emotional behaviour. The administration of CCK attenuates, whereas the selective CCK_2 receptor antagonists enhance, morphine-induced antinociception in rodents (Faris et al. 1983; Lavigne et al. 1992; Noble et al. 1995). The pretreatment of mice with the CCK_2 receptor oligonucleotide antisense significantly increases morphine-induced antinociception (Vanderah et al. 1994). On the other hand, the CCK_2 receptor antagonists can also facilitate the rewarding properties of morphine in the conditioned place preference test (Valverde et al. 1996). Moreover, a morphine-induced increase in locomotor activity can be antagonised by the pretreatment with CCK (Schnur et al. 1991). These findings suggest that CCK may act, via CCK_2 receptors, as an endogenous anti-opioid peptide. It is also noteworthy that the distribution of both CCK and CCK_2 receptors parallels with that of endopioid peptides and opioid receptors in the brain (Saito et al. 1980; Gall et al. 1987; Ghilardi et al. 1992).

Recently targeted disruption of CCK_2 receptor genes has been achieved in mice (Nagata et al. 1996). The animals without CCK_2 receptors display disturbances in the development of gastric mucosa (Nagata et al. 1996) and in the learning abilities (Sebret et al. 1999). The activity of the dopaminergic system is also affected in mice with corrupted function of CCK_2 receptors (Daugé et al. 2001; Kõks et al. 2001). Pommier et al. (2002) have shown that mice without CCK_2 receptors display hyperalgesia and a reduced response to morphine-induced analgesia in the hot plate test. They have applied the jumping behaviour as a sign of nociceptive response in mice. This is in an obvious contradiction with the data of the above-cited pharmacological studies, where the CCK_2 receptor antagonists potentiate morphine-induced analgesia (Faris et al. 1983; Lavigne et al. 1992; Noble et al. 1995). However, according to various authors, the jumping response is not the true threshold of pain sensitivity, but rather reflects tolerance to pain in the hotplate test (Perissin et al. 2000; Wilson and Mogil 2001). Therefore, the present study focused on pain sensitivity in the plantar analgesia and hotplate test, using the hind limb withdrawal and licking/shaking of a hind paw as thresholds of nociception. The jump latency was used as a measure of pain tolerance. The changes in morphine-induced antinociception were also evaluated in mice without CCK_2 receptors. Additionally, the density of opioid receptors was studied in the forebrain structures of mutant and wild-type mice.

Materials and methods

Animals

CCK_2 receptor-deficient 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 at the Department of Physiology, University of Tartu. Genotyping was carried out by means of polymerase chain reaction (PCR) using two pairs of primers HE2F (TGG AGT TGA CCA TTC GAA TCA C) and LacZrev (GTG CTG CAA GGC GAT TAA GTT G) were designed to detect the mutant allele, and HE3F (TAT CAG TGA GTG TGT CCA CTC T) and HE3R (ACA TTT GTT GGA CAC GTT CAC) were designed for the wild-type allele. For PCR, we used the following protocol: 96°C for 10 min (initial denaturation); 96°C for 50 s, 60°C for 50 s and 72°C for 2 min (25 cycles); and 72°C for 10 min (final amplification). PCR products were stored at 4°C until electrophoresis. Mutant mice were back-crossed six times to the C57/BL6 background to minimize the possible genetic effects from the 129sv strain. The mice were kept in the animal house at 20±2°C under a 12/12-h light/dark cycle (lights on at 0700 hours). Tap water and food pellets were available ad libitum. The male mice (3 months old) were used throughout the experiments and animals were used only once. Altogether, 377 homozygous (–/–) CCK_2 receptor-deficient, 374 heterozygous mutant (+/–) and 355 wild-type (+/+) mice were used in the behavioural and radioligand binding studies. All animal procedures were approved by the University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

Plantar analgesia test

The plantar analgesia test was performed according to the method of Hargreaves et al. (1988). The instrument for the measurement of plantar analgesia consists of a movable infrared generator, platform, mouse enclosure assembly and controller (TSE Technical & Scientific Equipment GMBH, Germany). In order to avoid the confounding effects of novelty and handling stress, prior handling of mice and habituation to the test equipment was performed. The animals were adapted for three consecutive days to the equipment, a 60-min session each day. Before the experiment a sufficient time (30 min) for exploration of the equipment, without presentation of stimulus, was allowed to each mice. After the adaptation period, the animals stayed quietly in a resting position, with occasional bouts of grooming, allowing to direct a beam of infrared light (intensity equal to 50°C) to the skin of a hind paw. The equipment measured latency to withdraw the hind paw automatically. The stimulation of the hind paw was repeated up to four times and the medium latency was calculated. At least a 2-min interval was kept between two measurements. Saline and morphine (1 mg/kg IP) were administered 15 min before the test. The morphine dose was chosen according to the pilot studies where the lower doses (0.05–0.5 mg/kg IP) of morphine did not cause a statistically significant effect in wild-type animals.

Hotplate test

An animal was placed on a surface (30×50 cm) maintained at 52±0.3°C and a glass funnel (diameter 15 cm, height 12.5 cm) was used to restrict the movements of the animal. The time when a mouse began to lick or shake its hind paw was noted as the pain threshold in seconds. Animals not exhibiting such aversive behaviour were removed at 40 s from the hotplate. Morphine (5; 7.5; 10 mg/kg IP) and saline were administered 15 min before the test. The effect of saline was also studied 90 min after the administration. In the isolation experiment the animals were taken from the home cage and placed singly into boxes for 30 min. The hotplate test was performed after the isolation period. In one experiment, the animals were divided into two groups. One group was treated with the repeated injections of saline (once daily) for 10 days in the experimental room, whereas the other half did not receive any manipulations and was kept in the animal facility. Twenty-four hours after the last injection of saline, both groups of animals were subjected to the hotplate test.

The other experiment measured the latency to jump. A 2-min cut-off time was chosen according to our pilot studies, where we noticed that the majority of wild-type and CCK_2 receptor-deficient mice responded during that period. After the experiment mice, when released to the home cage, did not show any behaviour to conclude that they had been damaged in any way by the noxious exposure. All pain measurements were carried out between 1100 and 1800 hours.

Radioligand binding studies

After decapitation the brains were quickly dissected on ice. The cerebral cortex (including the frontal and parietal cortices), striata, mesolimbic structures and hippocampus were dissected (Franklin and Paxinos 1997). The brain structures from six mice were pooled. The radioligand binding studies were performed according to the method of Kõks et al. (1997). For the labelling of opioid receptors [^{3}H]-diprenorphine (specific activity 58.0 Ci/mmol; Amersham Radiochemicals) was used. The parameters of opioid receptors were determined in the presence of 0.05–2.5 nM [^{3}H]-diprenorphine at 23°C for 60 min. Naloxone hydrochloride (RBI, 1 μM), an antagonist of opioid receptors, was added to determine the non-specific binding at opioid receptors. The brain tissue was homogenized in 20 vol ice-cold 50 mM Tris-HCl (pH 7.4 at 4° C) using a Potter-S glass-teflon homogenizer (1000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48,000 *g* for 20 min) and re-suspension. After the last centrifugation, the crude brain membranes were suspended in the incubation buffer (50 mM Tris-HCl, pH 7.4 at 4°C) for the binding assay. The protein content was measured according to the method of Bradford (1976). The saturation curves of [^{3}H]-diprenorphine binding were analysed using GraphPad Prism (Version 3.00) for Windows software. The experiment was repeated four times.

Statistics

Results are expressed as mean±SEM. The results were analysed by using one- and two-way analyses of variance (ANOVA). Post hoc comparisons between the individual groups were performed by means of the Tukey HSD test. The Statistica for Windows software was used.

Results

Plantar analgesia test

The testing of heterozygous (+/–) and homozygous (–/–) mice in the plantar analgesia test established a significantly increased hind paw withdrawal latency in these mice compared to wild-type (+/+) littermates [$F(2,135)$= 28,59, $P>0.01$, Fig. 1A]. The latency was, on average, twice longer in both homozygous (–/–) and heterozygous (+/–) animals compared with wild-type (+/+) littermates. The pretreatment of mice with saline induced a decrease of nociceptive thresholds in mice [two-way ANOVA: treatment, $F(1,42)=22.1$, $P>0.01$; genotype $F(2,42)=8.21$, $P>0.05$; genotype x treatment, $F(2,42)=1.99$, $P>0.1$; Fig. 1B]. However, the subsequent statistical analysis established a significant reduction only in mutant mice (Tukey HSD test: wild-type (+/+) $P>0.1$; heterozygous (+/–) $P>0.01$; homozygous (–/–) $P>0.01$). The administration of morphine (1 mg/kg), a μ-opioid receptor agonist, induced a significant decrease of pain sensitivity in wild-type (+/+) mice compared to the injection of saline. However, the antinociceptive effect of morphine (1 mg/kg) was significantly stronger in homozygous (–/–) CCK2 receptor deficient mice compared to heterozygous (–/+) and wild-type (+/+) mice [two-way ANOVA: treatment $F(1,42)=38,8$, $P>0.01$; genotype $F(2,42)=5.65$, $P>0.01$; genotype x treatment, $F(2,42)= 3.32$, $P>0.05$, Fig. 1C].

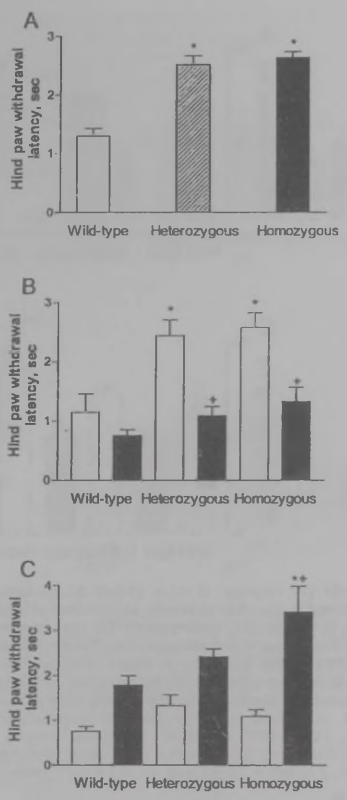


Fig. 1A–C The response of mice without CCK_2 receptors in the plantar analgesia test. A Baseline response. n=46 for each group. *P <0.01 (compared with wild-type mice, Tukey HSD test after the significant one-way ANOVA). B Effect of saline. n=8 for each group. *White bars* baseline, *black bars* saline treatment. *P<0.05 (compared with the baseline response of wild-type mice, Tukey HSD test after the significant one-way ANOVA); +P<0.05 (compared with the baseline response of heterozygous and homozygous mice). C Effect of morphine (1 mg/kg). n=8 for each group. *White bars* saline treatment, *black bars* morphine (1 mg/kg IP), *P<0.05 (compared with morphine-treated wild-type mice, Tukey HSD test after the significant two-way ANOVA), +P<0.05 (compared with saline-treated homozygous mice)

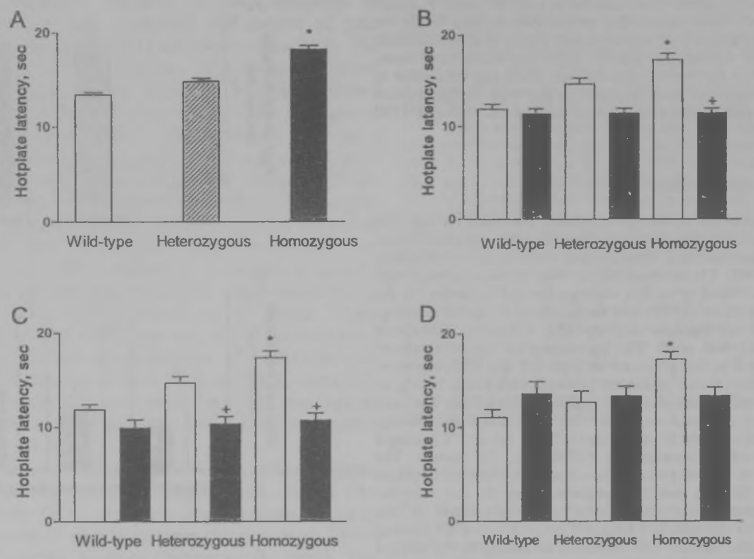


Fig. 2A–D The response of mice without CCK_2 receptors in the hot plate test. **A** Baseline response. The number of animals was as follows: wild-type 118, heterozygous 131 and homozygous 139. **P*<0.01 (compared with wild-type mice, Tukey HSD test after the significant one-way ANOVA). **B** Effect of saline. The number of animals as follows: wild-type 58, heterozygous 62 and homozygous 60. *White bars* baseline response, *black bars* saline treatment. **P*<0.05 (compared with the baseline response of wild-type animals, Tukey HSD test after significant two-way ANOVA), +*P*<0.05 (compared with baseline response of homozygous mice). **C** Effect of isolation for 30 min. *n*=24 for each group. *White bars* animals kept in the home cage, *black bars* isolated mice. **P*<0.05 (compared with the baseline response of wild-type animals, Tukey HSD test after significant two-way ANOVA), +*P*<0.05 (compared with baseline response of heterozygous and homozygous mice). **D** Effect of repeated treatment with saline. Saline was administered once daily for 10 days and the experiment was performed 24 h after the last injection of saline. *n*=10 for each group. *White bars* untreated mice, *black bars* repeated treatment with saline. **P*<0.05 (compared with the untreated wild-type animals, Tukey HSD test after significant two-way ANOVA)

Hotplate test

In the hotplate test homozygous (–/–) mice also displayed a delayed hind paw licking/shaking time compared to wild-type (+/+) littermates [one-way ANOVA, $F(2,385)=55{,}44$ $P>0.01$, Fig. 2A]. The pretreatment of mice with saline reversed this phenomenon in mutant mice if a measurement was performed 15 min after the injection [two-way ANOVA: genotype $F(2,174)=10.58$, $P>0.05$, treatment $F(1,174)=38.93$, $P>0.01$; genotype x treatment, $F(2,174)=9.95$, $P>0.01$, Fig. 2B]. However, the testing of mice conducted 90 min after the treatment with saline did not reveal any differences between the saline-treated and untreated homozygous (–/–) mice (data not shown). Also, isolation of mice for 30 min attenuated the reduced nociception in homozygous (–/–) mice [two-way ANOVA: genotype $F(2,132)=9.10$, $P>0.01$; isolation $F(1,132)=50.74$, $P>0.01$; genotype x isolation, $F(2,132)=5.00$, $P>0.01$, Fig. 2C]. The experiment performed 24 h after the last injection of saline (once daily for 10 days) demonstrated that this manipulation abolished the reduced nociception in homozygous (–/–) mice [two-way ANOVA: genotype $F(2,54)=4.68$, $P>0.05$; treatment $F(1,54)=0.08$, $P>0.5$; genotype x treatment, $F(2,54)=5.23$, $P>0.01$, Fig. 2D]. Differently from the plantar test, a significantly higher dose of morphine (7.5 mg/kg) was necessary to induce a significant antinociceptive effect. However, the antinociceptive effect of morphine (5–10 mg/kg) did not differ in mutant and wild-type (+/+) mice [two-way ANOVA: genotype $F(1,168)=1.04$, $P>0.35$; treatment $F(3,168)=88.4$, $P>0.01$; genotype x treatment, $F(6,168)=0.49$, $P>0.8$, Fig. 3]. If the jumping latency was used as a measure of nociception, then the results were totally opposite to those established by the measuring of hind paw licking/shaking response. Namely, the jump latency was significantly longer in wild-type (+/+) mice compared to heterozygous (–/+) and homozygous (–/–) animals [one-way ANOVA, $F(2,24)=6.80$, $P>0.01$, Fig. 4].

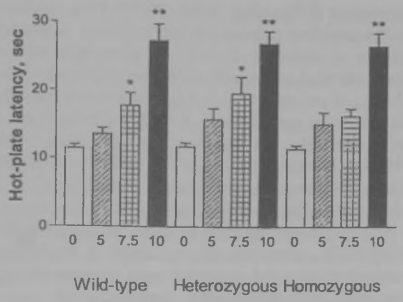


Fig. 3 The effect of morphine (5–10 mg/kg) on the response of mice without CCK_2 receptors in the hot plate test. n=15 for each group. *White bars* saline, *striped bars* morphine 5 mg/kg, *checked bars* morphine 7.5 mg/kg and *black bars* morphine 10 mg/kg. *P<0.05; **P<0.01 (compared with the respective saline-treated group, Tukey HSD test after significant one-way ANOVA)

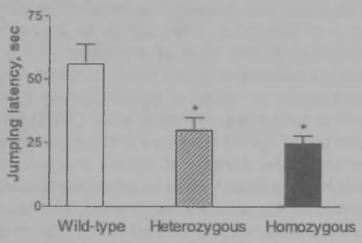
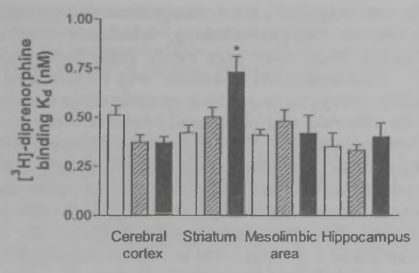


Fig. 4 The jump latency of mice without CCK_2 receptors in the hot plate test. The numbers of animals were as follows: wild-type 18, heterozygous 22 and homozygous 18. *P<0.01 (compared with wild-type mice, Tukey HSD test after significant one-way ANOVA)



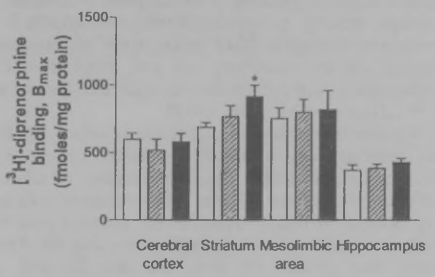


Fig. 5 The parameters of [^{3}H]-diprenorphine binding in the brain structures of mice without CCK_2 receptors. n=24 for each group. The brains of six mice were pooled, and the mean is a result of four experiments. *White bars* wild type, *striped bars* heterozygous, *black bars* homozygous. *P<0.05 (compared with wild-type group, Tukey HSD test after significant one-way ANOVA)

Radioligand binding studies

The radioligand binding studies with [^{3}H]-diprenorphine demonstrated that the affinity of opioid binding sites in the cerebral cortex of mutant mice tended to be increased compared to wild-type (+/+) littermates [F(2,6)=4.59, P=0.062] (Fig. 5). However, a decreased affinity [F(2,6)=6.82, P>0.05] and increased density [F(2,6)= 5.82, P>0.05] of opioid binding sites was established in the striatum of homozygous (–/–) mice. The parameters of [^{3}H]-diprenorphine binding were not affected in the mesolimbic structures and hippocampus.

Discussion

A major finding of the current study is that the nociceptive response of mice, lacking CCK_2 receptors, depends on the paradigm used for the measurement of pain sensitivity. In the plantar analgesia test pain sensitivity of heterozygous (+/–) and homozygous (–/–) mice is significantly reduced compared to wild-type (–/–) littermates. However, in the hotplate test, the response of mutant mice was related to the endpoint used to determine the threshold of nociceptive behaviour. If the hind paw licking/shaking was used as the endpoint, then homozygous (–/–) mice had a significantly delayed response to the noxious influence. The situation was completely different when we used the jump latency as the threshold for determining of nociceptive behaviour. The jump latency of wild-type mice (+/+) is significantly longer compared to heterozygous (+/–) and homozygous (–/–) mutant animals. It is important that heterozygous (+/–) animals, having 50% of CCK_2 receptors left, have a clear phenotype in the plantar analgesia and hotplate tests. Differently from the homozygous (–/–) mice, heterozygous (+/–) animals do not have any disturbances in the development of gastric mucosa (Nagata et al. 1996), which may potentially interfere with the pain sensitivity in mice without CCK_2 receptors. The reduced jump latency is in good agreement with a recent study of Pommier et al. (2002), where wild-type (+/+) and homozygous (–/–) mice were compared. Nevertheless, it is unclear whether the jump latency is a true threshold to the painful stimuli. Wilson and Mogil (2001) believe that

the best endpoint in the mouse is almost always hind paw licking or vigorous shaking, whichever occurs first. Hotplate-naive mice very rarely jump until long after they have responded in another way (Wilson and Mogil 2001). The jump response is considered as a measure of pain tolerance, and it is heavily influenced by morphine (Frederickson et al. 1977; Bar-Or and Brown 1989). This behavioural response is clearly an escape from the noxious stimuli and therefore involves more complex neural circuits (probably involving fear and anxiety circuits) than hind paw licking/shaking. The distinct behavioural responses in the models of pain sensitivity are also established in the animals lacking the other genes related to the regulation of nociceptive behaviour. The animals lacking pre-proenkephalin, tachykinin-1 and cannabinoid receptor CB1 genes were all reported to display altered sensitivity on the hotplate test but unchanged sensitivity on the tail-flick test (Konig et al. 1996; Zimmer et al. 1998, 1999).

Another interesting finding of the present study is that the reduced pain sensitivity in mice, lacking CCK_2 receptors, is affected by the administration of saline and isolation of mice for 30 min. The treatment with saline and isolation of mice attenuates the reduced pain sensitivity in mutant mice. It is worth noting that the effect of saline was present 15 min, but not 90 min, after administration in the hotplate test. Therefore, it is likely that the injection of saline induces the release of unknown substances attenuating the reduced nociceptive response in mutant mice. Evidence suggests that CCK receptor antagonist proglumide antagonises nocebo hyperalgesia induced by saline in humans (Benedetti et al. 1997). Moreover, Drago et al. (2001) have found that the administration of saline increases the immobility of rats in the forced swimming test. It has been suggested that catecholamines and endorphins may play a role in the effect of saline in the forced swimming test (Drago et al. 2001). Nevertheless, the background of saline-induced reversal of reduced pain sensitivity remains unclear, but could also be attributed to the previous findings that stressful factors affect the interaction between CCK and morphine in behavioural experiments. It has been shown that the administration of CCK antagonises morphine-induced antinociception in a novel but not in a familiar environment (Wiertelak et al. 1992). The same is true regarding the potentiation of morphine-induced antinociception by the CCK_2 receptor antagonists (Lavigne et al. 1992). This could be linked to the fact that CCK is also involved in the regulation of anxiety (Harro et al. 1993). Indeed, CCK agonists induce an anxiogenic-like action in an unfamiliar stressful, but not in a safe, environment (Kõks et al. 2000). The combination of sub-threshold doses of CCK-4 and opioid antagonist naloxone induces a significant neophobia in rats in the elevated plus-maze (Kõks et al. 1998). Therefore, it is possible that CCK and endopioid peptides play an opposite role in the adaptation to a novel environment. CCK signals that the environment is unsafe, whereas the endopioid system mediates the information that there is no danger in the surrounding environment (Kõks et al. 1998, 2000). This could explain why the baseline pain sensitivity of mice without CCK_2 receptors is reduced. Repeated treatments with saline increase alcohol consumption in low preference mice and the effect of saline is blocked by the administration of the CCK_2 receptor antagonist CAM1028 (Little et al. 1999). This is probably not linked to the anxiolytic action of CAM1028 because diazepam (1 mg/kg) was ineffective under these circumstances. In agreement with this finding, we found that repeated treatment with saline (once daily for 10 days) abolished the reduced nociception in mice lacking CCK_2 receptors. Accordingly, it is obvious that the repeated stressful manipulations reverse the reduced nociception in CCK_2 receptor deficient mice.

The effect of morphine, μ-opioid receptor agonist, also depends on the approach used. In the plantar analgesia test, the antinociceptive effect of morphine (1 mg/kg) in homozygous (–/–) mice is significantly increased compared to wild-type (+/+) littermates. This is in good agreement with previous studies where the administration of CCK_2 receptor antagonists or use of CCK_2 receptor antisense potentiates morphine-induced antinociception in rodents (Vanderah et al. 1994, 1996). In the hotplate test, a significantly higher dose of morphine (7.5 mg/kg) is necessary to induce the antinociceptive effect, and there is no difference in the action of opioid agonist in wild-type (+/+) and mutant mice. Pommier et al. (2002) established the reduction of opioid receptors at the supra-spinal level. We measured the density of opioid receptors by [^{3}H]-diprenorphine in four forebrain structures (cerebral cortex, hippocampus, striatum and mesolimbic area) and at least in these structures no decline in the density of opioid receptors was observed. Indeed, the number of opioid receptors is elevated in the striatum of mutant mice, whereas the other structures show no difference in the density of receptors between the homozygous (–/–) and wild–type (+/+) animals. On the other hand, the affinity of opioid receptors is decreased in the striatum. This could argue in favour of the initial idea of Pommier et al. (2002) that the levels of endopioid peptides are increased in certain brain regions and therefore the affinity of opioid receptors is decreased. A micro-array study was performed in the striatum in order to reveal differences between the wild-type (+/+) and homozygous (–/–) mice in the gene expression (our unpublished data). According to this study, the expression of the μ-opioid receptor gene was increased, whereas the expression of pre-proenkephalin, a precursor molecule of enkephalins, and nociceptin genes was reduced. By contrast from the striatum the affinity of opioid receptors tended to be increased in the cerebral cortex of mutant mice compared to wild-type (+/+) littermates. Accordingly, the targeted mutation of CCK_2 receptors affects differently the parameters of opioid receptors in the forebrain structures. The distinct changes in the sensitivity of opioid receptors in various brain structures may explain why the effect of morphine is increased in the plantar analgesia test, but remains unchanged in the hotplate test in CCK_2 receptor deficient mice. Pommier et al. (2002) showed that mutant mice

revealed not only a reduced latency to jump, but their response to the anti-jumping effect of morphine was also reduced. This finding may reflect the increased function of anti-opioid systems in the brain. Indeed, Pommier et al (2002) demonstrated the role of NMDA receptors because MK-801, an antagonist of NMDA receptors, effectively reversed the increased response of CCK_2 receptor deficient mice.

In conclusion, the present study established a reduced pain sensitivity of CCK_2 receptor deficient mice in the plantar analgesia and hotplate tests. However, CCK_2 receptor deficient mice seem to have a reduced tolerance to painful stimuli as measured by the jump latency in the hotplate test. Moreover, a clear dissociation of morphine effects is present in mice lacking CCK_2 receptors. In the plantar analgesia test the antinociceptive action of morphine is significantly stronger in mutant mice, whereas in the hotplate test the effect of μ-opioid receptor agonist remains unchanged compared with wild-type (+/+) littermates. In the jump latency test, a measure of pain tolerance, the effect of morphine is significantly reduced in CCK_2 receptor deficient mice compared to wild-type (+/+) littermates (Pommier et al. 2002). The molecular background of these phenomena is unclear but could be explained in the light of data showing that the targeted mutation of CCK_2 receptors induces distinct changes in the properties of opioid receptors in various brain structures.

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References

Bar-Or A, Brown GM (1989) Pineal involvement in the diurnal rhythm of nociception in the rat. Life Sci 44:1067–1075

Benedetti F, Amanzio M, Casadio C, Oliaro A, Maggi G (1997) Blockade of nocebo hyperalgesia by the cholecystokinin antagonist proglumide. Pain 71:135–140

Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Daugé V, Beslot F, Matsui T, Roques BP (2001) Mutant mice lacking the cholecystokinin2 receptor show a dopamine-dependent hyperactivity and a behavioural sensitization to morphine. Neurosci Lett 306:41–44

Drago F, Nicolosi A, Micale V, Lo Menzo G (2001) Placebo affects the performance of rats in models of depression: is it a good control for behavioral experiments. Eur Neuropsychopharmacol 11:209–213

Faris PL, Komisaruk BR, Watkins LR, Mayer DJ (1983) Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. Science 219:310–312

Franklin KBJ, Paxinos G (1997) The mouse brain in stereotaxic coordinates. Academic Press, San Diego

Frederickson RCA, Burgis V, Edwards JD (1977) Hyperalgesia induced by naloxone follows a diurnal rhythm in responsitivity to painful stimuli. Science 198:756–758

Gall C, Lauterborn J, Burks D, Seroogy K (1987) Co-localization of enkephalin and cholecystokinin in discrete areas of rat brain. Brain Res 403:403–408

Ghilardi JR, Allen CJ, Vigna SR, McVey DC, Mantyh PW (1992) Trigeminal and dorsal root ganglion neurons express CCK receptor binding sites in the rat, rabbit, and monkey: possible site of opiate-CCK analgesic interactions. J Neurosci 12:4854–4866

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77–88

Harro J, Vasar E, Bradwejn J (1993) Cholecystokinin in animal and human research of anxiety. Trends Pharmacol Sci 14:244–249

Kõks S, Vasar E, Soosaar A, Lang A, Volke V, Võikar V, Bourin M, Männistö PT (1997) Relation of exploratory behavior of rats in the elevated plus-maze to brain receptor binding properties and serum growth hormone level. Eur Neuropsychopharmacol 7:289–294

Kõks S, Soosaar A, Võikar V, Volke V, Ustav M, Männistö PT, Bourin M, Vasar E (1998) Opioid antagonist naloxone potentiates anxiogenic-like action of cholecystokinin antagonists in elevated plus-maze. Neuropeptides 32:235–240

Kõks S, Männistö PT, Bourin M, Shlik J, Vasar V, Vasar E (2000) Cholecystokinin-induced anxiety in rats: relevance of pre-experimental stress and seasonal variations. J Psychiatr Neuroci 25:33–42

Kõks S, Volke V, Veraksits A, Rünkorg K, Sillat T, Abramov U, Bourin M, Huotari M, Männistö PT, Matsui T, Vasar E (2001) Cholecystokinin2 receptor deficient mice display altered function of brain dopaminergic system. Psychopharmacology 158:198–204

Konig M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ, Zimmer A (1996) Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. Nature 383:535–538

Lavigne GJ, Millington WR, Mueller GP (1992) The CCK–A and CCK-B receptor antagonists, devazepide and L-365,260, enhance morphine antinociception only in non-acclimated rats exposed to a novel environment. Neuropeptides 21:119–129

Little HJ, O'Callaghan MJ, Butterworth AR, Wilson J, Cole J, Watson WP (1999) Low alcohol preference among the "high alcohol preference" C57 strain of mice; preference increased by saline injections. Psychopharmacology 147:182–189

Nagata A, Ito M, Iwata N, Kuno J, Takano H, Minowa O, Chihara K, Matsui T, Noda T (1996) G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proc Natl Acad Sci USA 93:11825–11830

Noble F, Blommaert A, Fournie-Zaluski MC, Roques BP (1995) A selective CCKB receptor antagonist potentiates μ-, but not δ–opioid receptor-mediated antinociception in the formalin test. Eur J Pharmacol 273:145–151

Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, Roques BP (1999) International Union of Pharmacology. XXI. Structure, distribution, and function of cholecystokinin receptors. Pharmacol Rev 51:745–781

Perissin L, Facchin P, Porro CA (2000) Diurnal variations in tonic pain reactions in mice. Life Sci 67:1477–1488

Pommier B, Beslot F, Simon A, Pophillat M, Matsui T, Daugé V, Roques BP, Noble F (2002) Deletion of CCK2 receptor in mice results in an upregulation of the endogenous opioid system. J Neurosci 22:2001–2011

Roques BP, Noble F (1998) Cholecystokinin receptors. In: Girdlestone D (ed) The IUPHAR receptor compendium of receptor characterization and classification. IUPHAR Media, London, pp 128–133

Saito A, Sankaran H, Goldfine ID, Williams JA (1980) Cholecystokinin receptors in the brain: characterization and distribution. Science 208:1155–1156

Savasta M, Palacios JM, Mengod G (1988) Regional localization of the mRNA coding for the neuropeptide cholecystokinin in the rat brain studied by in situ hybridization. Neurosci Lett 93:132–138

Schnur P, Cesar SS, Foderaro MA, Kulkosky PJ (1991) Effects of cholecystokinin on morphine-elicited hyperactivity in hamsters. Pharmacol Biochem Behav 39:581–586

Sebret A, Lena I, Crete D, Matsui T, Roques BP, Daugé V (1999) Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. J Neurosci 19:7230–7237

Shlik J, Vasar E, Bradwejn J (1997) Cholecystokinin and psychiatric disorders: role in aetiology and potential of receptor antagonists in therapy. CNS Drugs 8:134–152

Valverde O, Fournie-Zaluski MC, Roques BP, Maldonado R (1996) The CCKB antagonist PD-134,308 facilitates rewarding effects of endogenous enkephalins but does not induce place preference in rats. Psychopharmacology 123:119–126

Vanderah TW, Lai J, Yamamura HI, Porreca F (1994) Antisense oligonucleotides to the CCKB receptor produces naltrindole- and [Leu5]enkephalin antiserum-sensitive enhancement of morphine antinociception. Neuroreport 5:2601–2605

Vanderah TW, Bernstein RN, Yamamura HI, Hruby VJ, Porreca F (1996) Enhancement of morphine antinociception by a CCKB antagonist in mice is mediated via opioid delta receptors. J Pharmacol Exp Ther 278:212–219

Vanderhaegen J-J, Schiffmann SN (1992) Distribution of brain neuronal CCK: an in situ hybridization study. In: Dourish CT, Cooper SJ, Iversen SD, Iversen L (eds) Multiple cholecystokinin receptors in the CNS. Oxford University Press, New York, pp 38–56

Wiertelak EP, Maier SF, Watkins LR (1992) Cholecystokinin antianalgesia: safety cues abolish morphine analgesia. Science 256:830–833

Wilson SG, Mogil JS (2001) Measuring pain in the (knockout) mouse: big challenges in a small mammal. Behav Brain Res 125:65–73

Zimmer A, Zimmer AM, Baffi J, Usdin T, Reynold K, Konig M, Palkovits M, Mezey E (1998) Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. Proc Natl Acad Sci USA 95:2630–2635

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc Natl Acad Sci USA 96:5780–5785

IV

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Research report

Distinct changes in the behavioural effects of morphine and naloxone in CCK_2 receptor-deficient mice

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Abstract

The effects of morphine, μ-opioid receptor agonist, and naloxone, a non-selective opioid receptor antagonist, in the locomotor activity and place conditioning tests were studied in the CCK_2 receptor-deficient male mice. The exposure of mice to the motility boxes for 3 consecutive days induced a significant inhibition of locomotor activity in the wild-type (+/+) mice compared to homozygous (−/−) animals. The administration of naloxone (10 mg/kg i.p.) to animals, adapted to the motility boxes, induced a significant reduction of locomotor activity in the homozygous (−/−), but not in the wild-type (+/+) mice. Treatment of habituated mice with morphine (10 mg/kg i.p.) caused a stronger increase of locomotor activity in the wild-type (+/+) mice compared to the homozygous (−/−) littermates. In the place preference test the pairing of the preferred side with naloxone (1 and 10 mg/kg i.p.) induced a dose-dependent place aversion in the wild-type (+/+) mice. The treatment with naloxone was less effective in the homozygous (−/−) mice, because the high dose of naloxone (10 mg/kg) tended to shift the preference. The pairing of morphine (3 mg/kg i.p.) injections with the non-preferred side induced a significant place preference both in the wild-type (+/+) and homozygous (−/−) mice. The increased density of opioid receptors was established in the striatum of homozygous (−/−) mice, but not in the other forebrain structures. In conclusion, the targeted invalidation of CCK_2 receptors induces a dissociation of behavioural effects of morphine and naloxone. Morphine-induced place preference remained unchanged, whereas hyper-locomotion was less pronounced in the mutant mice compared to the wild-type (+/+) littermates. By contrast, naloxone-induced place aversion was weaker, but naloxone caused a stronger inhibition of locomotor activity in the homozygous (−/−) mice than in the wild-type (+/+) animals. These behavioural alterations can be explained in the light of data that the targeted mutation of CCK_2 receptors induces distinct changes in the properties of opioid receptors in various brain structures.

Keywords: CCK_2 receptors; Targeted mutagenesis; Mouse; Wild-type; Homozygous; Opioid receptors; Morphine; Naloxone; Place conditioning; Locomotor activity

1. Introduction

Various studies support the antagonistic interaction between two neuropeptide systems, cholecystokinin (CCK) and endogenous opioid peptides, in the regulation of behaviour. The administration of CCK attenuates, whereas the selective CCK_2 receptor antagonists enhance, morphine-induced anti-nociception in rodents [6,17,22]. CCK_2 receptor antagonists, but not CCK_1 receptor antagonists, facilitate the antidepressant-like effect induced by endopioid peptides in the conditioned suppression of the motility test in mice [30]. Moreover, the selective CCK_2 antagonists potentiate the rewarding effect of morphine in the place conditioning paradigm [10,33]. The CCK_2 receptor antagonists L-365,260 and PD-134,308 are shown to attenuate the place aversion induced by naloxone in morphine-dependent rats, whereas the CCK_1 receptor antagonist devazepide is ineffective [34]. A morphine-induced increase in the locomotor activity can also be antagonised by the pre-treatment with CCK [26]. These findings suggest that CCK may act, via CCK_2 receptors, as an endogenous anti-opioid peptide. It is also noteworthy that the distribution of both CCK and CCK_2 receptors parallels with that of endopioid peptides and opioid receptors in the brain [8,9,24]. Recently, the mice with targeted disruption of CCK_2 receptor gene have been generated [21]. The animals without CCK_2 receptors display

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disturbances in the development of gastric mucosa and in learning abilities [21,27]. The activity of the dopaminergic system is also affected in mice with a corrupted function of CCK_2 receptors, because the sensitivity of dopamine D_2 receptors is increased in mutant mice [5,16]. Recent evidence suggests the altered function of the endogenous opioid system in mice without CCK_2 receptors. Pommier et al. demonstrated that mice, lacking CCK_2 receptors, displayed hyperalgesia and a reduced response to morphine-induced analgesia in the hotplate test [23]. According to this study, mutant mice exhibit hypersensitivity to morphine-induced locomotor activity and develop a more severe withdrawal syndrome after chronic morphine treatment [23]. Therefore, Pommier et al. stated that the deletion of CCK_2 receptors resulted in an up-regulation of the endogenous opioid system [23].

Morphine, like psychostimulants (cocaine, amphetamine, and methamphetamine), induces conditioned place preference and motor activation [3]. Maldonado et al. showed that morphine-induced hyperlocomotion and conditioned place preference could be dissociated in the genetically modified mice [19]. They found that in dopamine D_2 receptor-deficient mice morphine did not induce conditioned place preference, but increased the locomotor activity. Our previous study established that animals without CCK_2 receptors had an increased sensitivity of dopamine D_2 receptors [16]. Therefore, the aim of the present study was to investigate whether morphine-induced hyperlocomotion and conditioned place preference were also dissociated in mice without CCK_2 receptors. Also, we studied the effect of naloxone, a non-specific antagonist of opioid receptors, on locomotor activity and on the place conditioning paradigm of CCK_2 receptor-deficient mice. Pommier et al. found that the affinity and density of opioid receptors was not changed in the whole brain of mice without CCK_2 receptors [23]. However, the interaction of CCK with endogenous opioid peptides and opioid receptors is different in various brain structures, and that was an obvious reason why we performed the radioligand studies with [^{3}H]-diprenorphine in the distinct forebrain regions, including the cerebral cortex, striatum, mesolimbic area, and hippocampus.

2. Materials and methods

2.1. Animals

Nagata et al. generated CCK_2 receptor-deficient mice by replacing a part of exons 2–5 [21]. Breeding and genotype analysis were performed at the Department of Physiology of the University of Tartu. Genotyping was carried out by means of polymerase chain reaction (PCR) using two pairs of primers—HE2F (TGG AGT TGA CCA TTC GAA TCA C) and LacZrev (GTG CTG CAA GGC GAT TAA GTT G) were designed to detect the mutant allele, and HE3F (TAT CAG TGA GTG TGT CCA CTC T) and HE3R (ACA TTT GTT GGA CAC GTT CAC) were designed for the wild-type allele. For PCR we used the following protocol: 96 °C for 10 min (initial denaturation); 96 °C for 50 s, 60 °C for 50 s, and 72 °C for 2 min (25 cycles); and 72 °C for 10 min (final amplification). PCR products were stored at 4 °C until electrophoresis. Altogether, 176 male homozygous (−/−) CCK_2 receptor-deficient and 176 male wild-type (+/+) (3-month-old) mice were used in the behavioural and radioligand binding studies. Mutant mice were crossed back six times to the C57Bl/6 background to minimise any possible genetic effects from the 129sv strain. The mice were kept in the animal house at 20 ± 2 °C under a 12:12-h light/dark cycle (lights on at 07:00 h). Tap water and food pellets were available ad libitum. All animal procedures were approved by the Animal Care Committee of the University of Tartu in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

2.2. Drugs

Morphine sulphate (Boehringer-Ingelheim) and naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved in sterile, pyrogen-free, 0.9% solution of NaCl (saline). Solutions of morphine and naloxone were injected intraperitoneally in a volume 10 ml/kg. The doses of naloxone (1 and 10 mg/kg) and morphine (3 and 9 mg/kg) were selected according to the data available in the literature. These doses are shown to induce a significant action in the place conditioning studies. In the locomotor activity studies, the higher doses of morphine were applied since the pilot study revealed that only 10 mg/kg of morphine induced a significant increase of locomotor activity in mice with 129sv/C57Bl/6 background.

2.3. Behavioural testing

The animals were brought into the experimental room one hour before the experiment. All behavioural experiments were performed between 11:00 and 19:00 h. The motility and conditioned place preference tests were performed on separate groups of animals. Since the behavioural experiments lasted 6–8 h, precautions were taken to control the possible daily fluctuations in the locomotor activity of animals. Therefore, the experiments were always performed in randomised order, that is, wild-type (+/+) mice were always used in parallel with mutant animals. In the radioligand binding study, we used animals, which had not been exposed to behavioural testing.

2.4. Motility test

For the study of locomotor activity, the animals were placed singly into the photoelectric motility boxes (448 mm × 448 mm × 450 mm) connected to a computer (TSE Technical & Scientific Equipment GMBH, Germany). The illumination level of the transparent test boxes was ~750 lux. After removing a mouse from the box the floor

was cleaned by using 5% alcohol solution. Time in movement (s), the total distance of movement (m), the number of rearing and corner entries were registered during the 30-min observation period. The locomotor effects of morphine (5 and 10 mg/kg) and naloxone (1 and 10 mg/kg) were studied in the separate groups of animals. Morphine and naloxone were injected intraperitoneally 15 min before the experiment. Naloxone is shown to reduce the locomotor activity in rodents. Pommier et al. described that naloxone selectively suppressed the locomotor activity in CCK_2 receptor-deficient mice not habituated to the experimental environment [23]. The effect of naloxone was studied in two different groups of mice. One group of animals was not exposed to the motility boxes, whereas the other group was adapted to the experimental environment for 3 days before naloxone was administered. Similarly to psycho-stimulants morphine stimulates the locomotor activity in rodents, and the effect of motor stimulants is stronger in animals adapted to the experimental conditions [3]. This was a reason why the animals were habituated to the motility boxes for 3 days before morphine was administered.

2.5. Place preference and avoidance tests

Place preference and avoidance experiments were performed in three identical shuttle boxes (50 cm × 25 cm × 30 cm). Each shuttle box was divided into two compartments of equal size by a sliding door having a partition. The door allows free movement of a test animal between the two compartments if opened or restricts the movement of a mouse to the other compartment if closed. These compartments were distinguished by wall and floor colour (dark green versus pale green). The illumination level in the shuttle boxes was ~250 lux. The place conditioning experiment was performed according to the method described by Bespalov et al. [1]. Each experiment consisted of pre-conditioning, conditioning, and post-conditioning periods. During the pre-conditioning period mice were placed three times into the apparatus for 15 min. The first and the second pre-conditioning tests were held on Day 1. The third pre-conditioning test was held on Day 2 of the experiment, and the time spent in the pale green compartment was measured. During the pre-conditioning and post-conditioning tests, animals were allowed to explore both compartments freely. Each pre- and post-conditioning session began with initial placement of an animal into the dark green compartment. The shuttle boxes were cleaned carefully by 5% ethanol solution after each animal. The conditioning period consisted of 30-min experimental session performed twice a day for 4 consecutive days (Days 3–6). On each day, animals received a saline injection before being placed into one compartment and were then injected with morphine (3 and 9 mg/kg) or naloxone (1 and 10 mg/kg) before placement into the opposite compartment. Injections were given immediately before the conditioning sessions. One half of the animals received saline as the first injection, whereas the other half were at first treated with morphine or naloxone. The morphine injections were paired with the placement of a mouse into the pale green compartment avoided in the pre-conditioning test (conditioning of place preference) and the naloxone injections were paired with the placement of an animal into the preferred compartment (dark green, conditioning of place avoidance). The control animals received the injection of saline in both compartments. Fifteen-minute post-conditioning tests were performed on the Day 7 of experiments and the time spent in the pale green side of the shuttle box was measured. The behaviour of animals was recorded on the videotape and it was analysed by the experienced person not aware about the previous conditioning sessions and genotype of mice. On the Day 8, we studied also the effect of morphine administration on the place preference, but the response of the animals did not differ from that in the drug-free state. Therefore, these data were not included in the further analysis.

2.6. Radioligand binding studies

After decapitation, the brains were quickly dissected on ice. The cerebral cortex (including the frontal and parietal cortices), striata, mesolimbic structures (*nucleus accumbens* and *tuberculum olfactorium*) and hippocampus were dissected [7]. The brain structures from six mice were pooled. The radioligand binding studies were performed according to the method of Kõks et al. [12]. For the labelling of opioid receptors [^{3}H]-diprenorphine (specific activity: 58.0 Ci/mmol, Amersham Radiochemicals) was used. The parameters of the opioid receptors were determined in the presence of 0.05–2.5 nM [^{3}H]-diprenorphine at 23 °C for 60 min. Naloxone hydrochloride (RBI, 1 μM), an antagonist of opioid receptors, was added to determine the non-specific binding at opioid receptors. The brain tissue was homogenised in 20 volumes of ice-cold 50 mM Tris–HCl (pH 7.4 at 4 °C) using a Potter-S glass–teflon homogeniser (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 (Tris–HCl, pH 7.4 at 4 °C). The protein content was measured according to the method of Bradford [2]. The saturation curves of [^{3}H]-diprenorphine binding were analysed using GraphPad Prism (Version 3.00) for Windows software. The experiment was repeated four times.

2.7. Statistics

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

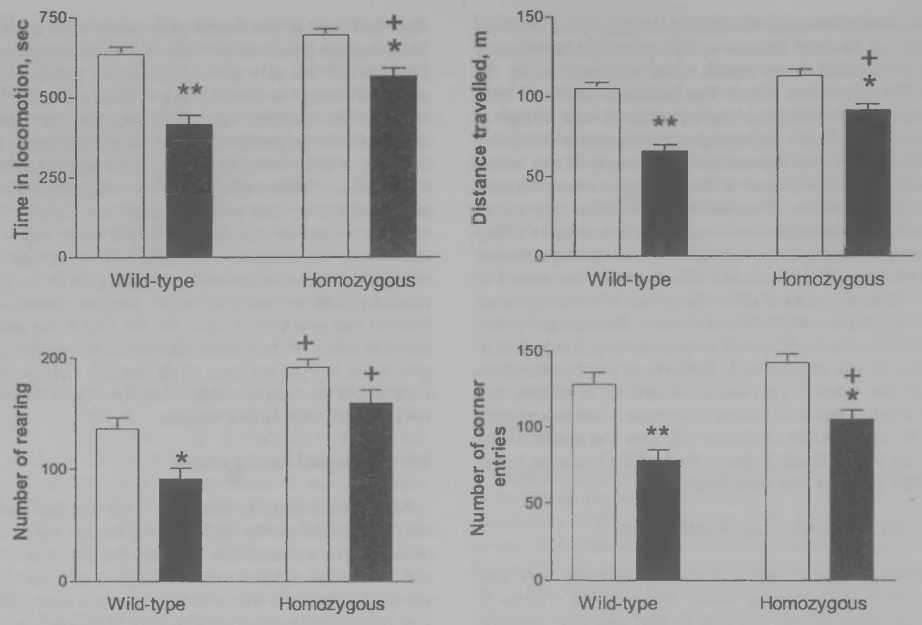

Fig. 1. Different adaptation of wild-type mice and mice without CCK_2 receptors in motility boxes. The number of animals in each group was 25. White bars—motor activity of mice during the first experiment, black bars—the response of animals during the third experiment. (*) $P < 0.05$; (**) $P < 0.001$ (compared with the first experiment, Tukey HSD test after the significant two-way ANOVA); (+) $P < 0.05$ (compared with the respective group of wild-type mice).

3. Results

3.1. Locomotor activity

In the first experiment, the locomotor activity of mutant mice did not differ from that of wild-type (+/+) littermates (Fig. 1). Only the number of rearing was higher in the homozygous (−/−) animals. However, during the third experiment some obvious differences became evident. There was significantly stronger inhibition of locomotor activity in the wild-type mice (+/+) compared to the homozygous (−/−) littermates. The application of two-way ANOVA demonstrated that the wild-type (+/+) animals displayed stronger reduction of time in locomotion (two-way ANOVA: genotype: $F_{1,96} = 18.2$, $P < 0.001$; experiment: $F_{1,96} = 51.7$, $P < 0.001$; genotype × experiment: $F_{1,96} = 4.06$, $P < 0.05$) and distance travelled (two-way ANOVA: genotype: $F_{1,96} = 14.7$, $P < 0.001$; experiment: $F_{1,96} = 53.5$, $P < 0.001$; genotype × experiment: $F_{1,96} = 4,32$, $P < 0.05$) compared to the homozygous (−/−) mice (Fig. 1). By contrast, the frequency of rearing (two-way ANOVA: genotype: $F_{1,96} = 32.0$, $P < 0.001$; experiment: $F_{1,96} = 15.3$, $P < 0.001$; genotype × experiment: $F_{1,96} = 0.11$, $P = 0.73$) and number of corner entries (two-way ANOVA: genotype: $F_{1,96} = 11.0$, $P < 0.01$; experiment: $F_{1,96} = 45.3$, $P < 0.001$; genotype × experiment: $F_{1,96} = 1.52$, $P = 0.22$) did not differ in these two groups of mice according to two-way ANOVA. However, the using of post-hoc analysis established that these two parameters of locomotor activity were also reduced in the wild-type (+/+) mice compared to the homozygous (−/−) animals (Tukey HSD test: frequency of rearing, $P < 0.001$ and number of corner entries, $P < 0.01$) (Fig. 1). The administration of opioid receptor antagonist naloxone (1 and 10 mg/kg) to the non-habituated mice induced a dose-dependent reduction of locomotor activity in the wild-type (+/+) and homozygous (−/−) animals (Fig. 2). However, the application of two-way ANOVA did not reveal any significant differences if the action of naloxone was compared in the wild-type (+/+) and mutant animals. Naloxone induced a decrease of time in locomotion (two-way ANOVA: genotype: $F_{1,42} = 0.95$, $P = 0.76$; treatment: $F_{2,42} = 8.91$, $P < 0.01$; genotype × treatment: $F_{2,42} = 0.87$, $P = 0.42$), distance travelled (two-way ANOVA: genotype: $F_{1,42} = 0.09$, $P = 0.77$; treatment: $F_{2,42} = 12.13$, $P < 0.01$; genotype × treatment: $F_{2,42} = 1.21$, $P = 0.30$), number of rearing (two-way ANOVA: genotype: $F_{1,42} = 7.94$, $P < 0.01$; treatment: $F_{2,42} = 6.44$, $P < 0.01$; genotype × treatment: $F_{2,42} = 1.06$, $P = 0.35$)

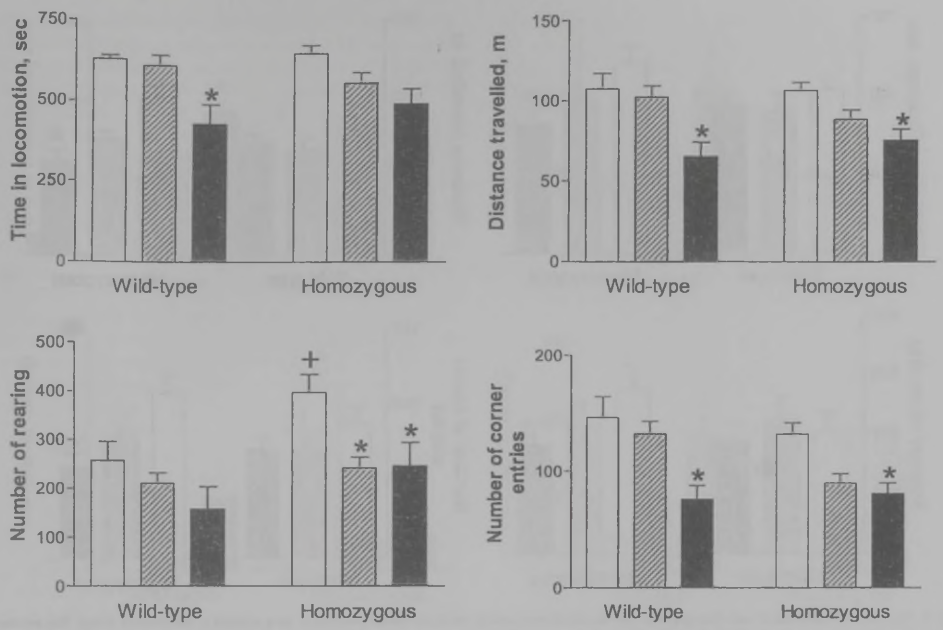

Fig. 2. The effect of naloxone (1 and 10 mg/kg i.p.) on the locomotor activity of CCK_2 receptor-deficient mice not adapted to the motility boxes. The number of animals in each group was eight. White bars—saline treatment, striped bars—naloxone (1 mg/kg), black bars—naloxone (10 mg/kg). (*) $P < 0.05$ (compared with saline-treated mice, Tukey HSD test after significant one-way ANOVA); (+) $P < 0.05$ (compared with saline-treated wild-type mice).

and frequency of corner entries (two-way ANOVA: genotype: $F_{1,42} = 3.05$, $P = 0.09$; treatment: $F_{2,42} = 12.6$, $P < 0.01$; genotype × treatment: $F_{2,42} = 1.79$, $P = 0.17$). Nonetheless, the using of post-hoc analysis demonstrated that the administration of naloxone (1 and 10 mg/kg) inhibited the increased frequency of rearing in the homozygous (−/−) mice. Also, the highest dose of naloxone (10 mg/kg) did not induce a significant reduction of time in locomotion in the homozygous mice (−/−) (Tukey HSD test: wild-type (+/+) mice $P < 0.05$ and homozygous (−/−) animals $P = 0.11$) (Fig. 2). In mice, habituated to the experimental environment, the application of two-way ANOVA did not distinguish the action of naloxone in the wild-type (+/+) and homozygous (−/−) mice [time spent in locomotion (two-way ANOVA: genotype: $F_{1,52} = 0.27$, $P = 0.61$; treatment: $F_{2,52} = 4.06$, $P < 0.05$; genotype × treatment: $F_{2,52} = 1.41$, $P = 0.25$); distance travelled (two-way ANOVA: genotype: $F_{1,52} = 0.48$, $P = 0.49$; treatment: $F_{2,52} = 6.44$, $P < 0.01$; genotype × treatment: $F_{2,52} = 2.26$, $P = 0.11$); frequency of rearing (two-way ANOVA: genotype: $F_{1,52} = 0.44$, $P = 0.51$; treatment: $F_{2,52} = 1.08$, $P = 0.35$; genotype × treatment: $F_{2,52} = 0.77$, $P = 0.47$) and number of corner entries (two-way ANOVA: genotype: $F_{1,52} = 0.38$, $P = 0.54$; treatment: $F_{2,52} = 5.77$, $P < 0.01$; genotype × treatment: $F_{2,52} = 1.06$, $P = 0.35$)] (Fig. 3). However, the situation was different after the post-hoc analysis. Naloxone (10 mg/kg) induced the inhibition of locomotor activity only in the homozygous (−/−), but not in the wild-type (+/+) mice (Tukey HSD test: time in locomotion, $P < 0.05$; distance travelled, $P < 0.01$, number of corner entries $P < 0.05$) (Fig. 3). The administration of μ-opioid receptor agonist morphine (5 and 10 mg/kg) induced a dose-dependent increase of locomotor activity in the wild-type (+/+) mice, adapted to the motility boxes (Fig. 4). Already the lower dose (5 mg/kg) of morphine tended to increase the parameters of horizontal locomotor activity in the wild-type (+/+) animals, but these changes were not statistically significant. By contrast, the higher dose (10 mg/kg) of morphine caused a significant hyper-locomotion in these animals. Morphine (5 and 10 mg/kg) suppressed the number of rearing in the wild-type (+/+) mice. Motor stimulation induced by morphine was significantly weaker in the homozygous (−/−) animals [two-way ANOVA: time in locomotion (two-way ANOVA: genotype: $F_{1,95} = 0.93$, $P = 0.33$; treatment: $F_{2,95} = 16.1$, $P < 0.001$; genotype × treatment: $F_{2,95} = 3.70$, $P < 0.05$), distance travelled (two-way

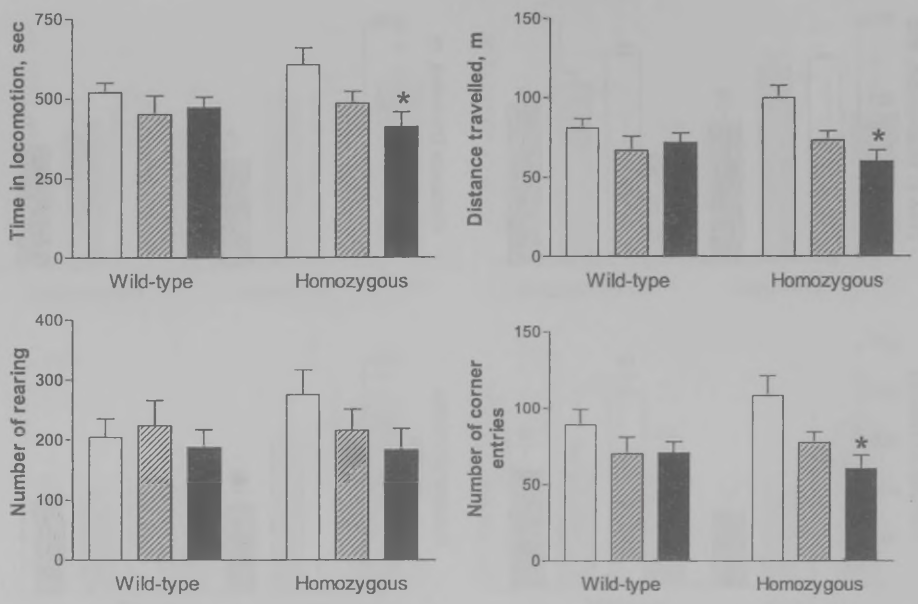

Fig. 3. The effect of naloxone (1 and 10 mg/kg i.p.) on the locomotor activity of CCK_2 receptor-deficient mice adapted to the motility boxes. The number of animals in each group was 9–10. White bars—saline treatment, striped bars—naloxone (1 mg/kg), black bars—naloxone (10 mg/kg). (*) $P < 0.05$ (compared with saline-treated homozygous mice, Tukey HSD test after significant one-way ANOVA).

ANOVA: genotype: $F_{1,95} = 1.67$, $P = 0.20$; treatment: $F_{2,95} = 28.2$, $P < 0.001$; genotype × treatment: $F_{2,95} = 3.48$, $P < 0.05$) and frequency of corner entries (two-way ANOVA: genotype: $F_{1,95} = 3.66$, $P = 0.059$; treatment: $F_{2,95} = 40.2$, $P < 0.001$; genotype × treatment: $F_{2,95} = 3.38$, $P < 0.05$)]. The only difference was the frequency of rearing. Two-way ANOVA established a stronger suppression of rearing in the homozygous (−/−) mice, because it was, like in the previous experiments, higher in the homozygous (−/−) mice (Fig. 4) (two-way ANOVA: genotype: $F_{1,95} = 0.07$, $P = 0.80$; treatment: $F_{2,95} = 56.4$, $P < 0.001$; genotype × treatment: $F_{2,95} = 6.71$, $P < 0.01$).

3.2. Place conditioning experiments

In the pre-conditioning test the wild-type (+/+) and homozygous (−/−) mice preferred to stay in the dark green compartment of shuttle box. The wild-type (+/+) animals spent only 115±15 s in the pale green compartment, whereas the respective value for the homozygous (−/−) animals was 177 ± 22 s. The pairing of the dark and pale green box with saline injections did not change the preference of animals compared to the pre-conditioning session. The pairing of the dark green box with naloxone (1 and 10 mg/kg) induced a significant shift of exploratory activity from the dark to the pale green compartment (Fig. 5A). The application of two-way ANOVA did not distinguish the action of naloxone in the wild-type (+/+) and mutant mice (two-way ANOVA: genotype: $F_{1,42} = 1.0$, $P > 0.3$; treatment: $F_{2,42} = 16.7$, $P < 0.01$; genotype × treatment: $F_{2,42} = 1.78$, $P > 0.15$). Nevertheless, the effect of naloxone was stronger in the wild-type (+/+) animals compared with the homozygous (−/−) mice. Already 1 mg/kg of naloxone tended to shift the preference in the wild-type (+/+) mice (Tukey HSD test, $P = 0.055$), whereas in the homozygous (−/−) mice even the highest dose (10 mg/kg) did not cause any significant effect (Tukey HSD test, $P = 0.16$). The highest dose of naloxone (10 mg/kg) induced a significant place aversion in the wild-type (+/+) mice, because they started to prefer the initially non-preferred side (Tukey HSD test $P < 0.001$). The pairing of morphine (3 mg/kg) with the pale green compartment induced a significant shift of exploratory activity from the dark green to the pale green part both in the wild-type (+/+) and homozygous (−/−) mice (two-way ANOVA: genotype: $F_{1,42} = 0.40$, $P > 0.8$; treatment: $F_{2,42} = 10.7$, $P < 0.01$; genotype × treatment: $F_{2,42} = 1.30$, $P > 0.25$) (Fig. 5B). However, differently from the wild-type (+/+) animals the higher dose of morphine (9 mg/kg) induced a significant effect only in the homozygous (−/−) mice.

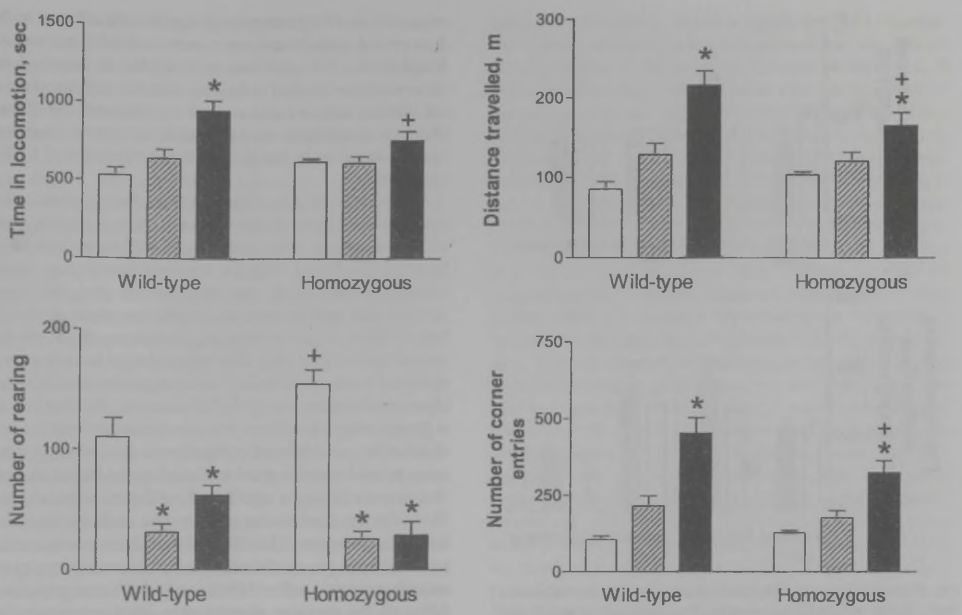

Fig. 4. The effect of morphine (5 and 10 mg/kg i.p.) on the locomotor activity of mice without CCK_2 receptors. The number of animals in each group was 17. White bars—saline treatment, striped bars—morphine (5 mg/kg), black bars—morphine (10 mg/kg). (*) $P < 0.05$ (compared with the respective saline-treated group, Tukey HSD test after significant two-way ANOVA); (+) $P < 0.05$ (compared with the respective group of wild-type mice).

3.3. Radioligand binding studies

The radioligand binding studies with [^{3}H]-diprenorphine demonstrated an increase in the affinity of the opioid binding sites in the cerebral cortex of mutant mice compared to the wild-type (+/+) littermates (Fig. 6). However, the decreased affinity and increased density of the opioid binding sites was established in the striatum of homozygous (−/−) mice. The parameters of [^{3}H]-diprenorphine binding were not affected in the mesolimbic structures and hippocampus.

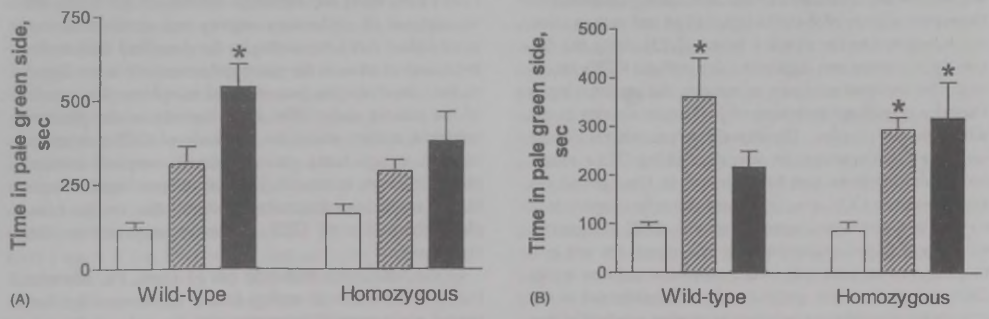

Fig. 5. The effect of naloxone (1 and 10 mg/kg) and morphine (3 and 9 mg/kg) on the place conditioning of mice without CCK_2 receptors. (A) *Naloxone-induced place aversion*. The number of animals in each group was eight. White bars—saline treatment, striped bars—naloxone (1 mg/kg), black bars—naloxone (10 mg/kg). (*) $P < 0.05$ (compared with saline-treated wild-type mice, Tukey HSD test after significant one-way ANOVA). (B) *Morphine-induced place preference*. The number of animals in each group was eight. White bars—saline treatment, striped bars—morphine (3 mg/kg), black bars—morphine (9 mg/kg). (*) $P < 0.05$ (compared with the respective saline-treated group, Tukey HSD test after significant one-way ANOVA).

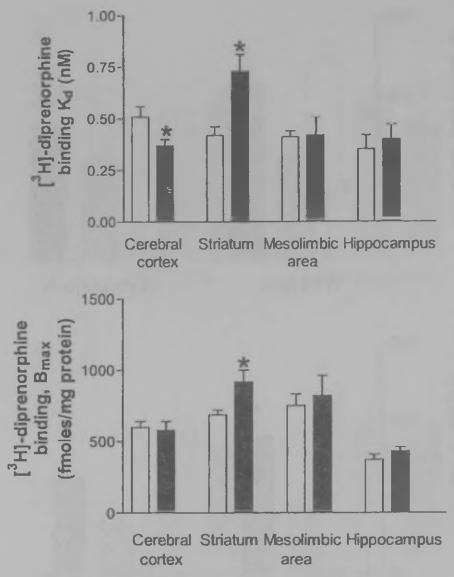

Fig. 6. The parameters of [^{3}H]-diprenorphine binding in the brain structures of mice without CCK_2 receptors. The number of animals in each group was 24, the brains of six mice were pooled, and the mean is a result of four experiments. White bars—wild type, black bars—homozygous. (*) $P < 0.05$ (compared with the wild-type group, Student's t-test).

4. Discussion

The present study yielded four major findings. First, the adaptation of CCK_2 receptor-deficient mice to a novel environment is impaired. Differently from the study of Daugé et al. and Pommier et al. we were unable to find any significant differences in the horizontal component of locomotor activity of the wild-type (+/+) and mutant mice, not habituated to the motility boxes [5,23]. Only the frequency of rearing was higher in mice without CCK_2 receptors. The repeated exposure of mice to the motility boxes caused a significant reduction of locomotor activity in the wild-type (+/+) mice. The described behavioural change was significantly weaker in animals, lacking CCK_2 receptors. This difference can be explained in the light of the suggestion that CCK is an important factor in adaptation of rodents to a novel environment. Namely, CCK antagonises morphine-induced antinociception in a novel, but not in a familiar environment [36]. Also, morphine induces a significant anxiolytic-like action in rats, not habituated to the experimental conditions, whereas morphine was ineffective in habituated rats [14,15]. Therefore, lack of CCK_2 receptors, a subtype of CCK receptors preferentially distributed in the brain, apparently attenuates a normal adaptation to the novel environment. Moreover, the learning ability of the mice without CCK_2 receptors is significantly affected in the T-maze and in the Morris water-maze test ([27], our unpublished data]. CCK_2-deficient mice display an impaired response in these learning tasks. However, it is unclear whether the affected adaptation to a novel environment and diminished learning abilities are linked to the same neurochemical changes in the brain due to the corrupted function of CCK_2 receptors.

Second, there is dissociation between the morphine-induced motor and motivational responses. Morphine (3 mg/kg) induces a similar effect both in the wild-type (+/+) and homozygous (−/−) mice in the conditioned place preference test, increasing time spent in the drug-associated compartment during the post-conditioning phase. It should be noted that the pre-conditioning preference of animals for one side was very strong. This biased design has often been criticised because it may lead to false-positive results in the place conditioning test [32]. For example, the drugs with a strong anxiolytic action may overcome the initial aversion for the non-preferred compartment. Several steps were taken to minimise the problem linked to the biased design. The animals having a significantly different response profile in the pre-conditioning session were excluded from the further experiments. The effect of morphine was not compared with the pre-conditioning session but with a group of animals receiving saline injections in both compartments. Although the response of mice after saline treatments did no differ from that seen during the pre-conditioning session. During the conditioning session half of animals received saline treatment as the first daily injection, whereas the other half were at first treated with morphine. Our previous studies showed that morphine induced an anxiolytic-like action in rats [13,14]. However, morphine was effective in the elevated plus-maze and zero-maze when its effect was studied in rats that were not handled and exposed repeatedly to the experimental room [15]. Therefore, we tested the action of morphine (3 mg/kg) in the non-habituated wild-type (+/+) mice using the dark-light exploration paradigm, and no increase of exploratory activity was established (our unpublished data). Accordingly, the described shift in the behaviour of mice in the place preference test is not linked to the anxiolytic-like properties of morphine. The results of the present study differ from the data of the pharmacological studies where the blockade of CCK_2 receptors induced a significant potentiation of morphine-induced place preference in rats [10,33]. The compensatory changes in the other neurotransmitter systems due to the complete inactivation of CCK_2 receptors may explain that discrepancy.

In the habituated wild-type (+/+) mice, the administration of morphine (5 mg/kg) tended to increase the horizontal component of locomotor activity and significantly inhibited the frequency of rearing. The higher dose of morphine (10 mg/kg) induced a significant hyper-locomotion in the wild-type (+/+) animals. By contrast, in the CCK_2 receptor-deficient mice this dose of morphine induced

significantly weaker motor activation. This finding contradicts with the study of Pommier et al. demonstrating that the administration of morphine and inhibition of enkephalin metabolism increases the locomotor activity in mice without CCK_2 receptors [23]. The reason for these differences is unclear but could be attributed to the different research design and distinct genetic background of mice used in these studies. Moreover, the current study established dissociation in the action of morphine in the motor activity and place preference tests. It is noteworthy that a significantly lower dose of morphine was effective in the place preference test compared to the locomotor activity study. It is possible that the distinct neural circuits are responsible for these two behavioural effects of morphine. The stronger effect of morphine on the locomotor activity in the wild-type (+/+) mice may be a reason why a higher dose of morphine causes a weaker effect in the place preference studies in the wild-type (+/+) mice compared to mutant animals. Increased motor stereotypy (psychosis-like behaviour) in the wild-type (+/+) mice under the influence of morphine may cause impaired perception of the surrounding environment and reduce the conditioning to the drug-paired environment in comparison with the mutant animals. Interestingly, Maldonado et al. have described dissociation between the opioid-mediated motor and motivational responses in mice lacking dopamine D_2 receptors [19]. They established that morphine-induced place preference was absent in these mice, whereas morphine-induced hyper-locomotion remained unchanged compared with the wild-type (+/+) mice. This study clearly demonstrates a role of the dopamine D_2 receptors of the nucleus accumbens in the mediation of the rewarding effects of morphine, whereas dopamine D_1 receptors and non-dopaminergic mechanisms are responsible for opioid-induced enhancement of locomotion [4,11,18,19].

Third, differently from the study of Pommier et al. only a high dose (10 mg/kg) of naloxone reduced the horizontal component of locomotor activity in mice, and this effect was similar in the wild-type (+/+) and mutant mice [23]. Nevertheless, the lower dose of naloxone (1 mg/kg) antagonised the increased frequency of rearing established in the homozygous (−/−) mice showing that the elevation of this behavioural parameter is probably due to the increased function of endopioid system in mice, lacking CCK_2 receptors. Moreover, we established that naloxone caused the different effect in the wild-type (+/+) and homozygous (−/−) mice adapted to the motility boxes. In these mice the high dose of naloxone (10 mg/kg) induced a significant inhibition of locomotor activity in the homozygous (−/−), but not in the wild-type (+/+) mice. It has been shown that naloxone at this high dose is lacking selectivity for the subtypes of opioid receptors [31]. Nevertheless, it is likely that the impaired adaptation of CCK_2 receptor-deficient mice to the novel environment is due to the increased function of endopioid system. In the place conditioning experiments a lower dose of naloxone (1 mg/kg) tended to cause a conditioned place aversion in the wild-type (+/+) mice, whereas a high dose of opioid antagonist (10 mg/kg) caused a significant place aversion. By contrast, naloxone-induced place aversion was weaker in mice without CCK_2 receptors, because the high dose of naloxone (10 mg/kg) only tended to shift the behaviour of mutant mice from the non-preferred to preferred side. Consequently, there is also dissociation of the behavioural effects of naloxone in the CCK_2 receptor-deficient mice. These data may also reflect an increased tone of the endopioid system in the neural circuits responsible for the development of place aversion in mutant mice. It was shown that naloxone (10 mg/kg) failed to produce conditioned place aversion in μ-opioid receptor-deficient mice, whereas the effect of μ-opioid agonist U50,488H remained unchanged [29]. This finding obviously supports the involvement of μ-opioid receptors in the mediation of naloxone-induced place aversion. This behavioural phenomenon can be induced by the local administration of naloxone into the ventral tegmental area and nucleus accumbens, but not into the striatum and medial prefrontal cortex [28]. Recent evidence suggests that naloxone-induced action is not linked only to the mesolimbic dopaminergic system, because the blockade of μ-opioid receptors in the dorsal periaquaductal gray matter caused the conditioned place aversion [25]. We did not find differences in the density of opioid and dopamine D_2 receptors [16] in the mesolimbic area, but we established a significant reduction of pain sensitivity in the CCK_2 receptor-deficient mice [35]. The role of periaquaductal gray matter in the regulation of pain sensitivity is well-known [20]. Therefore, we are tempting to speculate that the increased tone of endopioid system in this brain region could be linked to the reduced effect of naloxone in homozygous (−/−) mice. Moreover, to some extent this study tends to support the finding of pharmacological experiments showing that the CCK_2 receptor antagonists attenuate naloxone-induced place aversion in rats [34].

Fourth, we were unable to repeat the results of radioligand binding studies performed by Pommier et al. [23]. First, they found that the number of opioid receptors was decreased if measured in the whole brain of CCK_2 receptor-deficient mice in the in vivo conditions. Pommier et al [23] suggested that this effect was due to the increased levels of endopioid peptides in the brain of homozygous (−/−) mice. Second, in the in vitro studies they did not found any changes in the parameters of opioid receptors if the membranes were prepared from the whole brain [23]. We measured the density of opioid receptors by [^{3}H]-diprenorphine in four distinct forebrain structures (cerebral cortex, striatum, mesolimbic area, and hippocampus), and at least in these structures no decline in the density of opioid receptors was observed. Indeed, the number of opioid receptors was elevated in the striatum of mutant mice, whereas the other structures show no difference in the density of receptors between the homozygous (−/−) and wild-type (+/+) animals. On the other hand, the affinity of opioid receptors was reduced in the striatum. The reduced affinity of opioid receptors in the striatum could support the initial idea of Pommier et al.

[23] that the levels of endopioid peptides are increased in certain brain regions and therefore the affinity of opioid receptors is decreased. A micro-array study was performed in the striatum in order to reveal the differences between the wild-type (+/+) and homozygous (−/−) mice in the gene expression (our unpublished data). According to this, the expression of the μ-opioid receptor gene was increased, whereas the expression of pre-proenkephalin, a precursor molecule of enkephalins, and nociceptin genes was reduced. By contrast from the striatum the affinity of opioid receptors was increased in the cerebral cortex of mutant mice compared to wild-type (+/+) littermates. Accordingly, the targeted mutation of CCK_2 receptors affects differently the parameters of opioid receptors in the forebrain structures. These distinct changes in the sensitivity of opioid receptors in various brain structures may explain dissociation of behavioural effects of morphine and naloxone described in animals without CCK_2 receptors. The mesolimbic structures, and especially the *nucleus accumbens*, are targets for the rewarding effect of morphine [19]. However, we did not find any differences in the parameters of opioid receptors in the mesolimbic area, and no change in morphine-induced place preference was established when the wild-type (+/+) and mutant mice were compared. Maldonado et al. demonstrated the role of dopamine D_2 receptors in the *nucleus accumbens* for the motivational effects of morphine [19]. Our previous experiments did not reveal any differences in the density of dopamine D_2 receptors in the mesolimbic area of wild-type (+/+) and CCK_2 receptor-deficient mice [19]. Accordingly, the reduction of morphine-induced motor stimulation in mutant mice has to be explained by other mechanisms than opioid and dopamine D_2 receptors in the mesolimbic structures. It could be associated with changes in the parameters of opioid receptors in the striatum where the increased number and reduced affinity was found. This may reflect an increased tone of the endogenous opioid system in the striatum and could explain the reduced morphine-induced motor stimulation in mice without CCK_2 receptors. Altogether, the distinct changes of opioid receptors in the striatum and mesolimbic area are most likely linked to the dissociation of behavioural effects of morphine in CCK_2 receptor-deficient mice.

In conclusion, the present study to some extent supports the initial idea of Pommier et al. that the activity of the endogenous opioid system is increased due to the targeted invalidation of CCK_2 receptor gene in mice [23]. However, we established a clear dissociation of the behavioural effects of morphine and naloxone in mice, lacking CCK_2 receptors. Dissociation of behavioural effects of morphine was also established in the previous studies where the anti-nociceptive action of μ-opioid agonist was studied in mice with the corrupted function of CCK_2 receptors [23,35]. The molecular background of this phenomenon is unclear but could be explained in the light of data that the targeted mutation of CCK_2 receptors induces distinct changes in the properties of opioid receptors in various brain structures.

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References

[1] Bespalov AY, Tokarz ME, Bowen SE, Balster RL, Beardsley PM. Effects of test conditions on the outcome of place conditioning with morphine and naltrexone in mice. Psychopharmacology 1999;141:118–22.
[2] Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[3] Crawley JN. What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice. New York: Wiley.
[4] Daugé V, Rossignol P, Roques BP. Blockade of dopamine receptors reverses the behavioral effects of endogenous enkephalins in the nucleus caudatus but not in the nucleus accumbens: differential involvement of delta and mu opioid receptors. Psychopharmacology 1989;99:168–75.
[5] Daugé V, Beslot F, Matsui T, Roques BP. Mutant mice lacking the cholecystokinin$_2$ receptor show a dopamine-dependent hyperactivity and a behavioral sensitization to morphine. Neurosci Lett 2001;306:41–4.
[6] Faris PL, Komisaruk BR, Watkins LR, Mayer DJ. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. Science 1983;219:310–2.
[7] Franklin KBJ, Paxinos G. The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 1997.
[8] Gall C, Lauterborn J, Burks D, Seroogy K. Co-localization of enkephalin and cholecystokinin in discrete areas of rat brain. Brain Res 1987;403:403–8.
[9] Ghilardi JR, Allen CJ, Vigna SR, McVey DC, Mantyh PW. Trigeminal and dorsal root ganglion neurons express CCK receptor binding sites in the rat, rabbit, and monkey: possible site of opiate-CCK analgesic interactions. J Neurosci 1992;12:4854–66.
[10] Higgins GA, Nguyen P, Seller EM. Morphine place conditioning is differentially affected by CCK_A and CCK_B receptor antagonists. Brain Res 1992;572:208–15.
[11] Kalivas PW, Widerlow E, Stanley D, Breese G, Prange AJ. Enkephalin action on the mesolimbic system: a dopamine-dependent and a dopamine-independent increase in locomotor activity. J Pharmacol Exp Ther 1983;227:229–37.
[12] Kõks S, Vasar E, Soosaar A, Lang A, Volke V, Võikar V, et al. Relation of exploratory behavior of rats in the elevated plus-maze to brain receptor binding properties and serum growth hormone level. Eur Neuropsychopharmacol 1997;7:289–94.
[13] Kõks S, Soosaar A, Võikar V, Volke V, Ustav M, Männistö PT, et al. Opioid antagonist naloxone potentiates anxiogenic-like action of cholecystokinin antagonists in elevated plus-maze. Neuropeptides 1998;32:235–40.
[14] Kõks S, Soosaar A, Võikar V, Bourin M, Vasar E. BOC-CCK-4, CCK_B receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides 1999;33:63–9.
[15] Kõks S, Bourin M, Vasar E. Adaptation of rats to the experimental conditions modifies the behavioral effects of naloxone and morphine in the elevated zero-maze. Eur Neuropsychopharmacol 2000;10(suppl 2):S66.
[16] Kõks S, Volke V, Veraksitš A, Rünkorg K, Sillat T, Abramov U, et al. Cholecystokinin$_2$ receptor deficient mice display altered function of brain dopaminergic system. Psychopharmacology 2001;158:198–204.

[17] Lavigne GJ, Millington WR, Mueller GP. The CCK-A and CCK-B receptor antagonists, devazepide and L-365260, enhance morphine antinociception only in non-acclimated rats exposed to a novel environment. Neuropeptides 1992;21:119–29.
[18] Longoni R, Spina L, Di Chiara G. Dopaminergic D1 receptors: essential role in morphine-induced hypermotility. Psychopharmacology 1987;93:401–2.
[19] Maldonado R, Saiardi A, Valverde O, Sama TA, Roques BP, Borrelli E. Absence of opiate rewarding effects in mice lacking dopamine D_2 receptors. Nature 1997;388:586–9.
[20] Mason P. Central mechanisms of pain modulation. Curr Opin Neurobiol 1999;9:436–41.
[21] Nagata A, Ito M, Iwata N, Kuno J, Takano H, Minowa O, et al. G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proc Natl Acad Sci USA 1996;93:11825–30.
[22] Noble F, Blommaert A, Fournie-Zaluski MC, Roques BP. A selective CCK_B receptor antagonist potentiates μ- but not d-opioid receptor-mediated antinociception in the formalin test. Eur J Pharmacol 1995;273:145–51.
[23] Pommier B, Beslot F, Simon A, Pophillat M, Matsui T, Daugé V, et al. Deletion of CCK_2 receptor in mice results in an upregulation of the endogenous opioid system. J Neurosci 2002;22:2001–11.
[24] Saito A, Sankaran H, Goldfine ID, Williams JA. Cholecystokinin receptors in the brain: characterization and distribution. Science 1980;208:1155–6.
[25] Sante AB, Nobre MJ, Brandao ML. Place aversion induced by blockade of mu or activation of kappa opioid receptors in the dorsal periaquaductal gray matter. Behav Pharmacol 2000;11:583–9.
[26] Schnur P, Cesar SS, Federaro MA, Kulkosky PJ. Effects of cholecystokinin on morphine-elicited hyperactivity in hamsters. Pharmacol Biochem Behav 1991;39:581–6.
[27] Sebret A, Lena I, Crete D, Matsui T, Roques BP, Daugé V. Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. J Neurosci 1999;19:7230–7.
[28] Shippenberg TS, Bals-Kubik R. Involvement of the mesolimbic dopamine system in mediating aversive effects of opioid antagonists in the rat. Behav Pharmacol 1995;6:99–106.
[29] Skoubis PD, Matthes HW, Walwyn WM, Kieffer BL, Maidment NT. Naloxone fails to produce conditioned place aversion in mu-opioid receptor knock-out mice. Neuroscience 2001;106:757–63.
[30] Smadja C, Maldonado R, Turcaud S, Fournie-Zaluski MC, Roques BP. Opposite role of CCK_A and CCK_B receptors in the modulation of endogenous enkephalin antidepressant-like effects. Psychopharmacology 1995;120:400–8.
[31] Tsuda M, Suzuki T, Misawa M, Nagase H. Involvement of the opioid system in the anxiolytic effect of diazepam in mice. Eur J Pharmacol 1996;307:7–14.
[32] Tzschentke TM. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog Neurobiol 1998;56:613–72.
[33] Valverde O, Fournie-Zaluski MC, Roques BP, Maldonado R. The CCKB antagonist PD-134,308 facilitates rewarding effects of endogenous enkephalins but does not induce place preference in rats. Psychopharmacology 1996;123:119–26.
[34] Valverde O, Roques BP. Cholecystokinin modulates the aversive component of morphine withdrawal syndrome in rats. Neurosci Lett 1998;244:37–40.
[35] Veraksitš A, Rünkorg K, Kurrikoff K, Raud S, Abramov U, Matsui T, et al. Altered pain sensitivity and morphine-induced anti-nociception in mice lacking CCK_2 receptors. Psychopharmacology DOI 10.1007s00213-002-1333-6; 2003.
[36] Wiertelak EP, Maier SF, Watkins LR. Cholecystokinin anti-analgesia: safety cues abolish morphine analgesia. Science 1992;256:830–3.

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