DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

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BEHAVIOURAL AND NEUROGENETIC STUDY OF MECHANISMS RELATED TO CAT ODOUR INDUCED ANXIETY IN RODENTS

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ABBREVIATIONS

5-HT	5-hydroxytryptamine or serotonin
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
apoE	apolipoprotein E
BLA	basolateral nucleus of amygdala
BDZ	benzodiazepine
CCK	cholecystokinin
CCK-4	cholecystokinin tetrapeptide
CCK-8	cholecystokinin octapeptide
Cck	gene encoding cholecystokinin
CCK_1	cholecystokinin-1 receptor
CCK_2	cholecystokinin-2 receptor
Cckar	gene encoding CCK_1
Cckbr	gene encoding CCK_2
cDNA	complementary DNA synthesised from mature mRNA
cDNA-RDA	cDNA representational difference analysis
CEA	central nucleus of the amygdala
CNS	central nervous system
CPE	carboxypeptidase E
CRF	corticotropin releasing factor
DOR	δ-opioid receptor
DP	differential product
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4^{th} edition
EPM	elevated plus-maze
EST	expressed sequence tag
EZM	elevated zero-maze
GABA	γ-aminobutyric acid
$GABA_A$	GABA-A receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HPA	hypothalamic-pituitary-adrenal
KOR	κ-opioid receptor
LD	light-dark test
LPH	lipotrophin
LsAMP	limbic system-associated membrane protein
MC3R	melanocortin 3 receptor
Mc3r	gene encoding MC3R
MC4R	melanocortin 4 receptor
Mc4r	gene encoding MC4R
MOR	μ-opioid receptor
mRNA	messenger RNA

MSH	melanocortin
NA	noradrenalin
NMP35	neural membrane protein 35
Oprd1	gene encoding DOR
Oprk1	gene encoding KOR
Oprm1	gene encoding MOR
PAG	periaqueductal gray
PCR	polymerase chain reaction
PDYN	pre-pro-dynoprhin
Pdyn	gene encoding PDYN
PENK	pre-pro-enkephalin
Penk1	gene encoding PENK
POMC	pro-opio-melanocortin
Pomc1	gene encoding POMC
qRT-PCR	quantitative real-time PCR
RPC	*nucleus reticularis pontis caudalis*
RT-PCR	reverse transcription PCR
S.E.M.	standard error of mean values
TMT	2,5-dihydro-2,4,5-trimethylthiazoline
USV	ultrasound vocalisation
β-END	β-endorphin

I. INTRODUCTION

Interest concerning the molecular mechanisms of anxiety is driven by the fact that these disorders are highly prevalent in the population and are associated with high levels of morbidity as well as with great economic cost. It is estimated that anxiety disorders affect up to 25% of the population at some point in their lifetime, with an annual estimated cost of $44 billion in the United States alone (Greenberg *et al.* 1999; Hettema *et al.* 2001; Kessler *et al.* 1994). Although anxiety is a normal cognition at its entity, manifestation of pathological anxiety causes severe distress and disturbs severely ability to work and everyday life (Arikian & Gorman 2001). Patients with anxiety disorders develop cerebrovascular, cardiovascular, gastrointestinal, hypertensive, and respiratory disorders at a higher rate than does the general population (Bowen *et al.* 2000). Anxiety accompanying somatic illness further increases the disability experienced by sufferers, aggravates the rendering to therapy, and increases both direct and indirect expenses (Lecrubier 2001).

Studies about the mechanisms of anxiety and other emotions have lasted tens of years. Several neural networks and circuits have been mapped and several transmitter systems pointed out. Our current understanding of the molecular mechanisms of anxiety disorders is still very much based on the effectiveness of drugs, interacting with γ-aminobutyric acid (GABA) A-type receptor complex ($GABA_A$) and inhibiting serotonin (5-HT) and noradrenaline (NA) reuptake, in the treatment of pathological anxiety. However, drugs used for the treatment of anxiety are far from perfect. Benzodiazepine agonists, increasing the affinity of $GABA_A$ receptors for the endogenous GABA, have a high abuse potential, and antidepressant drugs, inhibiting 5-HT and NA reuptake, increase anxiety if too high doses are administered in the beginning of treatment. Although effective treatments are available, we still have only modest success in the treatment of these disorders, largely due to unknown molecular and neuronal mechanisms (Gorman 2003; Shelton & Brown 2001). Therefore, an attempt was made in the current study to extend our knowledge of the molecular mechanisms of anxiety. This kind of basic research can only be performed on laboratory animals. However, it means that relevant animal models have to be used for that purpose. Since mice and rats are commonly used for the search of new targets related to the regulation of anxiety, anxiety induced by cat odour seems to be a model of choice. Cat odour is a natural stimulus to induce strong emotional stress in rodents. This model engages specific neural pathways shaped by the mechanisms of natural selection and evolution. There is evidence that the amygdala plays a central and critical role in the neural circuits involved in the response to cat odour (LeDoux 2000). Nevertheless, in two parts of a study performed on Wistar rats and 129Sv/C57Bl6 mice we compared the effect of the cat odour exposure on the interaction of two antagonistic neuropeptide systems — opioid and cholecystokinin — in various brain structures, including

the amygdala. Indeed, these experiments supported the existing evidence and demonstrated that the cat odour exposure induced the biggest changes in the expression of the Pomc1 gene, playing a significant role in the mechanisms of stress, in the amygdala. Therefore, the final part of the study was performed on amygdala to establish by means of the cat odour exposure new molecular targets in the regulation of anxiety. For this purpose, we applied a method called cDNA representational difference analysis (cDNA-RDA). This method is advocated as highly sensitive helping to detect the smallest differences even for rare mRNA transcripts (Diatchenko *et al.* 1996)**.** Several genes (Wfs1, Lsamp and Gamm1) induced by cat odour were chosen for further studies in order to reveal their role in the regulation of emotional behaviour.

II. REVIEW OF LITERATURE

1. Neurobiology of anxiety

Wariness of threatening aspects of the environment is a protective, if at times uncomfortable, trait. In humans, the experience of such wariness is called anxiety, and it is typically accompanied by characteristic autonomic responses and defensive behaviours (Leonardo & Hen 2006). Anxiety is a normal response to threatening situations. Perceived threats that generate anxiety may be active and direct or indirect, such as the absence of people or objects that represent security. Anxiety is adaptive; it signals potential danger and can contribute to the mastery of a difficult situation and thus to personal growth (Kandel 2000). In its non-pathological form, anxiety can be divided into two categories: 'state' anxiety, a measure of the immediate or acute level of anxiety, and 'trait' anxiety, which reflects the long-term tendency of an individual to show an increased anxiety response (Belzung & Griebel 2001). Excessive anxiety on the other hand is maladaptive, either because it is too intense or because it is inappropriately provoked by events that present no real danger. Thus, anxiety is pathological when excessive and persistent, or when it no longer serves to signal danger (American Psychiatric Association 1994; Kandel 2000; World Health Organization 1992).

1.1. Neuroanatomy of anxiety

Neuroanatomical substrate of anxiety has been a target of scientific studies for a long time. The first implications about mapping different brain functions are about 80 years old. In 1937, James Papez (Papez 1995) proposed that the cortical machinery for feeling involves the *limbic lobe,* a region identified by Paul Broca (Broca 1878). The neural circuits underlying anxiety are organised at different levels, with each system having the ability to feed back onto those below it (Figure 1) (Finn *et al.* 2003). Simple automatic responses are mediated by the periaqueductal gray (PAG) and locus coeruleus. The PAG has been hypothesised to coordinate specific response patterns, based on the nature of threatening stimulus, via amygdaloid pathways (Behbehani 1995). The locus coeruleus, a critical component of efferent response systems, is activated by stress and aversive stimuli and has been implicated in the orchestration of the fundamental "alarm reaction" (Sullivan *et al.* 1999). The hypothalamus is a critical site for the integration of the autonomic and neuroendocrine responses to threat or anxiety, namely the activation of the hypothalamic-pituitary adrenal (HPA) axis and sympathoadrenal system, through the release of peptides and releasing hormones (Korte 2001). Intermediate levels that mediate more practiced responses include the amygdala and septohippocampal systems. The

amygdala has extensive connections to the cortex and locus coeruleus and projections to the striatum, midbrain, and brainstem and thus, plays a pivotal role in the assessment of, and response to, danger (Cahill & McGaugh 1998; Davis 1997; LeDoux 2000). The role of the hippocampus has been described as a 'comparator system' that can detect whether a threat is familiar or novel, requiring either a conditioned automatic response or higher order processing, respectively (Vinogradova 2001). Higher cortical regions, such as the paralimbic cortex (e.g. orbitofrontal, insular, anterior temporal and anterior cingulate) and prefrontal cortex manage more cognitively demanding stimuli. The orbitofrontal cortex is involved in determining the significance of anxiety producing sensory events, the choice and implementation of behaviours important for survival, and the extinction of conditioned fear responses via its reciprocal interactions with the amygdala (Bechara *et al.* 1994; Davidson 2002). The medial prefrontal cortex also has projections to the amygdala that are involved in the suppression of responses to anxiety related stimuli, helping to regulate the peripheral responses to stress (Gewirtz *et al.* 1997).

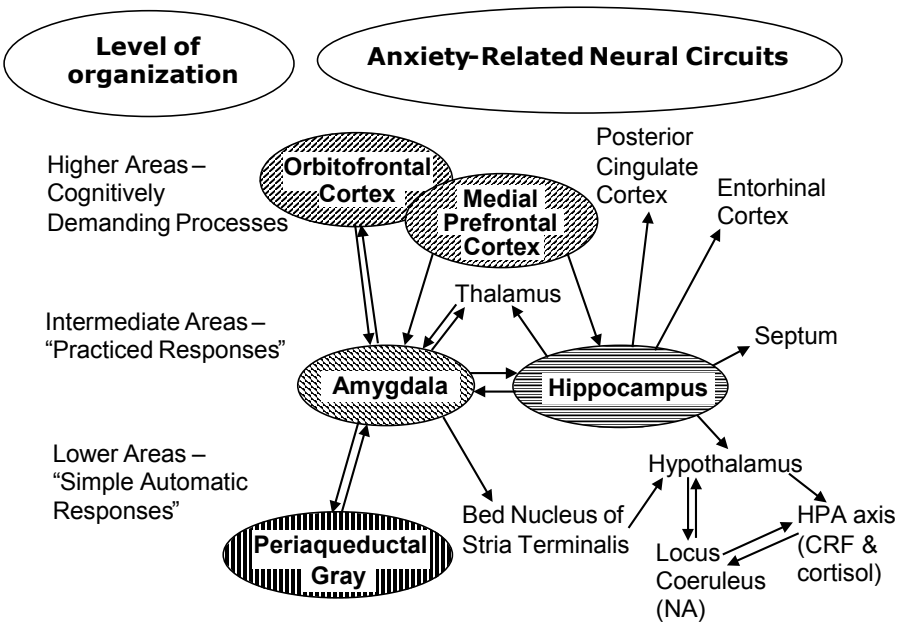

Figure 1. Organisation of neural circuits underlying anxiety (modified from Finn et al., 2003).

In the centre of anxiety regulating structures is a complex of nuclei at the temporal lobe, next to anterior end of the hippocampus, named amygdala. The amygdala plays an essential role in determining the emotional significance of

sensory stimuli, in the acquisition, consolidation, and retrieval of emotional memories, and in the generation of fear-related responses (Cahill & McGaugh 1998; Davis 1997; LeDoux 2000). It mediates both inborn and acquired emotional responses. The amygdala is a set of dense nuclei, the largest regions of which are generally divided into the central and lateral nuclei and also basal and accessory basal nuclei, often referred to as the basolateral nucleus (BLA). Numerous studies using electric shock have implicated the central nucleus of the amygdala (CEA) in playing a critical role in the production of conditioned fear behaviour (Davis & Whalen 2001; LeDoux 2000). Recently Rosenkranz and colleagues have presented that, contrary to conventional views, BLA stimulation primarily inhibits medial CEA neurons by a polysynaptic circuit, and showed that single medial CEA neurons respond to both BLA input and footshock in an opposite manner (Rosenkranz *et al.* 2006). The CEA is the main processing and output station to brain regions that regulate autonomic and behavioural responses in anxiety, such as the locus coeruleus, PAG and hypothalamus (Kim & Gorman 2005). The CEA is the interface with motor systems. Damage to the CEA interferes with the expression of conditioned fear responses (Gentile *et al.* 1986; Hitchcock & Davis 1986; Iwata *et al.* 1986; Kapp *et al.* 1979; Van de Kar *et al.* 1991), while damage to areas that the CEA projects to selectively interrupts the expression of individual responses. For example, damage to the lateral hypothalamus affects blood pressure but not freezing responses, and damage to the PAG interferes with freezing but not blood pressure responses (LeDoux *et al.* 1988).

1.2. Neurochemistry of anxiety

Symptoms of anxiety are mediated by the actions of specific neurotransmitters and neuropeptides. Most of the work on the neurochemistry of anxiety has focused on NA, 5-HT and GABA. This is due to the fact that the most effective drugs in the treatment of anxiety disorders — benzodiazepine anxiolytics and antidepressant drugs — primarily affect the function of these neurotransmitter systems (Asmundson *et al.* 2006). However, recent evidence suggests that various neuropeptides (corticotropin releasing hormone, cholecystokinin, opioid peptides, melanocortins, vasopressin, substance P and others) are also implicated in the regulation of emotional behaviour, and particularly anxiety (Holmes *et al.* 2003b; Kim & Gorman 2005; Strohle & Holsboer 2003).

1.2.1. Noradrenaline (NA)

NA-containing neurons in the locus coeruleus interact with the hypothalamic-pituitary-adrenal system. This NA system is responsible for producing the most immediately unpleasant symptoms of anxiety such as the fight or flight

response. A key component to the NA-ergic theory of anxiety is that increased NA release leads to anxiety, due to either excessive or dysfunctional arousal (Sandford *et al.* 2000). Animal studies have shown that stressful stimuli increase NA turnover in the locus coeruleus, hypothalamus, hippocampus, amygdala and cortex (Bremner *et al.* 1996b). Clinical studies have found that some chronic symptoms of anxiety disorders are consistent with increased NA-ergic function (Charney *et al.* 1984; Charney *et al.* 1987). Thus, neurons in the locus coeruleus appear to be activated in association with stressful stimuli and anxiety states (Bremner *et al.* 1996a). The release of NA from the locus coeruleus leads to the activation of the autonomic nervous system via the hypothalamus and adrenal gland, which releases adrenaline. Direct cardiovascular effects such as increased heart rate and blood pressure result accordingly.

1.2.2. Serotonin (5-HT)

5-HT has also been implicated, given the effectiveness of antidepressant drugs, to reduce symptoms of anxiety disorders. 5-HT receptors are primarily located in the dorsal raphe nuclei of the brainstem, but are also found throughout other parts of the brain and in other organs or tissues (such as the gut and platelets). Exposure to a variety of stressful stimuli in animals produces an increase in the level of 5-HT in the medial prefrontal cortex, nucleus accumbens, amygdala, and lateral hypothalamus (Inoue *et al.* 1994). The 5-HT-ergic innervation of the amygdala and hippocampus via the dorsal raphe is thought to mediate anxiogenic effects through an action at 5-HT_2 receptors, whereas the median raphe innervation of hippocampal 5-HT_{1A} receptors may provide resilience to aversive events (Graeff 1993; Grove *et al.* 1997). Although the general trend in research results is that the symptoms of anxiety can be associated with 5-HT-ergic dysfunction, the question of whether it is related to hyperactivity or hypoactivity of 5-HT is unclear (Jetty *et al.* 2001). Transgenic mice with the altered function of 5-HT, particularly with the 5-HT_{1A} receptor deletion, have also exhibited increased fear behaviours (Holmes *et al.* 2003a; Lesch *et al.* 2003).

1.2.3. Gamma-aminobutyric acid (GABA)

Benzodiazepines (BDZs) were developed in the 1960s and have been shown to bind with high affinity to an allosteric site on $GABA_A$ receptors (Braestrup & Squires 1978). This receptor complex is unique in that it demonstrates bi-directional agonism (Braestrup *et al.* 1983). In addition to agonists at the BDZ site, there are antagonists and inverse agonists (Barnard *et al.* 1998). Several lines of preclinical evidence support the hypothesis that alterations in the BDZ receptor function play a role in anxiety disorders (Guidotti *et al.* 1980). $GABA_A$

receptors are found throughout the brain, including regions implicated in anxiety disorders like the amygdala and the trajectory of the rest of the"fear" circuit, from the amygdala, downward via the ventral amygdalofugal pathway, through the anterior and medial hypothalamus, and down across the substantia nigra to the PAG and the *nucleus reticularis pontis caudalis* (the RPC), where fear modulation of the startle reflex occurs (Panksepp 1998). Transgenic mice with an altered function of GABA exhibit fear-related behaviours such as behavioural inhibition and increased response to fearful stimuli, particularly when the $GABA_A$ type receptor is involved (Crestani *et al.* 1999).

1.2.4. Cholecystokinin (CCK)

In 1975 Vanderhaegen and colleagues published a report concerning a new peptide in the vertebrate central nervous system (CNS) reacting with antigastrin antibodies (Vanderhaeghen *et al.* 1975). This gastrin-like immunoreactivity turned later out to be CCK (Dockray & Taylor 1976; Rehfeld 1985). Regional distribution of CCK varies widely with high levels (>4 ng CCK/mg protein) in the cerebral cortex, caudate-putamen, hippocampus, and amygdala; moderate levels (1–2 ng/mg protein) in the thalamus, hypothalamus and olfactory bulb; and low levels (<1 ng/mg protein) in the pons, medulla, and spinal cord. CCK is barely detectable in the cerebellum by radioimmunoassay (Beinfeld *et al.* 1981). CCK receptors exist as two major subtypes: CCK_1 and CCK_2, which differ in terms of molecular structure, distribution, and affinity for the natural ligands CCK and gastrin (Noble *et al.* 1999). The CCK_1 receptor was first characterised in pancreatic acinar cells (Sankaran *et al.* 1980), whereas the CCK_2 receptor was discovered in the brain (Innis & Snyder 1980). CCK_1 receptors bind cholecystokinin octapeptide (CCK-8) that is sulphated with high affinity, but CCK_2 receptors are less selective than CCK_1 receptors as they also bind desulphated CCK-8, gastrin and cholecystokinin tetrapeptide (CCK-4) with high affinity (Woodruff & Hughes 1991). Both receptors belong to the G protein-coupled super-family of receptors (Merritt *et al.* 1986; Roche *et al.* 1990). The CCK_2 receptors cloned from humans and mice have the same structural features as the rat CCK_2 receptor (Ito *et al.* 1993; Landgrebe *et al.* 2002). CCK_2 receptors, the predominant subtype in the CNS, are present with the highest concentration in the cerebral cortex, limbic system (the olfactory tubercles, hippocampus, nucleus accumbens, and amygdala), striatum, hypothalamus, ventral tegmentum, and dorsal raphe nuclei (Beinfeld 1983; Honda *et al.* 1993; Innis & Snyder 1980). CCK_1 receptors are located mainly in peripheral tissues. However, CCK_1 receptors occur in certain brain regions, including area postrema, nucleus of the solitary tract, and interpeduncular nucleus (Moran *et al.* 1986).

The ability of CCK to induce anxiety was discovered serendipitously by Jens Rehfeld. In 1969, when he injected 70 μg of tetragastrin (CCK-4) to volunteers

to assess insulin secretion, the subjects became immediately after the medication quiet and fearful (Rehfeld 1992). Early electrophysiological studies revealed that CCK-8 can produce marked excitation of the cortical and hippocampal neurons and this effect can be antagonised by benzodiazepine anxiolytics (Bradwejn & De Montigny 1984). Further investigation of the effects of CCK-4 in psychiatric patients and healthy volunteers confirmed the panic-like action of CCK-4 (De Montigny 1989; Koszycki *et al.* 1991; Shlik *et al.* 1997b). The anxiogenic like potency of CCK-related peptides was established in different species including mice, rats, cats and monkeys (Fekete *et al.* 1984; Harro *et al.* 1990; Harro & Vasar 1991; Palmour *et al.* 1992; Singh *et al.* 1991). The anxiogenic-like action of CCK agonists is mediated via CCK_2 receptors (Hernandez-Gomez *et al.* 2002; Noble *et al.* 1999; Shlik *et al.* 1997a). Anxiogenic manipulations, like social isolation, increase the density of CCK_2 receptors in the frontal cortex of rodents (Shlik *et al.* 1997a; Vasar *et al.* 1993). Female CCK_2 receptor deficient mice display reduced anxiety in ethological models compared to their wild-type littermates (Raud *et al.* 2003; Raud *et al.* 2005). Several lines of evidence demonstrate that CCK interacting with CCK_2 receptors in the brain antagonises the function of the GABA and opioid system (Acosta 2001; Siniscalchi *et al.* 2001; Wiesenfeld-Hallin *et al.* 1999). Therefore, CCK_2 receptor deficient mice display an increased function of the GABA and opioid system (Pommier *et al.* 2002; Raud *et al.* 2003; Raud *et al.* 2005; Veraksitš *et al.* 2003).

1.2.5. Opioid and melanocortin systems

Pro-opio-melanocortin (POMC) is a precursor peptide for several neurohormones: adrenocorticotropic hormone (ACTH), lipotrophins (LPHs), melanocyte-stimulating hormones also known as melanocortins (MSHs) and β-endorphin (β-END) (Chretien *et al.* 1979). Studies dedicated on the structure of the POMC encoding gene Pomc1 by restriction enzyme techniques have shown that at least 6 hormones are derived from one gene (Pritchard *et al.* 2002; Raffin-Sanson *et al.* 2003): ACTH, β-LPH, γ-LPH, α-MSH, β-MSH, γ-MSH, and endorphin (Figure 2). It has been shown that Pomc1 is expressed in certain brain regions, such as the amygdala, cerebral cortex, and mainly within the arcuate nucleus of hypothalamus (Civelli *et al.* 1982; Gee *et al.* 1983; Krieger & Liotta 1979). In the human, bovine, rat and mouse, the Pomc1 gene is similarly constituted of three exons and two introns (Cochet *et al.* 1982; Drouin *et al.* 1985; Nakanishi *et al.* 1981; Notake *et al.* 1983; Uhler *et al.* 1983).

ß-endorphin and μ-opioid receptors (MOR)

Martin and co-workers (Martin 1979) provided evidence for multiple opioid receptors when they demonstrated that a series of opioid peptides displayed different profiles of pharmacological activity *in vivo*. They proposed that opioid

peptides activated three different types of receptors, called μ, κ and δ. β-END as an endogenous ligand to opioid receptors was discovered by Hans Kosterlitz (Kosterlitz 1979). β-END appears to be an important mediator released from the brain and pituitary. This compound is equipotent at μ- and δ-receptors but with much lower affinity for κ-receptors (Corbett *et al.* 1993). The μ-opioid receptor is the primary site of action for the most commonly abused opiates, including morphine, heroin, fentanyl, and methadone. In the CNS, MOR encoding gene (Oprm1) expression has been ascertained using autoradiographic and mRNA expression studies in almost all rat brain areas, including higher expression areas like the amygdala, bed nucleus of stria terminalis, thalamus, hippocampus, nucleus accumbens, locus coeruleus and neocortex (Mansour *et al.* 1994; Tempel & Zukin 1987). MOR deficient mouse pups emit fewer ultrasonic vocalisations when removed from their mothers but not when exposed to cold or male mice odours (Moles *et al.* 2004). An endogenous tone of this receptor modulates nociception and mediates stress-induced analgesia (Gaveriaux-Ruff & Kieffer 2002). Most of the β-END within the brain arises from a clustered group of neurons within the medial hypothalamus and send projections to the PAG and to noradrenergic nuclei in the brain stem (Panksepp 1998). Both β-END and ACTH are released into the bloodstream in response to stress.

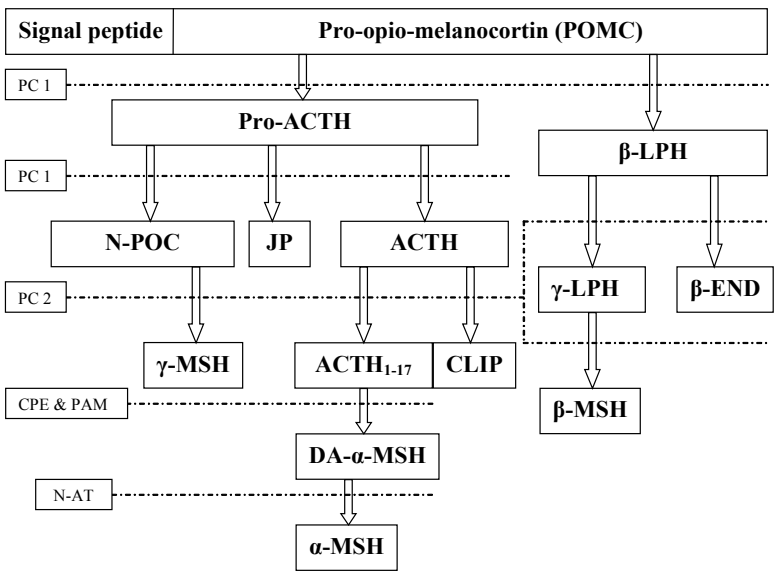

Figure 2. Peptides processed from the proteolytic cleavage of POMC. Pro-ACTH — ACTH prohormone, $ACTH_{1-17}$ — truncated ACTH with aminoacids 1-17 preserved, CLIP — corticotrophin-like intermediate protein, β-END — β-endorphin, LPH — lipotrophin, MSH — melanocortin, DA-α-MSH — desacetyl α-melanocortin, N-POC — N-terminal part of POMC, JP — joining peptide, PC — prohormone converting enzyme, CPE — carboxypeptidase E, PAM — peptidyl α-amidating mono-oxygenase, N-AT — n-acetyltransferase.

Pre-pro-enkephalin (PENK) and δ-opioid receptors (DOR)

The identification of the δ-receptor followed the discovery of the first endogenous opioid receptor ligands, [Met]- and [Leu]-enkephalin (Hughes *et al.* 1975), when it was shown that their pattern of agonist activity *in vitro* differed from that of prototypical opioid ligands (Lord *et al.* 1977). Precursor peptide pre-pro-enkephalin contains four copies of [Met]-enkephalin, one copy of [Leu]-enkephalin and one copy each of the octapeptide [Met]-enkephalyl-Arg-Gly-Leu and the heptapetide [Met]-enkephalyl-Arg-Phe, which forms the C-terminus of the precursor. [Met]- and [Leu]-enkephalins have high affinities for δ-receptors (Corbett *et al.* 2006). PENK is widely expressed in neuronal and non-neuronal sites, and its processing can differ markedly between tissues. PENK immunoreactivity is observed in the nucleus accumbens, striatum and olfactory tubercule that was rarely co-expressed with pre-pro-dynorphin immunoreactivity, but sometimes co-expressed with pre-pro-tachykinins (Furuta *et al.* 2002). PENK encoding gene Penk1 deficient mice are more anxious and males display increased offensive aggressiveness. Mutant animals show marked differences from wild-type littermates in supraspinal, but not in spinal, responses to painful stimuli (Konig *et al.* 1996). Based on behavioural studies with Penk1 deficient mice, Ragnauth and colleagues suggest that opioids, particularly Penk1 gene products, are acting naturally to inhibit fear and anxiety (Ragnauth *et al.* 2001).

Autoradiographic and mRNA expression studies have shown that the brain δ-opioid receptors are located in the neocortex, caudate-putamen, nucleus accumbens, olfactory tubercle, diagonal band of Broca, globus pallidus, ventral pallidum, septal nuclei, amygdala, pontine nuclei and ventromedial nucleus of hypothalamus (Mansour *et al.* 1994; Tempel & Zukin 1987). The δ-opioid receptor gene (Oprd1) contains 3 exons encoding a G protein-coupled receptor (Mayer *et al.* 2000). Filliol and colleagues (Filliol *et al.* 2000) generated Oprd1-deficient mice and compared the behavioural responses of mice lacking Oprd1, Oprm1, and gene encoding κ-opioid receptor (Oprk1) in several models of anxiety and depression. Their data showed no detectable phenotype in Oprk1 -/- mutants, suggesting that κ-receptors do not have a role in this aspect of opioid function. Opposing phenotypes in Oprm1 -/- and Oprd1 -/- mutants contrasted with the classic notion of similar activities of μ- and δ-receptors. Anxiogenic- and depressive-like responses in Oprd1 -/- mice indicated that DOR activity contributes to the suppression of negative emotions. It has been concluded that the Oprd1-encoded receptor, proposed to be a promising target for the clinical management of pain, should also be considered in the treatment of drug addiction and other mood-related disorders, and that δ-receptor opposes μ-receptor in several behaviours (Gaveriaux-Ruff & Kieffer 2002).

(Prepro)dynorphin (PDYN) and κ-opioid receptors (KOR)

In 1979, Goldstein and co-workers proposed the existence of 'dynorphin', a potent endogenous opioid peptide with the sequence of [Leu]-enkephalin at its

N-terminus, and this peptide was characterised as the full heptadecapeptide dynorphin A in 1981 (Goldstein *et al.* 1981). Soon afterwards the isolation of other endogenous [Leu]-enkephalin-containing peptides was reported; these peptides (dynorphin A, dynorphin A (1–8), dynorphin B, α- and β-neo-endorphins) were derived from a common precursor, pre-pro-dynorphin (PDYN). The opioid fragments of PDYN have high affinity for κ-receptors but also have significant affinity for μ- and δ-receptors (Corbett *et al.* 1993). It was revealed that dynorphin is widely distributed throughout the neuroaxis. Immunoreactive neuronal perikarya exist in the hypothalamic magnocellular nuclei, PAG, scattered reticular formation sites, and other brain stem nuclei, as well as in spinal cord (Khachaturian *et al.* 1982). Additionally, dynorphin-positive fibers or terminals occur in the cerebral cortex, olfactory bulb, nucleus accumbens, caudate-putamen, globus pallidus, hypothalamus, substantia nigra, PAG, many brain stem sites, and the spinal cord (Khachaturian *et al.* 1982). PDYN is co-localised with cocaine-amphetamine-regulated transcript peptides in both the substantia nigra and ventral tegmental area (Dallvechia-Adams *et al.* 2002).

KOR encoding gene Oprk1 expression has been identified by means of autoradiography and mRNA expression in the nucleus accumbens, caudate-putamen, olfactory tubercle, bed nucleus of striaterminalis, medial preoptic area, paraventricular, supraoptic, dorsomedial and ventromedial hypothalamus, amygdala, midline thalamic nuclei, PAG, raphé nuclei, parabrachial nucleus, locus coeruleus, spinal trigeminal nucleus, nucleus of the solitary tract, substantia nigra, pars compacta, ventral tegmental area and neural lobe of pituitary (Mansour *et al.* 1994; Tempel & Zukin 1987). KOR deficient animals showed no obvious alteration in the perception of thermal or mechanical pain, however, mutant mice displayed a strongly enhanced response to peritoneal acetic acid injections (Simonin *et al.* 1998). Based on studies with Oprk1 and PDYN encoding gene (Pdyn) knockout mice, the pronociceptive activity of Pdyn gene products in long-lasting neuropathic pain has been suggested (Gaveriaux-Ruff & Kieffer 2002).

Melanocortins and their receptors

Melanocortins (α, β and γ-melanocyte-stimulating hormone [MSH]) are ancient peptides that have changed little throughout evolution, being traced back to the appearance of the first vertebrates (Lipton & Catania 1997). The main peptide investigated is α-melanocyte-stimulating hormone (α-MSH), which corresponds to the first 13 amino acids of adrenocorticotrophic hormone ($ACTH_{1–39}$), being N-terminally acetylated and C-terminally amidated. The melanocortins all share a common amino acid sequence His-Phe-Arg-Trp, this being the minimum sequence required for receptor binding, activation and biological effects. Melanocortins exert their biological effects by binding to the family of G-protein-coupled 7 transmembrane receptors (Catania *et al.* 2004). To date 5 melanocortin receptors (MCR), termed MC1R to MC5R, have been cloned. In

the CNS, MC3R and MC4R are present, whereas only a small number of MC1 positive cells in neurons of the periaqueductal gray of rat and human brains can be detected by immunohistochemistry (Xia *et al.* 1995). At the MC3R, $ACTH_{1-39}$, α, β and γ-MSH are equipotent. MC3R is expressed in the CNS, particularly in the hypothalamus (Roselli-Rehfuss *et al.* 1993), peripheral tissues and immune cells, whereas initial studies showed expression in the brain, gut and placenta but not in the adrenal gland or melanocytes (Gantz *et al.* 1993). A potential role in energy metabolism has been postulated for the MC3R since MC3R null mice display increased fat mass and higher ratio of weight gain to food intake (Butler *et al.* 2000).

MC4R is solely expressed in many regions of the brain, including the cortex, hypothalamus, spinal cord and brainstem (Mountjoy *et al.* 1994). Many functions have been attributed to MC4R, including erectile dysfunction (Martin & MacIntyre 2004) and pain (Starowicz & Przewlocka 2003). However, most interest from the pharmaceutical industry has focused on its role in controlling food intake and energy expenditure, and it could also be an exciting target for controlling obesity (Adan *et al.* 2003). Less is known about the role of melanocortin system in the regulation of emotional behaviour. The neuropeptide α-MSH promotes camouflage-type pigmentary changes in many fish and reptiles. When such animals are scared, their skin tends to turn black. Although this peptide does not control skin pigmentation in higher vertebrates, a vigorous freezing/hiding pattern can be evoked in chicks by central administration of this peptide (Panksepp 1998). Rao and colleagues (Rao *et al.* 2003) reported that the anxiogenic-like effect induced by i.c.v. injection of α-MSH in the elevated plus-maze test was attenuated by diazepam as well as mucimol, suggesting the involvement of the GABAergic system in the anxiogenic-like effect of α-MSH. It has been shown that the administration of α-melanocyte stimulating hormone, an endogenous agonist of melanocortin receptors, into the amygdala of rats induces an anxiogenic-like action in the elevated plus-maze (Kokare *et al.* 2005). The administration of various MC4R antagonists induces anxiolytic-like and antidepressant-like effects in different models of stress and anxiety in rodents (Chaki & Okuyama 2005).

2. Animal models of anxiety

In order to understand the molecular mechanisms of anxiety, animal models have been widely employed. Several behavioural tests for assessing anxiety have been put into practice and several classifications of them exist (Finn *et al.* 2003; Hendrie *et al.* 1996; Rodgers 1997; Shekhar *et al.* 2001). The assumption underlying most of these is that anxiety/defence mechanisms are essential for survival and are a feature of mammals. While these tasks are based on putative parallels between human and rodent symptoms of anxiety, there is also growing

evidence that the measures of anxiety-related behaviour assessed in various tasks may reflect different aspects of anxiety-like behaviour in rodents (Shekhar *et al.* 2001). The purpose of each of these models is to attempt to activate endogenous anxiogenic mechanisms and/or to examine them. Applicable models must fulfil certain requirements (Belzung & Griebel 2001):

- *Predictive validity:* Predictive validity implies that the animal model should be sensitive to clinically effective pharmacological agents. Conversely, anxiogenic compounds should elicit opposite effects, while agents that have no effect in the clinic should have no effect in these tests.
- *Face validity:* This criterion implies that the anxiety response observed in the animal model should be identical to the behavioural and physiological responses observed in human. This indicates that the expression of a given emotion is supposed to be similar across species.
- *Construct validity:* This criterion relates to the similarity between the theoretical rationale underlying the animal model and the human behaviour. This requires that the aetiology of the anxiety behaviour and the biological factors underlying anxiety may be similar in animals and humans.

The possible classification of animal models of anxiety is presented in Table 1, where the animal models are considered as conditioned and ethological.

Table 1. Some commonly used animal models of anxiety.

Conditioned	*Unconditioned*
Active/passive avoidance tasks	Open field exploration test
Conditioned emotional responses	Elevated plus-maze and zero-maze
Conditioned taste aversion	Light/dark transition
Punished behaviour tasks	Dark/light emergence
Defensive burying	Social interaction
Stimulation of fear circuits	Ultrasonic vocalisation (pups)
Geller-Seifter conflict	Predator odours

2.1. Conditioned models

Conditioned models involve the pairing of an unconditioned response with (usually) an aversive stimulus and therefore may model reactions to specific aversive events (or stimuli paired with these). As such, the aetiology of anxiety under these conditions may be comparable to normal anxiety in humans as pathologic anxiety states are characterised by an overreaction to events that do not normally constitute a threat. The most back-reaching roots belong to Pavlovian fear conditioning (Davis 1990; Fendt & Fanselow 1999; Maren 2001) based on the classic work of Pavlov (Pavlov 1927). This model measures fear-

related behaviours that are induced by exposure to a previously innocuous stimulus (for example, auditory tone) that has been associated, through repeated pairings, with an innately aversive stimulus (for example, foot-shock). Evolution has favoured this form of learning as a means of rapidly ascertaining which environmental stimuli signal danger, and it is an essential component of many mammalian defensive behaviour systems (Maren 2001). In the laboratory, the degree of conditioned fear can be readily quantified through various behaviours, such as freezing, startle, tachycardia, defensive burying, and ultrasound vocalisations (USVs) (Davis 1990; Fendt & Fanselow 1999; Maren 2001; Stiedl *et al.* 2005). Punishment-based conflict procedures in rats have been used for more than 40 years in the identification and characterisation of anxiolytic agents (Geller & Seifter 1960). A more practical version of the punished-conflict test has been developed by Vogel and colleagues (Vogel *et al.* 1971).

2.2. Ethological models

Based on the assumption that the same neurobiological systems mediate both normal and abnormal anxiety, many animal models of anxiety disorders have therefore tapped knowledge of the natural behavioural patterns of rats and mice to develop ethologically based (unconditioned) behavioural tasks (Rodgers *et al.* 1997). These are 'spontaneous' or 'innate' behavioural patterns responding to certain environmental stimuli (object or subject), which arise without any conditioning and are difficult to eliminate by training. The most popular among these are the exploratory 'approach-avoidance' tasks. Some such more common behavioural models will be adduced here.

Open field test is the oldest and simplest measure of rodent emotional behaviour (Hall 1936; Henderson 1967). Spontaneous exploratory locomotion, proximity to the walls and central areas, and number of faecal boli deposited are quantified in a brightly lit, novel open arena for a period as short as 5–10 min. An animal exhibiting high perimeter and low centre activity would be interpreted as possessing high levels of anxiety. The simplicity of the task is a strength and it was the basis for the first rodent genetic animal model for anxiety, but pharmacological validation for this task is only modest.

One of the best-documented and pharmacologically validated animal models of anxiety is the *elevated plus-maze* (EPM) task (Lister 1987; Pellow *et al.* 1985). Mice and rats perform similarly on the EPM, and strain distribution analyses indicate that there is a genetic contribution to basal levels of anxiety measured on this task (Rodgers & Cole 1993; Trullas & Skolnick 1993). The number of entries and amount of time in the open and closed arms are measured. Rodents prefer the closed arms of the EPM, and the most commonly reported index of anxiety is the percentage of open arm entries or time. An animal exhibiting a decrease in open arm entries or time would be considered to possess an increased level of anxiety.

The *elevated zero-maze* task (EZM) is a variant of the EPM that was designed to eliminate the time spent in the centre area, which was a potential confound inherent with the EPM configuration (Shepherd *et al.* 1994). Interpretation is similar to that of the EPM, with a decrease in open arm entries and time reflecting increased anxiety.

Conceptually similar to the EPM, the *light/dark transition* and *dark/light emergence* tasks are based on the conflict between a rodent's tendencies to explore a novel environment versus the aversive properties of a brightly lit open field (Crawley 1985). Number of transitions and time spent in the light chamber or open fields are the most commonly used variables.

All these four afore-mentioned tasks are based on the conflict between a rodent's natural tendency to explore a novel environment for possible escape routes and its reluctance to venture away from a wall (to avoid a brightly lit open field) that offers relative safety from aerial predators (approach-avoidance task). The concept of conflict between opposing drives is also reminiscent of psychodynamic theories of human anxiety (Adler 1954; Freud 1966; Sato 2005).

2.3. Predator odour induced anxiety

A contemporary neurobiological analysis examining the relationship between odours and emotional behaviours is likely to provide novel information on the brain mechanisms involved in modulating fear. Odours of predators trigger a range of unconditioned behavioural and physiological responses in rodents and odours-induced fear behaviour is attracting increasing attention. Generally, predator models are based on the examination of the cognitive consequences of an exposure to stimuli indicating the presence of a predator. That is, in this test situation, animals are, in accordance with the DSM-IV definitions of anxiety disorders, reacting to the possibility of an aversive event occurring in the absence of external cues indicating that this is likely (Hendrie *et al.* 1996). Mammals respond readily to a variety of odours associated with predators both in the field (Apfelbach *et al.* 2005; Dickman & Doncaster 1984; Jedrzejewski & Jedrzejewska 1990; Müller-Schwarze 1972; Stoddart 1980; Weldon *et al.* 1990) and laboratory environment (Dell'Omo *et al.* 1994; Fendt *et al.* 2005; Zangrossi, Jr. & File 1992). Presentation of whole animal of different predator species, or odour by the means of faeces, urine, anal gland secretion, bedding, fur, cloth, block or collar has been used for eliciting anxiety-like behaviour in prey animals (Apfelbach *et al.* 2005). In the laboratory, the odours of predators most frequently employed to analyse fear in rodents include cat odour and 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; the major component of the anal gland secretions of the red fox) isolated from fox faeces (Vernet-Maury *et al.* 1984) but also adult anaconda cloacal gland secretion has been used (de Paula *et al.* 2005). In addition, auditory cues signalling the presence of predator like tawny

owl calls have been used to model anxiety (Hendrie 1991; Hendrie & Neill 1991). However, the propensity of rodents to engage in risk assessment appears to occur more frequently in tests involving cat odour (Hebb *et al.* 2004; Holmes & Galea 2002; Takahashi *et al.* 2005), in comparison to TMT (Rosen 2004).

2.3.1. Neurobiology of cat odour induced anxiety

A study by Blanchard and colleagues (Blanchard *et al.* 2003) showed that laboratory rats showed a clear avoidance response towards a cloth rubbed on a cat, cat faeces, cat urine and TMT. However, only the cat cloth was able to produce contextual conditioning of fear to the environment in which it was presented, suggesting a more profound anxiogenic effect of this odour source. There is another evidence favouring the hypothesis that sulphurous metabolites of meat digestion may be important for the repellent effects of predator odours to potential prey. Berton and colleagues (Berton *et al.* 1998) demonstrated that the diet of a cat strongly affects the behaviours of mice towards its faeces. Most studies indicate a negative effect of predator odour on the reproductive success of prey species. Apparently for the mouse or the rat it makes no difference whether the hunting cat is a female in oestrous or a dominant male: both represent a threat. Cohen and colleagues (Cohen *et al.* 2000) reported that the exposure to a cat odour impregnated cloth caused increased anxiety in rats in the elevated plus-maze 7 days later. Lasting effects of predator or predator odour exposure on plus-maze behaviour in rats have also been reported by others (Dielenberg & McGregor 1999). The main difference between odour and pheromone is that one learns to correlate odours with specific object or situation and output in behaviour can alter (e.g. after rewarding), in contrast, responses to pheromones are innate: a naive animal responds behaviourally to the presence of pheromones without any prior experience or exposure: pups suckle, males fight, and oestrus cycles are altered (Doving & Trotier 1998; Stowers & Marton 2005). Recent evidence suggests that in rodents both the vomeronasal organ and olfactory area are involved in the processing of pheromonal signals, including cat odour (Shepherd 2006).

2.3.2. Molecular mechanisms related to the cat odour induced anxiety

The exposure of mice to predator odour has been associated with elevated CCK mRNA levels in the ventral tegmental area, the medial and basolateral nuclei of the amygdala and increased anxiety-like behaviour in the light dark test (LD) and acoustic startle paradigms (Hebb *et al.* 2003). Acute exposure of CD-1 mice to the predator odour, TMT, is associated with enhanced freezing, anxiety in the LD test, and increased CCK mRNA levels in the prefrontal cortex, and BLA (Hebb *et al.* 2002). The same mice that showed excessive freezing in the home-

cage during TMT odour exposure had lower density of preproenkephalin mRNA levels in the BLA and CEA (Hebb *et al.* 2004). The exposure to TMT or fox urine odour has been reported to increase dopamine turnover in the prefrontal cortex (Hayley *et al.* 2001; Morrow *et al.* 2002; Morrow *et al.* 2000), and 5-HT turnover in the locus coeruleus, hippocampus, and prefrontal cortex. Whether cat odour generates a similar pattern of brain monoamine secretion remains to be established, especially in relation to its functional relevance.

3. Concluding remarks

According to the existing literature the cat odour induced anxiety is not a widely used model for the research of neurobiological background of anxiety. However, it is clear that the response of rodents to predator odour is strongly shaped by the mechanisms of natural selection and, therefore, may provide important cues for understanding the molecular machinery of anxiety. Wang and colleagues (Wang *et al.* 2003) studied the effect of the cat odour exposure in rats using gene chip technology. However, their study targeted the frontal cortex, playing probably an indirect role in the mechanisms of cat odour induced anxiety. Recent studies demonstrate that following the predator odour exposure, rodents display increased anxiety both in ethological and conditioned models. The increased anxiety is accompanied by an elevated level of CCK and reduced levels of PENK in the forebrain of rodents. Taking into account that the molecular architecture of cat odour induced anxiety has not been widely studied, the major goal for the present study was to use this approach for the detection of new molecular targets implicated in the regulation of anxiety. However, before that we decided to perform additional studies for the selection of right targets among the brain structures for gene expression studies. Taking into account that CCK and opioid system seem to have an opposite role in the regulation of anxiety (Kõks *et al.* 1998; Kõks *et al.* 1999) and there is already evidence that these systems are affected by predator odour, we decided to study changes in the opioid and CCK system in male Wistar rats in response to the cat odour. This study conducted on rats was further extended by using mice, lacking CCK_2 receptors. Since both of these studies demonstrated a strong involvement of POMC-linked mechanisms in the amygdala, we selected this brain structure for the study of gene expression changes induced by the cat odour exposure.

III. AIMS OF THE STUDY

The general goal of the present study was to establish new molecular targets implicated in the regulation of anxiety. For that purpose a model of anxiety induced by cat odour was used. The more specific tasks for the current study were the following:

1. To study further the effect of the cat odour exposure on the interaction of the opioid and CCK systems in Wistar rats. Also changes in morphine-induced increase of exploratory behaviour were studied in rats after the cat odour exposure.
2. To study the effect of the cat odour exposure on the exploratory behaviour and interaction of the opioid and CCK systems in female mice having different level of anxiety. Mice lacking CCK_2 receptors and their wild-type littermates were compared as for the effects induced by cat odour. CCK_2 deficient animals were selected because of their reduced anxiety compared to wild-type littermates.
3. To identify by means of cDNA representational difference analysis the genes induced by the cat odour exposure in the amygdala of male Wistar rats and to select from among them the most relevant targets for further studies. The amygdala was chosen, because of existing evidence concerning its role in the regulation of anxiety and due to the results of the previous parts of the present study where the biggest changes in a stress-related gene — Pomc1 — were established in this particular brain structure.

IV. MATERIALS AND METHODS

1. Animals

The experiments were performed in male Wistar rats and female transgenic mice of 129Sv/C57Bl6 background. In total 122 male Wistar rats (Han/Kuo: WIST) weighing 250–280 g at the time of testing were used (Papers I, III). Altogether 18 female wild-type (+/+) and 18 female homozygous CCK_2 receptor deficient mice were subjected to the study (Paper II). Animals were kept in the animal house at 21 ± 2 °C under a 12 h/12 h light/dark cycle (lights on at 0700 h). Tap water and food pellets were available *ad libitum*. All animal procedures were approved by the University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24th of November 1986 (86/609/EEC).

Characteristic features of CCK_2 receptor deficient mice

Since mice, lacking CCK_2 receptors, were used in the current study, more detailed information about this mouse line is given below. Nagata and colleagues (Nagata *et al.* 1996) generated CCK_2 receptor deficient mice by replacing a part of exon 2, and exons 3–5 by LacZ in-frame and a PGK-neo cassette. This replacement deleted most of the seven membrane-spanning CCK_2 receptor loops except for the first 108 amino acids containing the first membrane-spanning region. This deletion was expected to impair the entire function of the receptor. The targeting vector included a 1.2 kb upstream homologous region of exon 2 and a 7 kb downstream region of exon 5. Breeding and genotype analysis were performed at the Department of Physiology of the University of Tartu. Genetically modified mice were backcrossed six times to the C57Bl6 background to minimise possible genetic influence from the 129Sv strain. Genotyping was carried out as described in the study of Raud and colleagues (Raud *et al.* 2003). Mice with targeted disruption of CCK_2 receptor gene are fertile and without obvious behavioural abnormalities up to the age of 24 months (Nagata *et al.* 1996). In our previous experiments we found that female mice from the C57Bl6/129Sv background showed reduced aversion to the open arms of the plus-maze compared to male mice (Abramov *et al.* 2004; Raud *et al.* 2003). This was the reason why female mice were used in the present study. Kopin and colleagues (Kopin *et al.* 1999) were unable to establish differences between CCK_2 receptor deficient mice and their wild-type (+/+) littermates in the food intake, weight gain and pancreatic function. On the other hand, Miyasaka and colleagues (Miyasaka *et al.* 2002) and Weiland and colleagues (Weiland *et al.* 2004) have shown that the lack of CCK_2 receptors results in an increased energy expenditure, higher basal metabolic rate, increased body weight, water consumption, elevated body temperature and decreased locomotor activity in the dark phase. Miyasaka and colleagues

(Miyasaka *et al.* 2004) demonstrated that the increased gastric empting in mice, lacking CCK_2 receptors, may partly be responsible for the increased food intake, although the real mechanism is unknown. Sebret and colleagues (Sebret *et al.* 1999) revealed that mice with targeted disruption of CCK_2 receptor gene displayed impaired memory. In a two-trial memory task, receptor deficient mice spent significantly less time in the novel arm compared to wild-type mice (Sebret *et al.* 1999). Daugé and colleagues (Daugé *et al.* 2001) established that the behavioural activation in CCK_2 receptor deficient mice was suppressed by treatment with the selective dopamine D_2 antagonist sulpiride, suggesting an increased sensitivity of dopamine D_2 receptors in CCK_2 receptor deficient mice. Kõks and colleagues (Kõks *et al.* 2001; Kõks *et al.* 2003) demonstrated that an indirectly acting dopaminergic drug amphetamine caused a stronger hyperlocomotion in genetically modified mice compared to their wild-type littermates, also demonstrating an enhanced sensitivity of postsynaptic dopamine receptors in mice, lacking CCK_2 receptors. This finding was confirmed by radioligand studies where an increased density of dopamine D_2 receptors was established in the striatum of male mice, lacking CCK_2 receptors (Kõks *et al.* 2001). There is also evidence that the hyperactivity of mutant mice could be partly due to an increased function of the opioidergic system. Pommier and colleagues (Pommier *et al.* 2002) demonstrated that administration of morphine or inhibition of enkephalin metabolism induces a significantly stronger hyperlocomotion in homozygous (-/-) mice compared to wild-type (+/+) littermates. Recent evidence suggests that the function of the opioidergic system is significantly altered in mice, lacking CCK_2 receptors. Pommier and colleagues (Pommier *et al.* 2002) have found that these mice display hyperalgesia in the hotplate test. Reduced jumping latency of homozygous (-/-) mice in this test as well as in plantar analgesia test, pain sensitivity of CCK_2 receptor deficient mice was again significantly reduced compared to wild-type littermates as confirmed by our group (Veraksitš *et al.* 2003). In conclusion, it has to be underlined that the genetic invalidation of CCK_2 receptors induces distinct effects in male and female mice. In male mice, changes in the locomotor activity, pain sensitivity, and behavioural effects of amphetamine and activity of opioid and dopamine system are evident. In female mice, reduced anxiety and response to stress, but also increased activity of opioid and GABA system are prevailing.

2. Cat odour exposure

Half of the rats and mice were exposed to a cloth impregnated with the smell of a predator (cat) whereas another half of both species was exposed to a similar clean control cloth. The exposure was performed in two separate (one for cat odour and the other for clean cloth exposure), but similar rooms (lighting

conditions, humidity, ventilation, etc.). Animals were habituated to the rooms and experimenter three days (30 min each session) before the experiment.

Rats were exposed to the cloth in their home cages, groupwise (Papers I, III). After a 30-min habituation period in the exposure room, a cloth impregnated with cat odour or a clean cloth was placed on the cover grid of the cage for 30 min; each session was video recorded. Videotaped behavioural responses were analysed by an observer not aware of the manipulations performed with rats. At the end of each minute videotape was paused and the number of animals sniffing the cloth, in the proximity of the cloth or touching the cloth were recorded. We also evaluated the number of animals grooming in each group. These behavioural measures have been shown to reflect the avoidance of and anxiety towards an unpleasant object (Belzung *et al.* 2001).

The exposure of mice was performed in cages similar to animals' home cages (Paper II). Animals were placed singly into the cage and a cloth impregnated with cat odour or a clean cloth was placed in the opposite corner of the cage on the bedding. The exposure to cat odour lasted 30 min and the session was video recorded. An observer, not aware about the manipulations performed with mice, analysed the videotaped behavioural responses. The numbers of contacts with the cloth and stretch-attend postures were assessed.

3. Drugs (Paper I)

Sixty-six rats (33 animals in both groups) were used to study the effect of morphine on exploratory activity in a novel and stressful environment. As described above, the animals were exposed to cat odour for 30 min (five to six rats per cage). Immediately after that, the animals were separated and injections performed. The cat odour group and the control group were split. Eighteen animals from both groups received saline (0.9% NaCl) injection (1 ml/kg, subcutaneously) and 15 animals from both groups were treated with morphine (morphine sulphate [Boehringer-Ingelheim, Germany] 1 mg/kg dissolved in 0.9% NaCl, subcutaneously). The dose of morphine was chosen according to our previous studies where 1 mg/kg of morphine was the only dose to induce a significant increase in the exploratory activity of rats in an unfamiliar environment (Kõks *et al.* 1999). In a pilot study we explored also the effect of 2.5 mg/kg of morphine. However, this dose suppressed exploratory activity in both cat odour group and control group. After the injection, rats were kept separately for 15 min and, thereafter, the motility box test, lasting 15 min, was performed.

4. Behavioural studies

All behavioural experiments as well as the exposure of animals to a cloth were conducted between 1000 and 1800 h. The behavioural tests were performed in a room separate from the exposure procedures. Since behavioural experiments lasted up to 35 min per animal and the whole experiment up to 8 h, precautions were taken to control for possible daily fluctuations in the exploratory behaviour of animals. Therefore, the experiments were performed in randomised order, that is, all groups were used in parallel.

4.1. Test of exploratory activity — motility box (Paper I)

In this test, rats underwent morphine/saline injections respectively, and were not previously adapted to the experimental room or motility boxes. Animals were placed singly into computer-connected photoelectric motility boxes (448 mm × 448 mm × 450 mm, TSE Technical & Scientific Equipment, GMBH, Germany). Fifteen infrared sensors with a distance of 3 cm to each other were installed outside the transparent walls of the box in both *x* and *y* dimensions. The infrared sensors for recordings in the *z* dimension were mounted 10 cm above those that record in the *x* and *y* dimensions. The illumination level of the transparent test boxes was ~500 lx. After removing the rat from the box, the floor was cleaned with 5% alcohol solution. Time in locomotion (s), distance travelled (m) and number of rearings were registered during the 15-min observation period.

4.2. Elevated plus-maze test (Paper II)

Immediately after the exposure of mice to a cat odour impregnated cloth or a clean cloth the elevated plus-maze test was performed. The plus-maze consisted of two opposite open (17.5 cm × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14-cm-high sidewalls and an end wall. The arms extended from a common central square (5 cm × 5 cm) and were angled at 90° to each other, making the shape of a plus sign. The entire plus-maze apparatus was elevated to a height of 30 cm. The experiment was performed in a dimly lit room (illumination level: ~40 lx) to increase the exploratory activity of mice. In order to encourage open arm exploration, a slightly raised edge (0.25 cm) was put around the perimeter of the open arm, providing a grip for the animals. Testing began by placing an animal on the central platform of the maze facing an open arm. The mice clearly preferred the enclosed arms. An arm entry was counted only when all four limbs were within a given arm. Standard 5 min test duration was employed (Lister 1987; Pellow *et al.* 1985), and the maze was

wiped with damp and dry towels between the subjects. Test sessions were video recorded and the videotapes were subsequently blind-scored by a trained observer. The following measures were taken: (1) time spent on the open arms of the plus-maze and (2) the number of closed and open arm entries. It has been established that changes in exploratory activity in the plus-maze are indicative of anxiety in rodents (Rodgers 1997). Time spent on the open arms and the number of open arm entries are the conventional measures of anxiety in the elevated plus-maze (Lister 1987; Pellow *et al.* 1985). The frequency of closed arm entries was used to detect the locomotor activity of mice (Rodgers 1997).

4.3. Elevated zero-maze test

In total 16 rats (8 from both exposition groups) underwent elevated zero-maze (EZM) test. EZM exploration test is an unconditioned test of anxiety-like behaviour originally described by Shepherd and colleagues (Shepherd *et al.* 1994). The apparatus comprised a black perspex annular platform (105 cm diameter, 10 cm width) elevated to 65 cm above ground level. Two opposite quadrants were enclosed by black metal walls (27 cm high, closed part), while a 1 cm high lip surrounded the remaining two opposite quadrants. Both open parts were divided by five lines into four parts. A naive observer, in the same room 2 m away from the maze, recorded behaviour data. The behaviour of rats was recorded for 5 min and the following parameters were taken: number of open part entries, time spent in the open part, number of line crossings, number of head-dips and the number of closed part entries. The experiments were carried out in a dimly lit room (illumination 70 lux).

5. Gene expression studies

5.1. Dissection of brain structures, mRNA isolation and cDNA synthesis (Papers I–III)

Immediately following the cat odour exposure twenty rats from both groups (40 animals in total) were decapitated and their brains were sliced. Dissection of the amygdala, frontoparietal cortex, mesolimbic area (includes *nucleus accumbens* and *tuberculum olfactorium*) and striatum was performed according to the coordinates of the rat brain atlas of Swanson (Swanson 1998). Dissection of the amygdala was performed using a round-shape puncher (Figure 3). All samples were frozen in liquid nitrogen. For the quantitative PCR study (Paper I), the tissue samples from eight animals in both groups were pooled as follows: four pools (two rats per pool) were formed per tissue per group ($n = 4$). For the cDNA-RDA study (Paper III), the amygdala from 12 rats in both exposure

groups were used. The preparations from 6 animals were pooled. The poolings were made to minimise fluctuations resulting from individual differences. mRNA was purified from the tissue samples of the amygdala (QIAGEN RNeasy and Oligotex mRNA kit, Hilden, Germany) followed by cDNA synthesis (First Strand cDNA Synthesis Kit, Fermentas, Lithuania, Paper I or GIBCO BRL Superscript Choice System for cDNA Synthesis, Paper III).

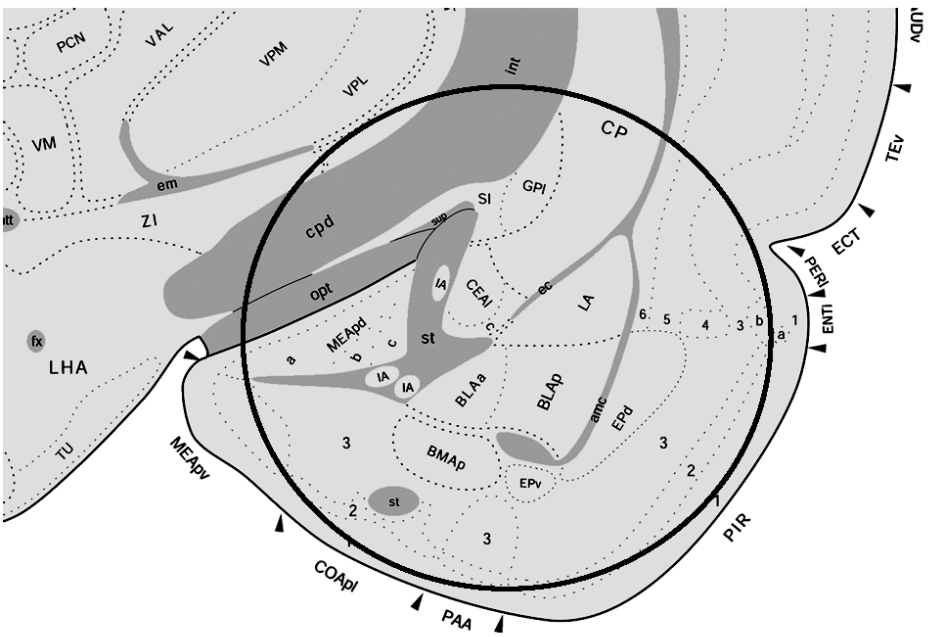

Figure 3. Preparation of amygdala to screen for genes induced after the exposure to the predatory odour.

Mice were decapitated immediately after the behavioural tests. Thereafter brains were quickly dissected into four parts: frontal cortex, mesolimbic area (including *nucleus accumbens*, *tuberculum olfactorium* and mesocortex), temporal lobe (including amygdala) and mesencephalon (including PAG) and frozen in liquid nitrogen. Dissection was performed according to the coordinates obtained from the mouse brain atlas (Franklin & Paxinos 1997). The pooling was performed to minimise fluctuations resulting from individual differences. Every experimental group ($n = 9$) was pooled as follows: three pools including samples from two mice and one pool including samples from three animals. Consequently the number of independent samples in each case was four. Sixty-four different pools (four brain structures, four groups of

animals and four samples) were received. Total RNA was extracted using Total RNA Isolation Spin-Kit (AppliChem), thereafter mRNA was separated using Oligotex mRNA Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, followed by first strand cDNA synthesis (First Strand cDNA Synthesis Kit, Fermentas, Lithuania).

5.2. Quantitative real-time PCR (qRT-PCR, Papers I–II)

The quantitative real-time PCR (qRT-PCR) was performed using ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, USA) equipment and ABI PRISM 7000 SDS Software. Hypoxanthine-guanine phosphoribosyl transferase (Hprt1) was used as a housekeeper gene in both rats and mice. Hprt1 was chosen as the endogenous reference gene, because it is a constitutively expressed gene in the mammalian brain. The use of this gene has been advocated in various studies (de Kok *et al.* 2005; Jiralerspong & Patel 1996). Primers were principally designed to cover exon-exon junctions, to exclude amplification of possible remains of genomic DNA or cDNA transcribed from possible remains of total RNA or antisense RNA. As a rule, the reactions with samples of different experimental groups were conducted in parallel. Every reaction was made in four parallel samples to minimise possible errors. All primers for mice and primers with a probe for rat Hprt1 were designed with the Primer Express™ software (PE Applied Biosystems, USA). For rat (Paper I) Hprt1 the primers were 5′-GCAGTACAGCCCCAAAATGG-3′ (forward) and 5′-AACAAAGTCTGGCCTGTATCCAA-3′ (reverse) and the amplified product was 85 base pairs in length. The probe was 5′ VIC-AAGCTTGCTGGTGAAAAGGACCTCTCG-TAMRA 3′. Primers and probes for rat gene encoding CCK (Cck), Pomc1 and Oprm1 were ordered from PE Applied Biosystems as Assay-On-Demand. Assay ID numbers were Rn00563215 for Cck, Mm00435874 for Pomc1 and Mm00440568 for Oprm1. All reactions with rat samples were performed by using TaqMan® Universal PCR Master Mix (Roche, USA). We used multiplex PCR measuring amplification characteristics for both rat Hprt1 and the gene of interest from the same reaction.

In mice (Paper II) we studied the expression levels of the following genes:[pre–pro-cholecystokinin (Cck), CCK_1 receptor (Cckar), CCK_2 receptor (Cckbr), pro-opio-melanocortin (Pomc1), μ-opioid receptor (Oprm1), δ-opioid receptor (Oprd1), κ-opioid receptor (Oprk1), pre-pro-enkephalin (Penk1), pre-pro-dynorphin (Pdyn), melanocortin-3 receptor (Mc3r), melanocortin-4 receptor (Mc4r)]. The selection of the above-mentioned cholecystokinin and opioid system genes was based on the results of previous studies where the effect of the exposure to predator odour was assessed in rodents (Bagnol *et al.* 1999; Hebb *et al.* 2002). The primer sequences are presented in Table 2. All reactions in mice samples were performed by using qPCR™ Core Kit for SYBR® Green I Master Mix (Eurogentec, Belgium).

Table 2. Primer sequences used for qRT-PCR

Cck	Forward: 5′–TGCCGAGGACTACGAATACC–3′; Reverse: 5′–CTTCCGACCACACAGCTAGG–3′
Cckar	Forward: 5′-GTGCTGATTCGAAACAAGAGG-3′; Reverse: 5′-AGATGGCTACCAGGTTGAAGG-3′
Cckbr	Forward: 5′-CACCCTTTATGCGGTGATCT-3′; Reverse: 5′-CTGTGCCGAAGATGAATGTG–3′
Pomc1	Forward: 5′-CTTTGTCCCCAGAGAGCTGC-3′; Reverse: 5′-AACAAGATTGGAGGGACCCC-3′
Oprm1	Forward: 5′-CGACTGCTCTGACCCCTTAG-3′; Reverse: 5′-TCCAAAGAGGCCCACTACAC-3′
Oprk1	Forward: 5′-GCAGCCTGAATCCTGTTCTC-3′; Reverse: 5′-TCATCCCTCCCACATCTCTC-3′
Oprd1	Forward: 5′-GCATCGTCCGGTACACCAA-3′; Reverse: 5′-AAAGCCAGATTGAAGATGTAGATGTTG-3′
Penk1	Forward: 5′-ATGCAGCTACCGCCTGGTT-3′; Reverse: 5′-GTGTGCACGCCAGGAAATT-3′
Pdyn	Forward: 5′-TTGGCAACGGAAAAGAATCTG-3′; Reverse: 5′-TGTGCGGCTTCATCATTCAT-3′
Mc3r	Forward: 5′-CATTGCCATCGACAGGTACGT-3′; Reverse: 5′-CTGTGGTACCGAAGGGCATAG-3′
Mc4r	Forward: 5′-AGCCTGGCTGTGGCAGATAT-3′; Reverse: 5′-GGTTTCCGACCCATTCGAA-3′
Hprt1	Forward: 5′-GCAGTACAGCCCCAAAATGG-3′; Reverse: 5′-AACAAAGTCTGGCCTGTATCCAA-3′

Instructions of the equipment and reagents manufacturers were always followed. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected in case of SYBR Green. All samples to be compared were run in the same experiment. The amount of the target gene was compared to that of the housekeeper gene in the groups by means of the ΔC_T method (Livak & Schmittgen 2001). To avoid possible errors, linear regression analysis of amplification slopes was performed using free-ware LinRegPCR (Ramakers *et al.* 2003). Besides that, visual inspection of amplification slopes was always performed. Every reaction was made in four parallels to minimise possible errors. The mRNA level in wild-type control group animals, exposed to the clean cloth, was always defined as 1 and the variations of mRNA amounts in other groups are shown as the fold change. Additionally, the basal expression levels of all genes in all brain structures were compared in mice. For that purpose, ΔC_T values in control groups (neither cat odour exposed nor CCK_2 deficient) were compared, the lowest expression is defined as 1 and the variations in other tissues or genes are shown as fold change.

5.3. cDNA Representational Difference Analysis (cDNA-RDA, Paper III)

In the beginning, mRNA was purified from the tissue samples of the amygdala (QIAGEN RNeasy and Oligotex mRNA Kit) followed by cDNA synthesis (GIBCO BRL Superscript Choice System for cDNA Synthesis). Differential gene expression analysis was performed in two directions. In "forward" subtraction experiment, the animals with fear response formed a "tester" group, in "reverse" subtraction, the "tester" group consisted of control animals. RDA was performed according to the protocol of Hubank and Schatz (Hubank & Schatz 1999) with minor modifications (O'Neill & Sinclair 1997; Pastorian *et al.* 2000). Briefly, double-stranded cDNA was digested with *Dpn II* (New England Biolabs) and ligated to annealed R-adaptors (sequences of primers are in Table 3). Amplicons for both "tester" and "driver" were generated with Vent DNA Polymerase in an appropriate buffer (New England Biolabs). For tester 5 and for driver 20 200 µl PCRs were performed. *Dpn II* digestion was used to remove the R-adaptors from both driver and tester amplicons followed by ligation of J-adaptors. Subtractive hybridisations were performed in 5 µl reactions at 67^{o}C for 24 hours in a thermocycler. To generate differential product 1 (DP1), 0.4 µg of tester cDNA was mixed with 40 µg of driver cDNA at a ratio of 1:100. DP1 was digested with *Dpn II* to remove J-adaptors before ligation of N-adaptors. To generate DP2 50 ng of tester, cDNA was mixed with 40 µg of driver cDNA at a ratio of 1:800. DP2 was digested with *Dpn II* to remove N-adaptors before ligation of J-adaptors. To generate a third difference product (DP3), 100 pg J-ligated DP2 was mixed with 40 µg driver (stringency 1:400,000). Another experiment was performed where 4 ng of tester was mixed with 40 µg of driver cDNA to get a ratio of 1:10,000 for DP3. DP3 was digested finally with *Dpn II* to obtain *Bam HI* compatible ends. For removal of digested adapters, spin column purification with Qiagen PCR purification kit was applied. The subtracted library was fractionated by agarose gel electrophoresis. Fractions in 1.5% low-melting agarose gels were cut and QIAEX II Gel Extraction was performed. Fractions were ligated into the *Bam HI* site of vector pGEM-7. The libraries were plated onto agar plates (with 50 µg/ml ampicillin) and incubated at 37^{o}C overnight. After brief incubation at 4^{o}C the blue/white staining became clearly distinguishable. The plasmids were purified from 2-ml cultures of the white colonies by alkaline lysis protocol. 300 ng of each plasmid DNA was used to perform cycle sequencing on ABI310 sequencer (Perkin-Elmer) with M13 forward primers according to the manufacturer's instructions. Sequence alignments were performed with the UK-HGMP software NIX (Williams *et al.* 1998).

Table 3. Sequences of primers used for RDA

Name	Sequence of primer
R-Bgl-24	5'-AGCACTCTCCAGCCTCTCACCGCA
R-Bgl-12	5'-GATCTGCGGTGA
J-Bgl-24	5'-ACCGACGTCG-ACTATCCATGAACA
J-Bgl-12	5'-GATCTGTTCATG
N-Bgl-24	5'-AGGCAACTGTGCTATCCGAGGGAA
N-Bgl-12	5'-GATCTTCCCTCG

5.4. Dot-blot analysis of clones (Paper III)

After sequencing and alignment, only clones containing different inserts were used for dot blot analysis to confirm the results of RDA. 100 µl of DNA with 0.4 M NaOH and 10 mM EDTA was denaturised (10 min at 100^{o}C) and dotted onto a Hybond N^{+} nylon membrane, followed by UV cross-linking. DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) was used for the generation of hybridisation probes and for the hybridisation of membranes. Equal amount of cDNA was used for the synthesis of DIG labelled probes. Each set of clones was dotted onto two identical membranes. One membrane was hybridised with starting cDNA from "cat odour" group; another was hybridised with starting cDNA from "control" group. The membranes containing different sets of clones ("cat odour" or "control" group) were hybridised with an identical probe in one hybridisation tube in identical conditions. Each experiment was repeated twice to avoid fluctuations caused by the experimental situation. Dot blots were scanned and analysed with Quantity One Software (GS 710 Calibrated Imaging Densitometer, BioRad). To correct for the gray value, a small area in between dots was measured as a local reference. Each gray value of the measured areas was corrected for this local reference. Resulting optical densities were compared to the membrane hybridised with a different probe and to the local references. Results are expressed as fold changes of respective dots.

5.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR, Paper III)

RT-PCR was applied to find the differential expression of the c-fos, corticotrophin-releasing hormone (CRH), GAMM1 and carboxypeptidase E (CPE) in separate samples (not used for differential cloning). In addition, to confirm the efficiency of subtractive hybridisation, we performed RT-PCR analysis of both drivers (amplified cDNA pools) and DP3 products. Total RNA was extracted, DNase-treated and subjected to RT-PCR using specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeper to

control the amount of RNA), c-fos and CRH. Primers were the following: GAP1: 5'-CCC TCA AGA TTG TCA GCA ATG C-3', GAP2:5'-GTC CTC AGT GTA GCC CAG GAT-3' for GAPDH; CFOS2: 5'-TCA CAG GGC TAG CAG TGT GG-3', CFOS3: 5'-ATG ACC TGG GCT TCC CAG AG-3' for c-fos; CRF1S 5'-TGG GTG AAG AAT ACT TCC TC-3', CRF2AS 5'-AGA GAG ATG GGC GGC TCC TC-3' for corticotropin releasing factor (CRF); GAMM1F: 5'-CTG ACC AAG ACA CTG AGG CAG G-3', GAMM1R: 5'-GCT CCT TGG GCA CAC ACT GG-3' for GAMM1; CPEF: 5'-GCT CTC TGC CAA CCTGCA CG-3', CPER: 5'-CTC AAA GCA GTT GCT GCT CAG G-3' for CPE. First strand synthesis was performed with Superscript II (Gibco BRL) according to the manufacturer's guidelines. Briefly, 2 pmol of random primers and 500 ng DNase treated RNA were used. Primers were designed to anneal on the sequences of different exons to avoid the influence of contaminating genomic DNA. PCR was performed from serial dilutions of the first-strand reaction mix and 10 µl of products were separated by agarose gel electrophoresis with the results digitally stored. Optical densities of fragments were compared by the densitometric analysis using QUANTITY ONE Software. The amount of mRNA product for c-fos, CRF, GAMM1 and CPE was calculated as the ratio to GAPDH mRNA product.

6. Statistical analysis

The results of rats' behavioural responses during the cat odour exposure were analysed using Mann-Whitney U-test (Paper I, III) and during EZM test by using Student's t-test. The statistical analysis of the motility box results was performed by using two-way ANOVA (the cat odour exposure and treatment with morphine as independent factors), followed by a Tukey HSD *post hoc* test (Paper I). Gene expression data of rats were analysed by Student's t-test (Paper I). The results of behavioural studies and gene expression studies of mice (Paper II) were analysed by means of two-way ANOVA (genotype and the cat odour exposure as independent factors). Multiple testing comparisons were done *post hoc* if the two-way ANOVA established a significant effect. The *post hoc* analysis of behavioural studies was performed by using Newman-Keuls test, whereas Scheffe test was applied for the analysis of gene expression data.

V. RESULTS

1. Experiment I: Changes in rat brain CCK and opioid systems after cat odour induced anxiety and morphine injection

1.1. Cat odour exposure

Cat odour containing cloth induced a robust anxiety-like behaviour in rats (Figure 4). Cat odour-exposed rats expressed behavioural suppression because they spent less time in the close vicinity of the cloth (7.0 ± 0.4 versus 3.1 ± 0.2) and avoided contact with the cloth (1.3 ± 0.2 versus 0). In addition, the grooming behaviour was markedly reduced (1.5 ± 0.3 versus 0.2 ± 0.1) and they sniffed the cloth less (3.6 ± 0.3 versus 1.5 ± 0.2). Differences in all these measures reached statistically significant levels ($p < 0.01$, Mann-Whitney *U* test). Cat odour exposed rats also displayed increased freezing as compared to the control group. This type of behavioural suppression is considered to reflect anxiety rather than fear, as this behavioural reaction persists for some time after the odour exposure (McGregor *et al.* 2002).

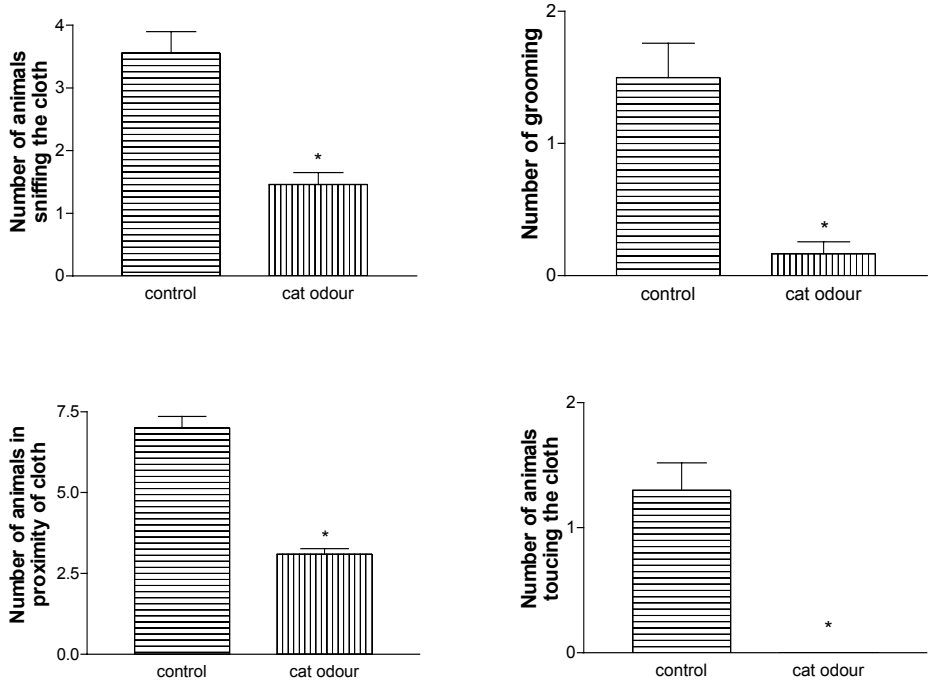

Figure 4. Behavioural changes in rats during the exposure to cat odour. Rats were exposed to the cloth impregnated with cat odour for 30 minutes and the behaviour was recorded. At the end of each minute the videotape was paused and the number of animals performing certain behavioural patterns was evaluated, mean values ± standard error of mean values (S.E.M.) *p<0.01, Mann-Whitney U-test.

After the cat odour exposure, 16 animals were tested in EZM, where the cat odour group showed also clear behavioural differences reflecting anxiety as compared to the control group (Table 4).

Table 4. The effect of cat odour on the behaviour of male Wistar rats in the elevated zero-maze, mean values ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 as compared to control odour group, Student's t-test.

	Control group (n=8)	Cat odour group (n=8)
Number of line crossings	41.5±4.28	3.0±0.85***
Time in open arms, s	137.4±10.80	46.1±19.37**
Number of open arm entries	9.1±1.13	0.8±0.25***
Time of stretch-attend posture, s	0.1±0.13	1.6±0.63*
Number of head-dips	16.4±1.31	2.0±0.38***
Latency to entry to open arm, s	14.8±2.14	155.3±44.79**

1.2. Exploratory activity after morphine injection

The injection of morphine (1 mg/kg) apparently increased the exploratory activity of control animals in an unfamiliar environment (Figure 5). Time in locomotion and distance travelled were significantly increased in control rats treated with morphine as compared to saline injected group (two-way ANOVA: time in locomotion: exposure × treatment: $F_{1,62} = 5.09$, $p < 0.05$; exposure: $F_{1,62} = 4.96$, $p < 0.05$; treatment: $F_{1,62} = 3.83$, $p = 0.055$; distance travelled: exposure × treatment: $F_{1,62} = 4.74$, $p < 0.05$; exposure: $F_{1,62} = 5.43$, $p < 0.05$; treatment: $F_{1,62} = 3.26$, $p = 0.076$). There was a tendency for increase in the number of rearings (two-way ANOVA: exposure × treatment: $F_{1,62} = 1.19$, $p = 0.28$; exposure: $F_{1,62} = 6.68$, $p < 0.05$: treatment: $F_{1,62} = 1.71$, $p = 0.20$). The exposure to cat odour did not alter the exploratory activity of saline-treated animals, but completely abolished the stimulating action of morphine.

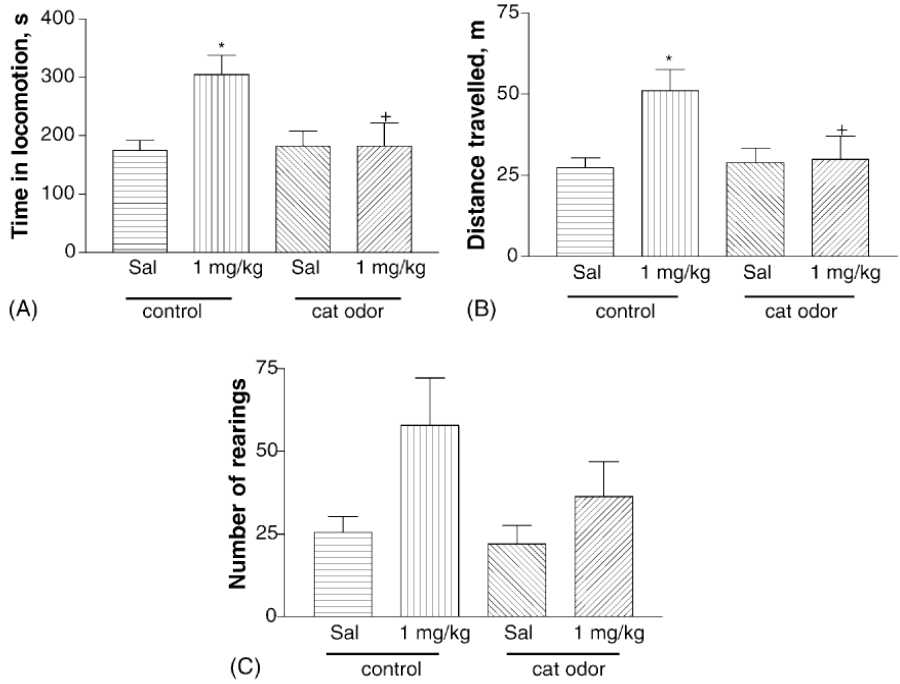

Figure 5. The cat odour exposure and the action of morphine on the exploratory behaviour in motility boxes, Tukey HSD after two-way ANOVA.
(A) Time spent in locomotion (s, mean values ± S.E.M.). Morphine injection significantly (*$p < 0.01$) increased the time in locomotion in the control group, whereas the previous cat odour exposure abolished the effect of morphine (+$p < 0.05$, morphine injected control rats compared to respective cat odour-exposed animals). (B) Distance travelled (m, mean values ± S.E.M.). Morphine injection significantly (*$p < 0.01$) increased the distance travelled in the control group, whereas the previous cat odour exposure eliminated the effect of morphine (+$p < 0.05$). (C) Number of rearings (mean values ± S.E.M.). Number of rearings also tended to be increased in the control group after morphine injection ($p = 0.051$) and the previous cat odour exposure decreased the effect of morphine, but this was not statistically significant ($p = 0.37$).

1.3. The effects of the cat odour exposure on gene expression

Gene expression study revealed that the cat odour exposure induced a significant over-expression of CCK, POMC and MOR genes in the forebrain structures. The CCK mRNA expression was significantly ($p< 0.05$) increased (1.6-fold increase) in the mesolimbic area after the cat odour exposure. There were no significant changes in the CCK mRNA expression level in the amygdala, frontoparietal cortex and striatum (Figure 6). The expression of the POMC

mRNA was increased in the amygdala (5.8-fold increase, $p < 0.001$), fronto-parietal cortex (1.4-fold increase, $p < 0.05$), and mesolimbic area (2.1-fold increase, $p < 0.01$), whereas in the striatum no significant change was found (Figure 7). The expression of the MOR mRNA was significantly elevated in the frontoparietal cortex (2.2-fold increase, $p < 0.01$), mesolimbic area (3.8-fold increase, $p < 0.01$) and striatum (2.1-fold increase, $p < 0.01$), but not in amygdala (Figure 8).

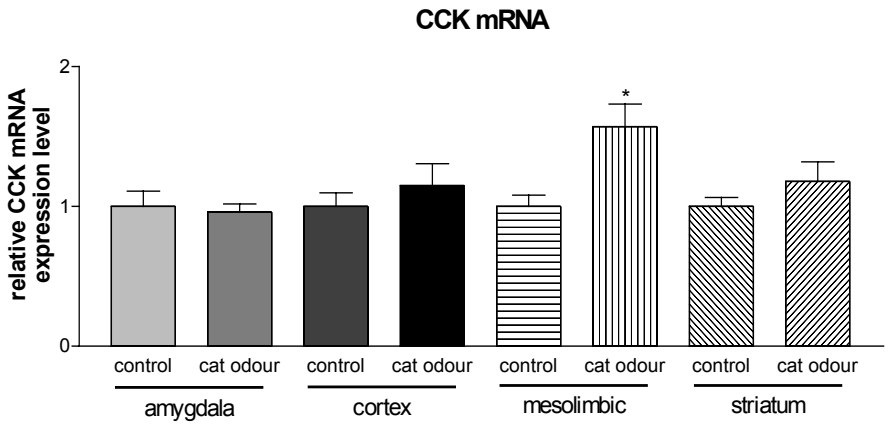

Figure 6. The effect of the cat odour exposure on cholecystokinin (CCK) mRNA expression in the forebrain structures. Relative CCK mRNA expression level in the rat amygdala, frontoparietal cortex, mesolimbic area and striatum, mean values ± S.E.M *$p < 0.05$, Student's t-test.

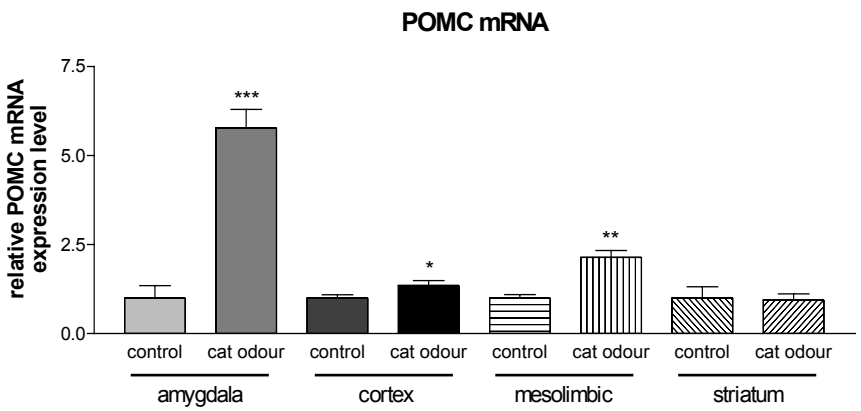

Figure 7. The effect of the cat odour exposure on pro-opio-melanocortin (POMC) mRNA expression in the forebrain structures. Relative POMC mRNA expression level in the rat amygdala, frontoparietal cortex, mesolimbic area and striatum, mean values ± S.E.M *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Student's t-test.

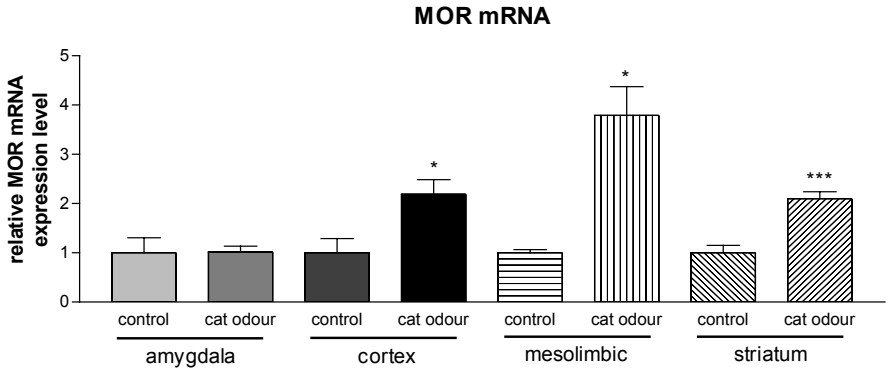

Figure 8. The effect of the cat odour exposure on μ-opioid receptor (MOR) mRNA expression in the forebrain structures. Relative MOR mRNA expression level in the rat amygdala, frontoparietal cortex, mesolimbic area and striatum, mean values ± S.E.M *$p < 0.05$; ***$p < 0.001$, Student's t-test.

2. Experiment 2: The effects of the cat odour exposure on the exploratory activity and neuropeptide gene expression in CCK_2 receptor deficient mice

2.1. Behavioural experiments

2.1.1. Cat odour exposure

The analysis of behavioural recordings from videotapes demonstrated that the number of contacts with the cloth was significantly reduced in the case of cat odour both in wild-type and CCK_2 receptor deficient mice [two-way ANOVA: $F_{1,32} = 0.14$, $p = 0.7$ (genotype); $F_{1,32} = 13.4$, $p < 0.001$ (stimulus); $F_{1,32} = 0.28$, $p = 0.6$ (genotype × stimulus)] (Figure 9). The same was true about the increase of stretch-attend postures [two-way ANOVA: $F_{1,32} = 1.20$, $p = 0.25$ (genotype); $F_{1,32} = 4.91$, $p < 0.05$ (stimulus); $F_{1,32} = 0.02$, $p = 0.85$ (genotype × stimulus)].

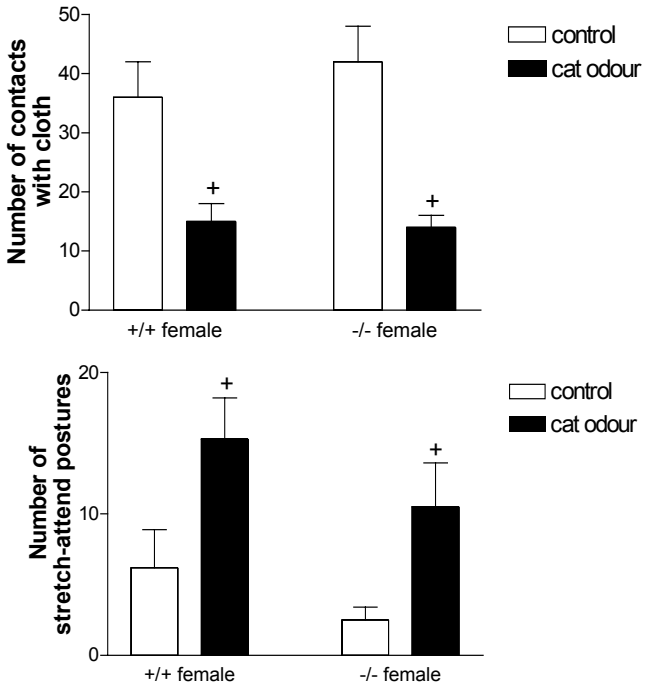

Figure 9. Behaviour of CCK_2 receptor deficient mice and their wild-type littermates during the cat odour exposure, mean values ± S.E.M. Number of mice was nine in each group. $+p < 0.05$, Newman–Keuls test after significant two-way ANOVA, compared to the mice of respective genotype exposed to a clean cloth.

2.1.2. Elevated plus-maze

The analysis of exploratory behaviour of wild-type and CCK_2 receptor deficient mice in the elevated plus-maze supported the assumption of previous studies that the female mice of C57Bl6/129Sv background with a corrupted function of CCK_2 receptors display an increased exploratory activity compared to their wild-type littermates (Raud *et al.* 2003). Indeed, the number of open arm entries and time spent on open arms were significantly elevated in homozygous animals compared to wild-type mice (Figure 10). By contrast, the number of closed arm entries, reflecting the locomotor activity of mice, did not differ in the two genotypes. The exposure of mice to cat odour induced a significant anti-exploratory action in CCK_2 receptor deficient mice, but not in their wild-type littermates. Time spent on the open arms was reduced in CCK_2 receptor deficient mice to the level of wild-type littermates [two-way ANOVA: $F_{1,32} = 4.24$, $p < 0.05$ (genotype); $F_{1,32} = 4.32$, $p < 0.05$ (stimulus); $F_{1,32} = 5.94$, $p < 0.05$ (genotype × stimulus)] (Figure 10). The number of open arm entries

was also reduced, but this change was not statistically significant [two-way ANOVA: $F_{1,32} = 4.16$, $p < 0.05$ (genotype); $F_{1,32} = 1.56$, $p = 0.2$ (stimulus); $F_{1,32} = 0.83$, $p = 0.35$ (genotype × stimulus)]. By contrast, no differences were established in the frequency of closed arm entries [two-way ANOVA: $F_{1,32} = 0.05$, $p = 0.8$ (genotype); $F_{1,32} = 0.69$, $p = 0.4$ (stimulus); $F_{1,32} = 0.27$, $p = 0.6$ (genotype × stimulus)].

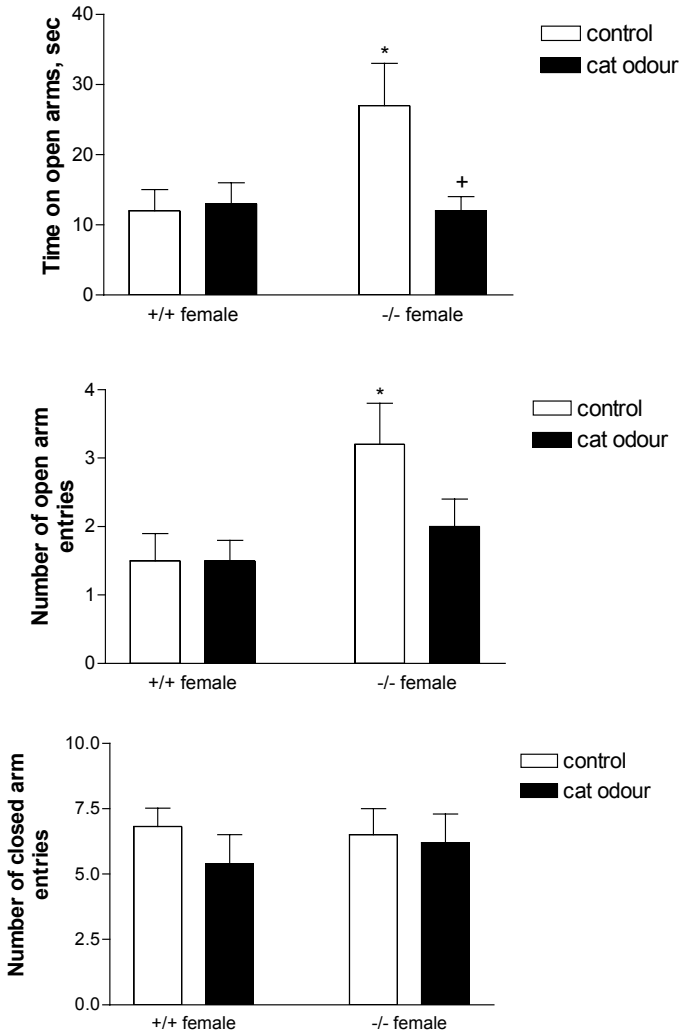

Figure 10. The effect of the cat odour exposure on the exploratory behaviour of CCK_2 receptor deficient mice in the elevated plus-maze, mean values ± S.E.M. The number of mice was nine in each group. *$p < 0.05$, Newman-Keuls test after significant two-way ANOVA compared to the respective group of wild-type mice; +$p < 0.05$, compared to mice of respective genotype exposed to a clean cloth.

2.2. Gene expression studies

As expected, the expression of the Cckbr gene was not detectable in the brain structures of genetically modified mice (Tables 5–8). Comparative basal gene expression of control exposed wild-type mice is shown in Table 9.

Frontal cortex (Table 5)
The comparison of wild-type and CCK_2 receptor deficient mice established that the expression of the following genes was significantly increased in homozygous mice: Cckar, Oprm1, Mc3r and Penk1. Especially, the elevation of Cckar and Oprm1 genes was remarkable. By contrast, the level of Cck expression was significantly reduced in the frontal cortex of homozygous mice. The cat odour exposure did not cause any marked changes in the expression levels of the selected genes in wild-type mice. In homozygous mice, the cat odour exposure caused an even stronger elevation of Mc3r gene expression and reduced somewhat the level of mRNA of Cckar and Oprm1.

Table 5. The effect of the cat odour exposure on neuropeptide gene expression in the frontal cortex of CCK_2 receptor deficient mice, mean values ± S.E.M.
Two-way ANOVA (genotype x stimulus): Cck (genotype $F_{1,12} = 54.4$, $p < 0.001$; stimulus $F_{1,12} = 1.10$, $p = 0.32$; genotype × stimulus $F_{1,12} = 0.001$, $p = 0.97$); Cckar (genotype $F_{1,12} = 71.8$, $p < 0.00001$; stimulus $F_{1,12} = 9.68$, $p < 0.01$; genotype × stimulus $F_{1,12} = 8.29$, $p < 0{,}01$); Cckbr (genotype $F_{1,12} = 716.4$, $p < 0.00001$; stimulus $F_{1,12} = 0.01$, $p = 0.92$; genotype × stimulus $F_{1,12} = 0.01$, $p = 0.92$); Oprm1 (genotype $F_{1,12} = 15{,}7$, $p < 0.01$; stimulus $F_{1,12} = 0.86$, $p = 0.38$; genotype × stimulus $F_{1,12} = 1.19$, $p = 0.31$); Penk1 (genotype $F_{1,12} = 84.7$, $p < 0.0001$; stimulus $F_{1,12} = 0.001$, $p = 0.97$; genotype × stimulus $F_{1,12} = 4.02$, $p = 0.07$); Mc3r (genotype $F_{1,12} = 56.4$, $p < 0.0001$; stimulus $F_{1,12} = 15.4$, $p < 0.01$; genotype × stimulus $F_{1,12} = 4.51$, $p < 0.05$). *$p < 0.05$, Scheffe test after significant two-way ANOVA, compared to the respective group of wild-type mice. +$p < 0.05$, compared to mice of respective genotype exposed to a clean cloth.

Gene	Wild-type, n=9	Wild-type+ Cat odour, n=9	Homozygous, n=9	Homozygous+ Cat odour, n=9
Cck	1.0±0.12	1.10±0.09	**0.50±0.01***	**0.58±0.03***
Cckar	1.0±0.11	0.93±0.16	**5.43±0.68***	**3.11±0.31*,+**
Cckbr	1.0±0.06	1.01±0.05	**0±0***	**0±0***
Pomc1	1.0±0.18	0.60±0.15	0.45±0.06	0.78±0.14
Oprm1	1.0±0.21	1.11±0.20	**3.04±0.54***	2.24±0.49
Oprd1	1.0±0.14	0.94±0.08	1.24±0.13	1.27±0.23
Oprk1	1.0±0.04	0.87±0.08	1.28±0.04	1.21±0.14
Penk1	1.0±0.04	1.10±0.04	**1.54±0.04***	**1.44±0.06***
Pdyn	1.0±0.04	1.01±0.02	1.25±0.02	1.14±0.10
Mc3r	1.0±0.06	1.22±0.12	**1.62±0.07***	**2.29±0.17*,+**
Mc4r	1.0±0.06	0.93±0.08	1.00±0.05	1.09±0.04

Temporal lobe (Table 6)

In the temporal lobe we did not find any differences in the level of gene expression between homozygous and wild-type mice. In the temporal lobe, the exposure to cat odour did not change the expression of genes in wild-type mice. However, in homozygous mice, the expression level of the Pomc1, Oprd1, Penk1 and Mc3r genes was significantly increased after the exposure to cat odour. It is notable that the elevation was the most significant in the Pomc1 gene, where we found an almost 5-fold increase in the expression level.

Table 6. The effect of the cat odour exposure on neuropeptide gene expression in temporal lobe of CCK_2 receptor deficient mice, mean values ± S.E.M.
Two-way ANOVA (genotype × stimulus): Cckbr (genotype $F_{1,12} = 731.7$, $p < 0.00001$; stimulus $F_{1,12} = 0.01$, $p = 0.92$; genotype × stimulus $F_{1,12} = 0.01$, $p = 0.92$); Pomc1 (genotype $F_{1,12} = 27.4$, $p < 0.001$; stimulus $F_{1,12} = 23.0$, $p < 0.01$; genotype × stimulus $F_{1,12} = 21.8$, $p < 0.01$); Oprd1 (genotype $F_{1,12} = 2.17$, $p = 0.17$; stimulus $F_{1,12} = 8.46$, $p < 0.05$; genotype × stimulus $F_{1,12} = 2.10$, $p = 0.19$); Penk1 (genotype $F_{1,12} = 2.90$, $p = 0.13$; stimulus $F_{1,12} = 17.7$, $p < 0.01$; genotype × stimulus $F_{1,12} = 2.91$, $p = 0.12$); Mc3r (genotype $F_{1,12} = 0.78$, $p = 0.40$; stimulus $F_{1,12} = 50.6$, $p < 0.0001$; genotype × stimulus $F_{1,12} = 0.03$, $p = 0.88$). *$p < 0.05$, Scheffe test after significant two-way ANOVA, compared to the respective group of wild-type mice. +$p < 0.05$, compared to mice of respective genotype exposed to a clean cloth.

Gene	Wild-type, n=9	Wild-type+ Cat odour, n=9	Homozygous, n=9	Homozygous+ Cat odour, n=9
Cck	1.0±0.08	1.11±0.06	1.01±0.20	1.81±0.41
Cckar	1.0±0.11	1.26±0.09	0.85±0.14	0.94±0.09
Cckbr	1.0±0.04	1.24±0.07	**0±0***	**0±0***
Pomc1	1.0±0.15	1.07±0.12	1.23±0.28	**4.64±0.64*,+**
Oprm1	1.0±0.10	1.01±0.02	1.05±0.11	0.92±0.13
Oprd1	1.0±0.06	1.21±0.17	1.01±0.12	**1.63±0.18***
Oprk1	1.0±0.06	1.24±0.05	1.03±0.04	1.03±0.04
Penk1	1.0±0.13	1.22±0.05	1.01±0.07	**1.50±0.04***
Pdyn	1.0±0.04	1.06±0.17	0.98±0.07	1.07±0.04
Mc3r	1.0±0.04	1.13±0.08	0.80±0.03	**1.42±0.05*,+**
Mc4r	1.0±0.04	1.22±0.02	0.83±0.05	0.81±0.06

Mesolimbic area (Table 7)

In the mesolimbic area the expression of the Cck gene was reduced and the Mc3r gene was increased if homozygous mice were compared to wild-type animals.

The exposure of mice to cat odour caused a significant decrease in the expression of the Cck gene in wild-type animals, and an elevation in the expression of the Pomc1 and Cckar gene in homozygous mice.

Mesencephalon (Table 8)

In the mesencephalon the expression of Oprm1 was significantly increased in homozygous mice compared to wild-type littermates. The exposure of mice to cat odour increased the expression of the Mc3r gene in wild-type mice and the Pomc1 gene in homozygous animals.

Table 7. The effect of the cat odour exposure on neuropeptide gene expression in mesolimbic area of CCK_2 receptor deficient mice, mean values ± S.E.M.
Two-way ANOVA (genotype × stimulus): Cck (genotype $F_{1,12} = 59.1$, $p < 0.0001$; stimulus $F_{1,12} = 21.9$, $p < 0.01$; genotype × stimulus $F_{1,12} = 0.18$, $p = 0.68$); Cckar (genotype $F_{1,12} = 24.1$, $p < 0.001$; stimulus $F_{1,12} = 0.40$, $p = 0.54$; genotype × stimulus $F_{1,12} = 0.04$, $p = 0.84$); Cckbr (genotype $F_{1,12} = 67.3$, $p < 0.00001$; stimulus $F_{1,12} = 0.63$, $p = 0.44$; genotype × stimulus $F_{1,12} = 10.7$, $p < 0.01$); Pomc1 (genotype $F_{1,12} = 10.3$, $p < 0.01$; stimulus $F_{1,12} = 2.24$, $p = 0.17$; genotype × stimulus $F_{1,12} = 7.92$, $p < 0.05$); Oprm1 (genotype $F_{1,12} = 18.7$, $p < 0.01$; stimulus $F_{1,12} = 2.10$, $p = 0.18$; genotype × stimulus $F_{1,12} = 0.60$, $p = 0.46$); Mc3r (genotype $F_{1,12} = 52.3$, $p < 0.0001$; stimulus $F_{1,12} = 1.17$, $p = 0.31$; genotype × stimulus $F_{1,12} = 4.03$, $p = 0.07$). *$p < 0.05$, Scheffe test after significant two-way ANOVA, compared to the respective group of wild-type mice. +$p < 0.05$, compared to mice of respective genotype exposed to a clean cloth.

Gene	Wild-type, n=9	Wild-type+ Cat odour, n=9	Homozygous, n=9	Homozygous+ Cat odour, n=9
Cck	1.0±0.02	**0.72±0.05+**	**0.56±0.05***	**0.33±0.08***
Cckar	1.0±0.27	0.93±0.13	1.85±0.26	**2.18±0.10***
Cckbr	1.0±0.06	0.67±0.08	**0±0***	**0±0***
Pomc1	1.0±0.18	0.76±0.30	1.07±0.08	**1.87±0.07*,+**
Oprm1	1.0±0.09	0.72±0.03	1.47±0.21	**1.38±0.11***
Oprd1	1.0±0.05	1.01±0.07	1.10±0.10	1.08±0.11
Oprk1	1.0±0.02	0.94±0.08	0.92±0.03	0.82±0.05
Penk1	1.0±0.19	0.89±0.21	0.90±0.10	0.60±0.14
Pdyn	1.0±0.18	0.74±0.11	0.78±0.19	0.66±0.03
Mc3r	1.0±0.06	0.92±0.04	**1.44±0.08***	**1.38±0.06***
Mc4r	1.0±0.6	1.13±0.14	1.11±0.11	0.88±0.08

Table 8. The effect of the cat odour exposure on neuropeptide gene expression in mesencephalon of CCK_2 receptor deficient mice, mean values ± S.E.M.
Two-way ANOVA (genotype × stimulus): Cckbr (genotype $F_{1,12} = 139.0$, $p < 0.000001$; stimulus $F_{1,12} = 1.29$, $p = 0.27$; genotype × stimulus $F_{1,12} = 1.40$, $p = 0.25$); Pomc1 (genotype $F_{1,12} = 35.2$, $p < 0.001$; stimulus $F_{1,12} = 71.8$, $p < 0.0001$; genotype × stimulus $F_{1,12} = 31.3$, $p < 0.0001$); Oprm1 (genotype $F_{1,12} = 39.8$, $p < 0.001$; stimulus $F_{1,12} = 0.32$, $p = 0.59$; genotype × stimulus $F_{1,12} = 2.74$, $p = 0.14$); Mc3r (genotype $F_{1,12} = 1.60$, $p = 0.24$; stimulus $F_{1,12} = 12.7$, $p < 0.01$; genotype × stimulus $F_{1,12} = 40.1$, $p < 0.001$). *$p < 0.05$, Scheffe test after significant two-way ANOVA, compared to the respective group of wild-type mice. +$p < 0.05$, compared to mice of respective genotype exposed to a clean cloth.

Gene	Wild-type, n=9	Wild-type+ Cat odour, n=9	Homozygous, n=9	Homozygous+ Cat odour, n=9
Cck	1.0±0.10	1.31±0.11	1.17±0.24	1.16±0.28
Cckar	1.0±0.15	1.28±0.11	1.03±0.09	0.96±0.11
Cckbr	1.0±0.14	0.85±0.08	**0±0***	**0±0***
Pomc1	1.0±0.13	1.44±0.12	1.07±0.14	**3.12±0.19*,+**
Oprm1	1.0±0.18	1.39±0.13	**2.30±0.04***	**2.13±0.23***
Oprd1	1.0±0.01	1.05±0.03	1.03±0.07	1.28±0.04
Oprk1	1.0±0.02	1.12±0.09	0.96±0.15	0.92±0.09
Penk1	1.0±0.14	0.95±0.05	0.70±0.07	0.68±0.11
Pdyn	1.0±0.10	1.20±0.15	1.46±0.12	1.48±0.13
Mc3r	1.0±0.02	**1.42±0.03+**	1.32±0.07	1.21±0.04
Mc4r	1.0±0.09	1.02±0.08	0.87±0.06	1.06±0.13

Table 9. Comparative basal gene expression in wild-type control group mice, mean values of fold increase shown.

Gene	Frontal cortex	Temporal lobe	Mesolimbic area	Mesencephalon
Cck	227245.0	91793.9	21849.0	13327.9
Cckar	1.0	46.2	92.7	95.3
Cckbr	3089.9	1478.6	276.9	409.4
Pomc1	388.8	580.3	3958.8	317.2
Oprm1	80.4	250.4	291.9	236.1
Oprd1	509.2	219.2	188.4	145.4
Oprk1	627.2	1376.4	1867.6	1056.9
Penk1	1693.9	5188.5	7103.8	1197.2
Pdyn	1976.3	3357.9	9391.1	515.1
Mc3r	8.64	58.1	109.6	59.4
Mc4r	155.6	275.8	386.8	234.3

3. Experiment 3: Establishing genes in rat amygdaloid area induced by the cat odour exposure

3.1. cDNA Representational Difference Analysis (cDNA-RDA) followed by dot-blot analysis of clones

cDNA-RDA was performed to find the genes expressed in the amygdala of Wistar rats in response to the cat odour exposure. When DP3 was generated at high stringency (1:400,000), no detectable bands were observed. Therefore, we applied much lower stringency for the DP3 subtraction (1:10,000) and at the end of differential cloning (DP3) clearly distinguishable bands were observed. This probably indicates that there are no robust changes in gene expression caused by anxiety response, and the resulting differences as compared to the relaxed condition reflect moderate quantitative differences between these two populations. We randomly isolated 288 up-regulated clones and 288 clones down-regulated during the anxiety response. All clones were sequenced and analysed by the NIX application (at the UK HGMP website). A database search (http://menu.hgmp.mrc.ac.uk/menu-bin/Nix/Nix.pl) revealed several genes with different functions. There were transcription factors, transporter molecules, enzymes, receptors, expressed sequence tags (ESTs) and recently cloned proteins with unknown function. Dot blot analysis confirmed 41 differentially expressed genes in anxiety responders and 26 differentially expressed genes in control animals (Tables 10 and 11). Examples of the most remarkable differences after dot-blot hybridisation are shown in Table 12. Moreover, semi-quantitative differences between different dots were measured (Tables 10 and 11). After forward subtraction we found evidence for up-regulation of carboxypeptidase E (CPE) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein (protein 14-3-3), the genes that are involved in the synthesis of neurotransmitters. However, the difference in the expression level was rather small (1.1- and 1.2-fold increase). With the highest expression level (1.8-fold increase) we found a gene with unknown function: Gamm1 or Myg-1 (melanocyte proliferating gene 1). The genes with moderate differences in expression levels were Rho-GTPase, CDCrel-1, NFB42, and Ca/calmodulin-dependent protein kinase, and Na+/K+-ATPase. The rest of the genes showed only small differences in the expression levels compared to the control group. After reverse subtraction the highest differences (1.4-fold increase) were found for nectadrin and to general control of amino acid synthesis-like 2 (also named as LOC303540, PCAF-B/GCN5). All other genes showed only small levels of differences. Most of the differentially expressed genes were with unknown function. However, in some cases there are limited data about their possible function and the results were interpreted according to the published data to understand a possible role for these genes in the regulation of anxiety.

3.2. RT-PCR analysis

Reverse transcriptase PCR was performed with the aim to find differences in the expression levels of c-fos and CRF genes. The ratio of c-fos/GAPDH and ratio CRF/GAPDH were used to quantify relative expression levels. We did not find any differences in the c-fos or CRF expression levels in rats after the exposure to cat odour (Figure 11, CRF data not shown). To confirm dot blot hybridisation, we performed RT-PCR also from the 3rd differential products (Figure 11). We did not find any signal of GAPDH and c-fos after the subtraction, which confirms that equally represented transcripts were subtracted "out". Interestingly, in cDNAs (drivers) we detected both Gamm1 and CPE signals without any significant differences. After subtraction, CPE signals were detectable in both samples (higher in the "cat odour" sample); Gamm1 signal was present only in the "cat odour" sample. This result shows that RDA is also sensitive to small quantitative differences in the starting material. However, precise measurements of the levels of transcripts in DP3 were impossible, as no housekeeper signal was detected after subtraction.

Table 10. Transcripts increased in amygdala of rat during anxiety response.
Each clone was dotted onto two identical membranes; equal amount of cDNA was used for the synthesis of DIG-labelled probes and hybridisation. The cDNA of six animals in each group was pooled and used as hybridisation probes. In order to minimise false positive results we used cDNA for hybridisation that was not used for RDA. Experiments were repeated twice, only genes giving positive signals in both experiments are listed. In order to evaluate semiquantitative differences,corrected gray values of the dots were compared to the membrane hybridised with a different probe.

Gene name	GenBank Acc	Fold increase
GAMM1 M. musculus protein, Myg-1	AF252871	1.8
CDCre1-1A	AB027143	1.5
KIAA0337 protein, Rho-specific guanine-nucleotide exchange factor 164 kDa	NM_014786	1.5
Rattus norvegicus Rho GTPase activating protein 4 (Arhgap4)	NM_144740	1.5
Neural F box protein NFB42	AF098301	1.4
Putative transmembrane protein 2c	AF282981	1.4
BEC1 protein	AF035814	1.3
Ca/calmoduline-dependent protein kinase alpha	AB023658	1.3
KIAA0408 protein	AL096711	1.3
Mus musculus expressed sequence AI428855	AI428855	1.3
Na^+,K^+-ATPase alpha(+) isoform catalytic subunit	M14512	1.3
NADH-ubiquinone oxidoreductase B17 subunit	XM_204158	1.3
NIR1	AF334586	1.3
Rattus norvegicus calmodulin III (Calm3)	AF231407	1.3

Gene name	GenBank Acc	Fold increase
Rattus norvegicus CaM-kinase II inhibitor alpha	AF271156	1.3
Rattus norvegicus neurochondrin, norbin	NM_053543	1.3
Apolipoprotein E gene	J02582	1.2
Carboxypeptidase E (EC 3.4.17.10.)	X51406	1.2
DXImx39e, M. musculus	AF229636	1.2
Homeobox protein HOX-4.4 and HOX-4.5	X62669	1.2
Limbic system associated membrane protein (LsAMP)	NM_017242	1.2
Myelin-associated glycoprotein precursor (l-MAG/S-MAG)	M14871	1.2
NCI-CGAP-Lu29, M. musculus cDNA clone 601102606F1	BE305755	1.2
ODZ3	AF195418	1.2
T-type calcium channel alpha-1 subunit	AF051947	1.2
WFS1, wolframin	AF136378	1.2
Beta-spectrin III	AB008551	1.1
EST 196451 Normalised rat kidney, cDNA clone RKIAV31	AA892648	1.1
EST349530 Rat gene index, cDNA clone RGIEO08	AW918226	1.1
Gelsolin (actin-depolymerizing factor — ADF), brevin	J04953	1.1
Homo sapiens cDNA clone IMAGE:2813462	AW303350	1.1
KIAA0429 protein	XM226503	1.1
KIAA0771 protein	AB018314	1.1
Mus musculus cDNA clone IMAGE:575778	AA120430	1.1
NCI-CGAP-Lu29, M. musculus cDNA clone IMAGE: 3989880, 601770406F1	BF161425	1.1
NIH_MGC_71 Homo sapiens cDNA clone	BE889795	1.1
Rattus norvegicus Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	NM_013053	1.1
Transketolase (EC 2.2.1.1.)	U09256	1.1
Rattus norvegicus similar to hypothetical protein MGC25696	XM229276	1.1
KIAA0014 protein	NM_014665	1.1

Table 11. Transcripts increased in the amygdaloid area of control rats compared to rats with anxiety response.

Gene name	GenBank Acc	Fold increase
Similar to general control of amino acid synthesis-like 2, LOC303540	XM220979	1.4
Rattus norvegicus heat stable antigen CD24, nectadrin	U49062	1.4
2,3-cyclic nucleotide 3-phosphodiesterase (CNPII)	L16532	1.3
Mus musculus signal recognition particle receptor beta subunit	U17343	1.3
Neural membrane protein 35	AF044201	1.3
Testican-3 protein	AJ278998	1.3
G protein gamma subunit (gamma7 subunit)	L23219	1.2
MAP kinase kinase	Z16415	1.2
Mus musculus, clone IMAGE:3987018	BC019714	1.2
Mus musculus, Similar to hypothetical protein clone MGC:7259 IMAGE:3484751	BC002144	1.2
Nischarin	AF315344	1.2
Plectin	X59601	1.2
Rattus norvegicus voltage-dependent anion channel	AF268469	1.2
rELO1 mRNA for fatty acid elongase 1	AB071985	1.2
Rev-Erb-alpha protein	AF291821	1.2
Sequence specific single-stranded DNA-binding protein 2	AY037837	1.2
Similar to stress-associated endoplasmic reticulum protein 1	BC029067	1.2
Cyclin-dependent kinase homologue	L37092	1.1
EST293447 Normalized rat brain cDNA clone RGIBE57	AW143151	1.1
Mus musculus, predicted gene ICRFP703B1614Q5.3	BC002208	1.1
NCI_CGAP_Mam2 Mus musculus cDNA clone IMAGE:5374091, 603346543F1	BI697150	1.1
Non-histone chromosomal protein HMG-14	X53476	1.1
Rab geranylgeranyl transferase component B beta	S62097	1.1
SIR2L2	AF299337	1.1
Splicing factor U2AF 65 kDa subunit	X64587	1.1
ZnBP gene for zinc binding protein	X64053	1.1

Table 12. Results of the dot-blot hybridisation.
Only samples with the most remarkable differences are shown. 100 µl of sample (contains 100 ng of unamplified plasmid DNA with 0.4 M NaOH and 10 mM EDTA) was denaturated (10 min at 100°C) and dotted onto a Hybond N^+ nylon membrane. Unsubtracted cDNA-s were used as probes and non-radioactive labeling (with DIG High Prime DNA Labeling and Detection Starter Kit I from Roche) reaction was performed.

Cat	Control	
		GAMM-1
		Rho GTPase activating protein 4
		CDCrel-1A
		Ca/calmodulin-dependent protein kinase alpha
		LOC303540
		Testican-3
		Rev-Erb-alpha protein
		Rab geranylgeranyl transferase

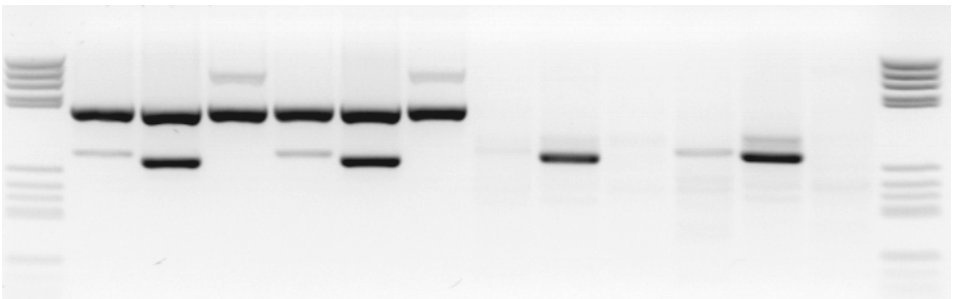

Figure 11. RT-PCR analysis of drivers (amplified cDNA) and 3rd differential product (DPIII). GAPDH primers were used to control the loading of DNA in all lanes (product size 409 bp).
Lane 1: pBR322 DNA/*Bsu*RI (*Hae*III) Marker; Lane 2: cDNA control, GAMM1 primers (product 303 bp); Lane 3: cDNA control, carboxypeptidase E (CPE) primers (product 289 bp); Lane 4: cDNA control, c-fos primers (product 520 bp); Lane 5: cDNA cat odour, GAMM1 primers; Lane 6: cDNA cat odour, CPE primers; Lane 7: cDNA cat odour, c-fos primers; Lane 8: DP3 Control, GAMM1 primers; Lane 9: DP3 Control, CPE primers; Lane 10: DP3 Control, c-fos primers; Lane 11: DP3 Cat odour, GAMM1 primers; Lane 12: DP3 Cat odour, CPE primers; Lane 13: DP3 Cat odour, c-fos primers; Lane 14: pBR322 DNA/*Bsu*RI (*Hae*III) Marker.

VI. DISCUSSION

1. Action of the cat odour exposure on the behaviour of rodents

1.1. Behavioural changes in rats during the cat odour exposure (Papers I, III)

The exposure of male Wistar rats to a natural unpleasant stimulus — cat odour — induced a robust anxiogenic-like response in all behavioural parameters measured. Numbers of grooming, sniffing and animals in the proximity of cloth were decreased significantly. Remarkably, none of the animals touched the cat odour impregnated cloth. Animals not exposed to the cat odour did not display freezing behaviour, whereas under the influence of predator odour this behaviour, reflecting fear and anxiety, was particularly increased. Moreover, after the exposure to cat odour the exploratory behaviour of male Wistar rats was significantly suppressed in the elevated zero-maze model of anxiety. This behavioural suppression replicates previously described results (Dielenberg & McGregor 2001) and is considered to reflect anxiety rather than fear, as this behavioural reaction persists for some time after the odour exposure (McGregor *et al.* 2002). Thus, the cat odour exposure caused an anxiety reaction in rats, which was taken as an assumption for further experiments. It has to be noted that the exposure of rats to cat odour for 30 minutes is a strong non-escapable stress for animals. It is unlikely in the natural circumstances that the rat would be exposed for such a long period of time to predator odour. Rat has to escape quickly or otherwise the cat will kill it.

1.2. The cat odour exposure antagonises the stimulating effect of morphine on the exploratory behaviour of rats (Paper I)

The exposure to cat odour completely abolished the increase of exploratory behaviour induced by opioid agonist morphine (1 mg/kg) in an unfamiliar environment. Similar changes after previous handling and adaptation of rats to the experimental environment have been reported earlier by our group (Kõks *et al.* 2000). Thus, the exposure of rats to cat odour induced significant adaptational changes in the opioid system. One possible mechanism of adaptation is the desensitisation of μ-opioid receptors, probably accomplished by an increase in the release of opioid peptides in the brain. This notion is indirectly supported by the study of Lester and Fanselow (Lester & Fanselow 1985) demonstrating that the exposure to cat odour produced significant analgesia in rats, reversible by naltrexone, an antagonist of opioid receptors. One the other hand, it has been shown that the administration of morphine (1–7.5 mg/kg) increases defensiveness in a situation associated with the cat odour stimuli (Blanchard *et al.* 1991),

but the opioid agonist eliminates ultrasonic vocalisations evoked by the cat odour in rats (Shepherd *et al.* 1992). These findings support the idea that the opioid system is involved in the regulation of anxiety. Alternatively, it is possible that the cat odour exposure raises the activity of opioid system to a higher level and thus morphine is unable to overcome the effect of endogenous opioid ligands at the μ-opioid receptors. However, this seems unlikely, because it should be accompanied by increased exploratory activity of rats. As a matter of fact, the exploratory behaviour of rats is significantly decreased in the exploratory models of anxiety after the cat odour exposure. Therefore, the increased activity of the opioid system is rather reflecting a compensatory response to the emotional stress induced by the cat odour.

1.3. Behavioural responses of wild-type and CCK_2 receptor deficient mice to the cat odour exposure (Paper II)

The behavioural analysis revealed that both wild-type and CCK_2 receptor deficient mice avoided the cloth impregnated with cat odour. Also, the frequency of risk assessment behaviour, as measured by the number of stretch-attend postures, was increased in both genotypes. Hebb and colleagues (Hebb *et al.* 2004) demonstrated that the exposure of CD1 mice to the fox pheromone TMT elevated their risk assessment behaviour and suppressed non-defensive behaviours. The exposure to TMT also increased subsequent anxiety in the light/dark exploration test (Hebb *et al.* 2004). However, it should be noted that the behavioural response of female 129Sv and C57Bl6 mice to the cat odour is significantly weaker compared to that of described for the male Wistar rats (our unpublished data). 129Sv animals display increased anxiety, but they do not seem to respond to the exposure of cat odour at all. By contrast, there is some evidence that the exposure to the cat reduces the exploratory behaviour of C57Bl6 mice. Belzung and colleagues (Belzung *et al.* 2001) have shown that the response of different mice strains to the cat odour is very variable. According to this study, C57Bl6 mice belong to the strong responders. Unfortunately, 129Sv strain was not explored in this study, but it is obvious from our experience that this strain belongs to the non-responders.

In our previous studies it was established that the exploratory behaviour of female mice of 129Sv/C57Bl6 background, lacking CCK_2 receptors, was increased in the elevated plus-maze compared to their wild-type littermates (Raud *et al.* 2003). The same was found in the present experiment, because homozygous mice displayed a two-fold increase in the number of open arm entries and spent significantly longer time on open arms compared to wild-type animals. The exposure to cat odour caused a significant anxiogenic-like action in CCK_2 receptor deficient mice exposed to the elevated plus-maze. This effect was not seen in wild-type animals, displaying significantly lower exploratory activity in the plus-maze. It is remarkable that a higher exploratory activity of

mice is also necessary for the detection of anxiogenic-like effect induced by genetic manipulations. Holmes and colleagues (Holmes *et al.* 2003a) demonstrated that the genetic disruption of the 5-HT transporter gene induces in the elevated plus-maze anxiety-like state in the C57Bl6, but not in the 129Sv strain. Indeed, the exploratory activity of C57Bl6 mice is significantly higher in the plus-maze compared to the 129Sv strain (Võikar *et al.* 2001). Therefore, the low exploratory activity of wild-type mice could be a likely reason why the cat odour exposure did not affect their behaviour in the plus-maze. However, this seems not to be the only explanation. Previously our group has found that the current level of exploratory activity (between 1.5 and 2 open arm entries) of wild-type mice is high enough to establish the anxiogenic-like effect of social isolation (Abramov *et al.* 2004). In the mentioned study, social isolation induced a statistically significant reduction of open arm entries in female wild-type mice, but not in CCK_2 receptor deficient mice. Social isolation of animals also decreased time spent in the exploration of open platform, located between the open and closed arms, in wild-type mice (from 46 ± 6 s in group housed mice to 25 ± 5 s in isolated animals). It means that after social isolation the exploratory activity of wild-type mice was even more shifted into the enclosed arms. By contrast, the cat odour exposure did not change this parameter in wild-type mice (58 ± 7 s in control group versus 70 ± 11 s in cat odour exposed mice). Consequently, one could state that the cat odour exposure is less challenging compared to social isolation in female wild-type mice having C57Bl6/129Sv background. Apparently, despite the numerous crossings into the C57Bl6 background, the genetic effect of 129Sv strain still dominates in wild-type mice and this dominating background suppresses the anxiogenic-like action of cat odour.

2. The effect of the cat odour exposure on the expression of opioid- and CCK-related genes in the brain structures of rats (Paper I)

Increased expression of the rat Cck, Pomc1 and Oprm1 genes was established after the cat odour exposure. It is worthy to note that the biggest changes in gene expression were established in the brain regions related to anxiety (amygdala — Pomc1) and motivation (mesolimbic area — Cck, Pomc1 and Oprm1). There was no change in the Cck expression in the amygdala, cortex or striatum. Wang and colleagues (Wang *et al.* 2003) established a 1.2-fold increase in the expression of the Cckbr gene in the frontal cortex of rats after the cat odour exposure. Chen and colleagues (Chen *et al.* 2006) produced a new transgenic line of mouse over-expressing CCK_2 receptors in the brain. A robust increase in anxiety is the dominating peculiarity in the behaviour of these mice. Another evidence for the role of the CCK system in the mechanisms of anxiety

comes from our recent study. Namely, we found that Wistar rats displaying low exploratory activity in the elevated plus-maze also show an increased expression of the Cckar and Cckbr genes in the amygdala compared to high exploratory and home-cage control rats (Nelovkov *et al.* 2006). Moreover, it has been shown that after stressors both CCK-like immunoreactivity and CCK_2 receptors density in the brain of rats are increased, indicating that the elevated CCKergic tone is associated with an increased anxiety due to stress (Harro *et al.* 1996; Siegel *et al.* 1987). The activation pattern in the amygdala probably reflects an increase in anxiety, whereas in the mesolimbic area established changes in the opioid genes may be responsible for the abolished action of morphine on the exploratory behaviour. However, it is not clear whether the over-expressed Pomc1 transcript will be processed to β-endorphin during the cat odour exposure or to someother peptides such as ACTH or β-MSH. Nevertheless, the increased expression of the Pomc1 and Oprm1 genes, and the abolished action of the μ-opioid agonist morphine leads us to assume that the processing of the Pomc1 transcript into β-endorphin is increased.

3. The effect of the cat odour exposure on the expression of CCK, opioid and melanocortin related genes in the brain structures of wild-type and CCK_2 receptor deficient mice (Paper II)

The exposure of wild-type mice to cat odour did not increase anxiety in the elevated plus-maze and caused only minor changes in the expression pattern of the selected genes in various brain structures. The expression of the Cck gene was significantly reduced in the mesolimbic area and the expression of the Mc3r gene was significantly increased in the mesencephalon of wild-type mice. This may reflect to some extent the suppression of exploratory behaviour established in wild-type mice in the presence of a cloth impregnated with cat odour. Indeed, the number of contacts with the cloth was decreased and the number of risk assessment behaviours was increased in the presence of predator odour. Nevertheless, this evidence is too premature to draw any definite conclusions from that.

The higher exploratory activity of CCK_2 receptor mice was accompanied by an increased expression of Oprm1 in the frontal cortex and mesencephalon. This finding is in good agreement with previous studies showing that CCK_2 receptor deficient mice have an increased tone of the opioid system (Pommier *et al.* 2002; Veraksitš *et al.* 2003). Motta and Brandao (Motta & Brandao 1993) established that systemic treatment with morphine, an agonist of μ-opioid receptors, at low doses or its injection into the dorsal periaqueductal gray, located in the mesencephalon, induced anxiolytic-like action in the plus-maze. At low doses, naloxone, an antagonist of opioid receptors, reversed the

anxiolytic-like effect of morphine. Kõks and colleagues (Kõks *et al.* 1998) have found that the coadministration of naloxone and CCK_2 receptor agonist BOC-CCK at subeffective doses induces a strong anxiogenic-like action in the elevated plus-maze. Moreover, pretreatment of rats with BOC-CCK-4 antagonised the anxiolytic-like action of morphine (Kõks *et al.* 1999). These studies demonstrate a significant functional interaction between the CCK and the opioid system in the regulation of anxiety and exploratory behaviour. Therefore, it is likely that the increased expression of Oprm1 could be partly attributed to the increased exploratory activity established in CCK_2 receptor deficient mice. Moreover, the expression of Cckar was also significantly increased in the frontal cortex and mesolimbic area of homozygous mice. It has been shown that the stimulation of CCK_1 and CCK_2 receptors by CCK-8 produces different effects. Branchereau and colleagues (Branchereau *et al.* 1992) demonstrated that the stimulation of CCK_1 receptors inhibits the brainstem solitary complex in rats, whereas the stimulation of CCK_2 receptors causes the opposite effect. The blockade of CCK_1 receptors with devazepide increases anxiety, whereas the blockade of CCK_2 receptors with L-365,260 induces an anxiolytic action in rats (Männisto *et al.* 1994). However, in certain cases the effect of the stimulation of the CCK_1 and CCK_2 receptors can be in the same direction. Recently we established that CCK-8 in low nanomolar concentrations increases the activity of Na^+/K^+-ATPase in the frontal cortex of mice (our unpublished data). This effect was potently antagonised by CCK_1 receptor antagonist devazepide and CCK_2 receptor antagonist L-365,260. In mice, lacking CCK_2 receptors, 100-fold lower concentrations of CCK-8 were effective in stimulating the activity of Na^+/K^+-ATPase. This increase in the action of CCK-8 was accompanied by a 5-fold increase in the expression of the Cckar gene. Therefore, one could state that the elevation of the Cckar gene in the frontal cortex is the result on an adaptation to the lack of CCK_2 receptors. Simultaneously with the increase of the Cckar gene the expression of the Cck gene was reduced in the frontal cortex and mesolimbic area of mice, lacking CCK_2 receptors. It is likely that this change in the expression of Cck gene, determining the synthesis of endogenous ligand CCK-8, is a compensatory response to the increased level of the Cckar gene. Noble and Roques (Noble & Roques 1999) described a CCK and opioid supra-spinal interaction model in which signals through CCK_1 receptor exert functional pro-opioid and signals through CCK_2 receptor anti-opioid activity. Thus, the pro-opioid action of CCK_1 receptor, as well as the up-regulation of the opioid system, may both contribute to the reduced anxiety in CCK_2 receptor deficient mice. This hypothesis seems to be supported by the fact that under the influence of cat odour the expression of the Cckar and Oprm1 genes was reduced to some extent in the frontal cortex of homozygous mice.

By contrast to wild-type mice, in the CCK_2 receptor deficient animals significant alterations in the gene expression were found after the exposure to cat odour. Especially, the increase of the Pomc1 gene was remarkable in the temporal lobe, mesencephalon and mesolimbic area. It has to be noted that an

increased expression of the Pomc1 gene was also detected in the amygdala and mesolimbic area of rats after the cat odour exposure (Paper I). Moreover, the cat odour exposure elevated the expression of the Mc3r gene in the temporal lobe and frontal cortex of homozygous mice. It has been shown that the administration of α-melanocyte stimulating hormone, an endogenous agonist of melanocortin receptors, into the amygdala of rats induces an anxiogenic-like action in the elevated plus-maze (Kokare *et al.* 2005). The administration of various Mc4r antagonists induces anxiolytic-like and antidepressant-like effects in different models of stress and anxiety in rodents (Chaki *et al.* 2003a; Chaki *et al.* 2003b; Shimazaki & Chaki 2005). These findings obtained from pharmacological studies are in favour of an anxiogenic action of α-melanocyte stimulating hormone in rodents. In the temporal lobe, cat odour also increased the expression of the Oprd1 and Penk1 genes in homozygous mice. The Penk1 gene is responsible for the synthesis of enkephalins, the endogenous ligands for δ-opioid receptors. In the light of this finding it is interesting to note that mice, lacking the Oprd1 and Penk1 genes, display increased anxiety- and depression-like symptoms (Gaveriaux-Ruff & Kieffer 2002). Accordingly, the established changes in the expression of the Pomc1 and Mc3r genes in the temporal lobe seem to contribute to the increased anxiety due to the cat odour exposure, whereas the elevation of the Oprd1 and Penk1 mRNA is a compensatory response to the increased anxiety.

4. Genes induced by the cat odour exposure in the amygdaloid area of Wistar rats (Paper III)

Two studies discussed previously help us to draw the following conclusions. First, male Wistar rats are significantly better responders to the cat odour compared to mice of 129Sv/C57Bl6 background. Second, both studies demonstrate the up-regulation of stress-related genes in the temporal lobe, particularly in the amygdala. Therefore, to identify new genetic markers induced by the cat odour, samples of the amygdala obtained from male Wistar rats were used.

We identified a number of genes that were over-expressed in the rat amygdala in response to the exposure to cat odour. Altogether they reflect that not only genes related to neurotransmission, but also other genes, responsible for the functioning of nerve and glial cells, are activated. Results from the "reverse" subtraction (control versus cat odour group) are considered to show the genes, which are down-regulated during the anxiety response. "Reverse" subtraction gives additional information to the results obtained from the "forward" subtraction and is useful as an "internal control". Complex analysis of the genes from both subtractions supports the findings from each of the experiments and helps to understand their function, especially when the different experiments have revealed genes with antagonistic functions. As we

did not get any detectable signals at the high stringency subtractions (1: 400,000), but found some signals in more relaxed conditions (1: 10,000), we can conclude that the changes in the abundance of these transcripts are moderate quantitative differences and not "all-or-none" qualitative ones. Similar effect of the subtraction stringency has been described earlier (Hubank & Schatz 1999). Another important issue is that subtractive hybridisation and following PCR enriches relatively the abundance of rare transcripts in the mRNA population. That can explain why we detected genes with rather small differences in their expression levels. Of course, it is possible that subtraction has not been sensitive and efficient enough. We confirmed our subtraction by means of RT-PCR. Transcripts equally expressed in both samples (housekeepers) should disappear after RDA. Indeed, we were not able to detect signals for GAPDH and cyclophilin. Even signals of c-fos disappeared after performing RDA. According to published data, the genes we were able to clone could be grouped into possible functional subgroups.

The first set includes genes directly related to neurotransmission. Carboxypeptidase E participates in the synthesis of neuropeptides involved in the regulation of emotional behaviour (Berman *et al.* 2001; Gabreels *et al.* 1998). Wolframin is a protein with unknown function, but it seems to be involved in the regulation of emotional behaviour. Mutations in the wolframin gene may predispose people to affective disorders (Swift *et al.* 1998). As the activation of wolframin gene occurs in parallel with the activation of carboxypeptidase E, it is tempting to speculate that wolframin is a protein participating in the synthesis of bioactive peptides, including neuropeptides (Kõks *et al.* 2002). Some supportive evidence for that speculation is coming from the recent study stating that Wolfram syndrome patients have defective vasopressin processing with no detectable staining for prohormone convertase 2 (Gabreels *et al.* 1998). Moreover, coimmunoprecipitation in cell culture revealed an interaction between transiently expressed N-terminal fragments of wolframin aminoacids 1-310 and carboxypeptidase E aminoacids 78-280 (Luuk 2004). However, this hypothesis needs the further investigations. Experiments with Wfs1 knockout mice are being done by our group.

The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein reflects that the synthesis of monoamines is activated. The up-regulation of these genes supports their possible role in stress coping.

On the other hand, we found that in control animals, the neural membrane protein 35 (NMP35) and G protein gamma 7 subunit genes were over-expressed. The expression of NMP35 was similarly 1.6-fold up-regulated in Sprague-Dawley rats that displayed less anxiety-like behaviour if compared to PVG hooded rat strain after live cat exposure (Wang *et al.* 2003). These genes are involved in signal transduction in many types of neurons. NMP35 is a novel protein expressed in dendrites and at the postsynaptic membrane (Schweitzer *et al.* 2002). These genes reflect the down-regulation of certain signalling pathways during the anxiety response.

Rho GTPase activating protein and Rho-specific guanine-nucleotide exchange factor are both involved in the growth cone guidance (Dickson 2001). We were able to detect the activation of several different genes involved in that pathway (Rho GTP-ase activating protein 4, Rho-specific guanine-nucleotide exchange factor) during the anxiety response.

In addition, we established that the Ca/calmodulin-dependent protein kinase α and neurochondrin (norbin) were upregulated during the exposure to cat odour. Both these genes have been shown to participate in the formation of LTP (Kasahara *et al.* 2001; Shinozaki *et al.* 1997). Inhibition of calcium/calmodulin-dependent protein kinase kinase by protein 14-3-3 has been suggested (Davare *et al.* 2004). Several genes, having suppressive action on the Rho GTP-ase pathway, are down-regulated during the anxiety response.

After "reverse" subtraction we found that the Rab geranylgeranyl transferase and nischarin genes were up-regulated in control rats compared to rats exposed to cat odour. These genes have been shown to inhibit the activity of the Rho GTP-ase pathway (Alahari *et al.* 2000; Holstein *et al.* 2002). The inhibition of these genes is in good agreement with the finding that the Rho GTP-ase pathway is activated during the anxiety response. Recent evidence suggests the involvement of Rho GTP-ase in the fear memory formation (Lamprecht *et al.* 2002). Therefore, it is likely that the exposure of rats to cat odour for 30 minutes also activates the memory pathways in the amygdala. Moreover, abnormal tyrosine phosphorylation of p190 RhoGAP, a GTPase-activating protein for Rho GTPase, will cause impairments in hippocampus-dependent contextual fear memory (Tamura *et al.* 2006).

Apolipoprotein E (apoE) is related to the pathogenesis of Alzheimer's disease. Recent findings suggest a role of apoE in the regulation of neurotransmission and especially in the synaptic plasticity. Namely, an apoE4-induced disruption of phosphoinositide hydrolysis has been established (Cedazo-Minguez & Cowburn 2001). Furthermore, *Apoe*$^{-/-}$ mice without human apoE or with apoE4, but not apoE3, showed increased measures of anxiety in the elevated plus-maze and heightened acoustic startle response if compared to wild-type mice, which were associated with reduced microtubule-associated protein 2-positive neuronal dendrites in the central nucleus of the amygdala (Robertson *et al.* 2005). Controversially, *Apoe*$^{-/-}$ mice strain with same C57Bl6/J background tested by another group in the elevated plus-maze was found less anxious compared to wild-type, whereat female apoE-deficient mice visited the open arm of the elevated plus-maze more often than their apoE-deficient male counterparts (McLachlan & Yi Xing 2005). Interestingly, we found the activation of gelsolin, actin-depolymerizing factor, in response to the cat odour exposure. Gelsolin has been shown to interact with apolipoprotein E and is related to amyloidosis (Fadika & Baumann 2002). On the other hand, Rev-Erb-alpha protein, down-regulated during the cat odour exposure, is an orphan nuclear hormone receptor and has been described as negative transcriptional regulator for the apoE gene (Coste & Rodriguez 2002).

Limbic system-associated membrane protein (LsAMP) is a glycoprotein expressed on the surface of somata and proximal dendrites of neurons in cortical and subcortical regions of the limbic system (Zacco *et al.* 1990) and is highly conserved in rodents and humans (Pimenta & Levitt 2004). Functional and biochemical studies indicate that LsAMP selectively promotes neurite outgrowth of neurons comprising limbic pathways and mediates proper circuit formation (Pimenta *et al.* 1995). LsAMP seems to be an important determinant of proper limbic system development and function. The present study demonstrates the over-expression of the LsAMP gene in the amygdala due to the anxiety response. Interestingly, the selection of rats according to their exploratory behaviour also revealed an increased expression of the LsAMP gene in the periaqueductal grey of anxious rats as revealed by reverse-transcriptase PCR (Nelovkov *et al.* 2003) and qRT-PCR (Nelovkov *et al.* 2006).

Another interesting finding was the robust overexpression of an unknown protein GAMM1 (also known as melanocyte proliferating gene 1, Myg1, AF252871). There are no data about its function other than it is a highly conserved gene related to the proliferation of melanocytes. Its high up-regulation in the amygdala during anxiety response is remarkable and needs further studies.

We could not find any differences in the expression levels of c-fos and CRF after the cat odour exposure in the amygdala of rats. Both these genes have been shown to be activated during the anxiety response induced by predatory odour (Adamec *et al.* 1998; Dielenberg *et al.* 2001). As c-fos activation has mostly been studied by immunohistochemistry and in our study we used mRNA detection, one possible explanation is the shorter lifetime of mRNA compared to the c-fos protein. On the other hand, we used in our study quite a large area of the amygdala. In addition to the central nucleus, our sample contained also basolateral, basomedial and medial nuclei and other subnuclei of the amygdala. Therefore the samples of different studies are quite different and in our case "dilution" of specific mRNA-s might be the case. In addition, gene expression profiles of different genes in the amygdala follow its anatomical subdivision (Zirlinger *et al.* 2001). In this study Affymetrix GeneChips comprising 34,325 murine genes and ESTs were used to identify amygdala-enriched genes. Authors found 33 genes to be enriched in the amygdala compared to other brain areas studied. The comparison of the list of genes (available online as supplementary information) revealed some similarities between the study of Zirlinger and colleagues (Zirlinger *et al.* 2001) and our study. For instance, we found up-regulation of homeobox protein HOX-4.4 after the cat odour exposure and SIR2L2 in control animals. Zirlinger and colleagues (Zirlinger *et al.* 2001) also found that both these genes were over-expressed in the amygdala (D49658 and TC17396, respectively).

5. Concluding remarks and future prospects

Cat odour induced anxiety is strongly shaped by the mechanisms of natural selection and, therefore, may provide important cues for the understanding of the molecular machinery of anxiety. Nevertheless, one should take into account the existing differences between species of rodents widely used in scientific laboratories. The comparison of behaviour of male Wistar rats and female 129Sv/C57Bl6 mice demonstrates that cat odour induces a significantly stronger anxiogenic-like action in rats compared to mice. Belzung and colleagues (Belzung *et al.* 2001) have demonstrated that different mice strains have different sensitivity to cat odour. According to this study C57Bl6 mouse strain belongs to the sensitive ones. In our recent unpublished study we compared the response of 129Sv and C57Bl6 strains to cat odour. 129Sv mice displayed significantly lower exploratory activity compared to C57Bl6 animals reflecting also the difference in the basal anxiety between these two strains. It is known that 129Sv strain is characterised by increased anxiety (Võikar *et al.* 2001). The cat odour exposure did not induce any changes in the behaviour of 129Sv mice. By contrast, in C57Bl6 mice, cat odour induced a moderate suppression of the exploratory behaviour. Therefore, male Wistar rats seem to display a more distinct behavioural profile for the study of neurobiology of cat odour induced anxiety compared to the two commonly used mouse lines — 129Sv and C57Bl6.

It is obvious from different studies that the anxiogenic effects of pharmacological and genetic manipulations can be studied in animals having the reduced anxiety (Holmes *et al.* 2003a). According to our previous studies in the exploratory models of anxiety the activity of female CCK_2 receptor deficient mice is significantly higher compared to their wild-type littermates (Raud *et al.* 2003; Raud *et al.* 2005). As a matter of fact, that was the main reason to include these mice into the present study. Indeed, their exposure to cat odour significantly reduced the exploratory behaviour of CCK_2 receptor deficient mice in the elevated plus-maze. However, this effect was not established in their wild-type littermates. Moreover, the cat odour exposure induced significant changes in the gene expression of homozygous mice, but not in wild-type animals. The most prominent change was established for the Pomc1 gene in the temporal lobe of homozygous mice. The cat odour exposure induced an almost 5-fold increase of this stress-related gene. Interestingly, we found a similar increase of this gene in the amygdala of Wistar rats after the cat odour exposure. Since the amygdala is located in the temporal lobe, one can assume that the increased anxiety due to the cat odour is accompanied by an increased expression of the Pomc1 gene in this particular structure both in mice and rats. POMC is a precursor of various biologically active neuropeptides and neurohormones. Subsequent analysis demonstrated that the anxiogenic effect of

the cat odour exposure could be related to the activation of the melanocortin system.

Based on the above-mentioned suggestions we tried to go further and establish new target genes implicated in the regulation of anxiety. For this purpose we applied cDNA-RDA analysis to detect genes over-expressed in the amygdala of male Wistar rats after the cat odour exposure. Interestingly, no genes of interest established in qRT-PCR studies were seen in the cDNA-RDA analysis. Possible explanations are that qRT-PCR with 'targeted' priming, which detects mRNA starting from a few copies, is much more powerful tool if compared to the semiquantitative cDNA-RDA. The latter detects genes with higher absolute quantities than the former. For the same reason there are several genes responsible for the functioning of nerve and glial cells amidst the differential products of the cDNA-RDA. The advantage of the cDNA-RDA is the ability to detect unknown genes with different expression. Nevertheless, this study revealed several target genes that are of great interest for the understanding of the mechanisms of physiological and pathological anxiety. Among these genes are Wfs1, Lsamp and Gamm1. The Wfs1 gene is responsible for the synthesis of a protein called wolframin. According to the existing studies, mutations in this gene seem to have an impact on the development of depression and diabetes. In our lab, we have developed transgenic mice, lacking the Wfs1 gene. Preliminary studies demonstrate that these transgenic mice display reduced tolerance to stress and increased anxiety. The Gamm1 gene is likely to be involved in the regulation of skin pigmentation (Smicun 2001). However, the potential interaction of Gamm1 with the melanocortin system also makes it an attractive target for the study of the molecular mechanisms of anxiety. LsAMP is a protein playing a significant role in the development of the limbic system (Pimenta *et al.* 1995), but the functional role of this particular protein is probably not limited with that. Recent studies demonstrate in rats, selected according to the exploratory behaviour in the elevated plus-maze, that low exploratory activity animals display increased expression of Lsamp in the amygdala and periaqueductal gray (Nelovkov *et al.* 2003; Nelovkov *et al.* 2006). This is in good agreement with the present study in that increased anxiety is accompanied by the elevated expression of Lsamp in the amygdala.

VII. CONCLUSIONS

1. The exposure of male Wistar rats to cat odour induced increased freezing and avoidance behaviour. These are species-specific signs of anxiety in rats. Moreover, the exploratory activity of rats was suppressed in the elevated zero-maze model of anxiety. Morphine-induced behavioural activation in rats was also absent after the exposure to cat odour. The exposure to cat odour increased the expression of the rat Cck, Pomc1 and Oprm1 genes in the forebrain structures. It is worthy to note that the biggest changes in gene expression were established in brain regions related to anxiety (amygdala — Pomc1) and motivation (mesolimbic area — Cck, Pomc1 and Oprm1). The increased expression of the Pomc1 and Oprm1 genes, and the abolished action of μ-opioid agonist morphine leads us to assume that the processing of Pomc1 transcript into β-endorphin is increased.
2. Female CCK_2 receptor deficient mice display reduced anxiety compared to their wild-type littermates in the elevated plus-maze. The higher exploratory activity of homozygous mice was accompanied by an increased expression of Oprm1 in the frontal cortex and mesencephalon. Moreover, the expression of Cckar was also significantly increased in the frontal cortex and mesolimbic area of homozygous mice.
3. The exposure to cat odour suppressed the exploratory behaviour of CCK_2 receptor deficient mice, but not in wild-type mice, in the plus-maze. Also in homozygous animals, but not in their wild-type littermates, significant alterations in gene expression patterns were established after the exposure to cat odour. Especially, an increase in the expression of the Pomc1 gene was remarkable in the temporal lobe, mesencephalon and mesolimbic area. Moreover, the cat odour exposure caused an elevation in the expression of the Mc3r gene in the temporal lobe and frontal cortex of homozygous mice. In the temporal lobe, cat odour also increased the expression of the Oprd1 and Penk1 genes in homozygous mice. The established changes in the expression of the Pomc1 and Mc3r genes in the temporal lobe probably contribute to the increased anxiety due to the cat odour exposure, whereas the elevation of Oprd1 and Penk1 mRNA is probably a compensatory response to the increased anxiety.
4. The gene expression studies performed by means of qRT-PCR established the biggest changes in the level of gene expression in the temporal lobe, especially in the amygdala. The following cDNA-RDA study showed that a significant number of genes were over-expressed in the amygdala of male Wistar rats in response to the predator odour. Altogether they reflect that not only genes related to neurotransmission, but also other genes responsible for the functioning of nerve and glial cells are activated. The biggest increase was demonstrated for the Gamm1 gene, related to skin pigmentation. Two more genes were selected for further studies: Wfs1 and Lsamp. There is evidence that the Wfs1 gene is linked to mood disorders and diabetes. Lsamp is a gene responsible for the development of the limbic system.

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SUMMARY IN ESTONIAN

Kassilõhna tekitatud ärevuse mehhanismide käitumuslik ja neurogeneetiline uurimus närilistel

Sissejuhatus

Huvi ärevuse mehhanismide vastu on seotud asjaoluga, et ärevushäired on elanikkonnas laialt levinud ning seotud kõrgenenud haigestumuse ja suremusega, aga ka suurte majanduslike kulutustega. Kuni 25 %-l inimestest esineb vähemalt korra elu jooksul mingi ärevushäire episood. Ärevus on oma olemuselt küll normaalne kogemus, kuid liigne ja patoloogiline (s.o. võimaliku ähvardava ohuolukorraga mitteseotud) ärevus põhjustab tõsiseid kannatusi ning tööga ja igapäevaeluga toimetuleku häirumist. Ehkki ärevuse ja teiste emotsioonide mehhanisme on uuritud aastakümneid, baseerub ärevuse ravi tänapäeval põhiliselt siiski vaid gammaaminovõihappe A-tüüpi retseptorkompleksi afiinsust suurendavate bensodiasepiinide ning serotoniini ja noradrenaliini tagasihaarde inhibiitorite kasutamisel. Neil ravimitel on aga olulisi kõrvaltoimeid ning kõrge väärkasutamise risk. Sellest tingituna on oluline selgitada uusi molekulaarseid sihtmärke, mis osalevad ärevuse regulatsioonis. Taolisi uuringuid on võimalik läbi viia vaid katseloomadel, mis tingib vajaduse asjakohaste ärevusemudelite rakendamiseks. Kindlasti üheks kõige spetsiifilisemaks ja liigiomasemaks mudeliks on närilistel kassilõhna poolt esilekutsutud ärevus.

Uurimuse põhieesmärgid

Käesoleva töö peamiseks eesmärgiks oligi selgitada uusi molekulaarseid sihtmärke ärevuse regulatsioonis. Ärevuse esilekutsumiseks katseloomadel kasutati kassilõhna mudelit. Uuringu täpsemad eesmärgid olid järgmised:

1. Uurida täpsemalt koletsüstokiniini (CCK) ja opioidide interaktsiooni Wistar liini rottidel kassilõhna poolt põhjustatud ärevuse mehhanismis, sealhulgas muutusi morfiini uudistamisaktiivsust suurendavas toimes.
2. Uurida kassilõhnale eksponeerimise mõju uudistamisaktiivsusele ja CCK-opioidide interaktsioonile erineva ärevuse tasemega emastel hiirtel. Kassilõhna mõju uuriti CCK_2 retseptori puudulikkusega hiirtel ja nende „metsikut tüüpi" pesakonnakaaslastel. CCK_2 retseptori puudulikkusega hiiri kasutati nende vähenenud ärevuse tõttu võrreldes „metsikut tüüpi" pesakonnakaaslastega.
3. Leida cDNA diferentsiaalanalüüsi abil isaste Wistar liini rottide mandelkehas kassilõhna toimel suurenenud ekspressiooniga geene ja valida neist asjakohased edasisteks uuringuteks. Mandelkeha kasuks otsustati vastavalt

varasemale informatsioonile selle rollist ärevuse regulatsioonis ning samuti käesoleva töö esimeste katsete alusel, mis näitasid suurimaid muutusi stressiga (ärevusega) seotud geeni — Pomc1 — ekspressioonis just mandelkehas.

Katseloomad ja meetodid

Katseloomadeks olid isased Wistar liini rotid ning emased 129Sv/C57Bl6 taustaga transgeensed CCK_2 retseptori puudulikkusega hiired ja viimaste „metsikut tüüpi" pesakonnakaaslased (Nagata jt. 1996).

Rotte eksponeeriti kiskjalõhnale pannes kassilõhnaga riidetüki puuri kattevõrele, hiirte puhul pandi riidetükk puuri ühte nurka. Nii hiirtel kui rottidel hinnati ka ekspositsiooniaegseid uudistamisaktiivsuse parameetreid, mis peegeldavad ärevust.

Ekspositsioonijärgset ärevust hinnati rottidel tõstetud null-puuri mudelis. Rottidel hinnati ka morfiini toimet uudistamisaktiivsusele, süstides morfiini kõhuõõnde peale ekspositsiooni kassilõhnale, seejärel uuriti loomade motoorset käitumist automatiseeritud jälgimissüsteemi abil. Hiirtel hinnati kassilõhnale eksponeerimise järgset ärevust tõstetud pluss-puuri mudelis.

Kassilõhna tekitatud ärevuse põhjustatud muutusi geeniekspressioonis uuriti rottidel mandelkehas, frontoparietaalses ajukoores, mesolimbilises alas ning juttkehas CCK, proopiomelanokortiini ning μ-opioidretseptori geenide osas. Hiirtel olid vaatlusalusteks ajupiirkondadeks otsmikukoor, mesolimbiline ala, oimusagar (s.h. mandeltuum) ja keskaju (s.h. ajuveejuhaümbrine hallaine) ning geenideks: CCK; CCK_1 ja CCK_2 retseptorite; proopiomelanokortiini; μ-, δ-, ja κ-opioidiretseptorite; pre-pro-enkefaliini; pre-pro-dünorfiini ning melanokortiin-3 ja -4 retseptorite geenid. Geeniekspressiooni hinnati kvantitatiivse reaalaja polümeraasi ahelreaktsiooni (qRT-PCR) meetodil, suhtelise ekspressioonina koduhoidjageeni hüpoksantiini-guaniini fosforibosüültransferaasi suhtes.

Mitteteadaolevate muutunud ekspressiooniga geenide leidmiseks roti mandelkehast kasutati cDNA diferentsiaalanalüüsi (cDNA-RDA). Muutunud ekspressiooniga kloonid kvantiteeriti dot-blot analüüsil.

Peamised tulemused

Kassilõhnale eksponeerimine põhjustas nii hiirtel kui rottidel ärevust peegeldavate käitumisparameetrite statistiliselt olulise suurenemise, kuid Wistar liini rottidele oli kassilõhna anksiogeenne toime tugevam kui 129Sv/C57Bl6 taustaga hiirtele. Varem on näidatud ka, et C57Bl6 hiired on tundlikumad kassilõhnale (Belzung jt., 2001) ja et 129Sv liin on suurenenud ärevusega (Võikar jt., 2001). Meie laboris hiljuti tehtud avaldamata uuringud näitavad samuti 129Sv

ja C57Bl6 liinide erinevat reageerimist kassilõhnale, kuid hiirte reaktsioon kiskjalõhnale on oluliselt vähem väljendunud, võrreldes Wistar liini rottidega.

Varasemast on teada, et anksiogeenseid efekte saab uurida vähenenud ärevusega loomadel. Meie töörühma poolt on tuvastatud CCK_2 retseptori puudulikkusega hiirte oluliselt suurenenud uudistamisaktiivsus võrreldes „metsikut tüüpi" pesakonnakaaslastega (Raud jt. 2003; 2005), see oli nimetatud transgeensete hiirte töösse kaasamise peamiseks põhjuseks. CCK_2 retseptori puudulikkusega hiired näitasidki peale kiskjalõhnale eksponeerimist tõstetud pluss-puuri mudelis üles vähenenud eksploratiivset aktiivsust, ehkki sellist mõju nende „metsikut tüüpi" pesakonnakaaslastel ei ilmnenud ning ka muutused geenieksprессioonis olid viimastel oluliselt tagasihoidlikumad. Suurim muutus CCK_2 retseptori puudulikkusega hiirtel oli proopiomelanokortiini ligi viiekordne ülesregulatsioon oimusagaras (kus asub ka mandelkeha), sarnane muutus esines kassilõhnale eksponeerimise järgselt ka Wistar rottide mandelkehas. Edasine analüüs näitas, et need muutused võivad olla seotud melanokortiinisüsteemi aktivatsiooniga.

Ülaltoodud viited olid aluseks edasisele katsele leida uusi sihtmärke ärevuse regulatsioonis. Selleks viidi läbi cDNA-RDA katse, leidmaks kiskjalõhnale eksponeerimise järgseid geenieksprессiooni muutusi roti mandelkehas. On tähelepanuväärne, et ühtegi qRT-PCR meetodil nähtud ekspressioonimuutust cDNA-RDA meetodiga ei tuvastatud. See on seletatav asjaoluga, et kindlat geeni uuriv qRT-PCR tuvastab juba mõne cDNA koopia, võrreldes suuremat hulka algmaterjali vajava poolkvantitatiivse cDNA-RDA'ga, see-eest viimane võimaldab leida erinevalt ekspresseerunud geene ilma neile konkreetselt fokuseerumata. Peamised edasisteks uuringuteks välja valitud geenid cDNA-RDA katsest on Wfs1, Lsamp ja Gamm1. Wfs1 geen kodeerib valku wolframiin ning mutatsioonid selles geenis on seotud depressiooni ja diabeediga. Meie töörühma poolt on välja arendatud Wfs1 geeni puudulikkusega hiireliin ning esialgsed katsed näitavad selle hiireliini suurenenud ärevust. Gamm1 tundub olevat seotud naha pigmentatsiooniga, seos melanokortiinisüsteemi ja ärevusega vajab edasist uurimist. Lsamp geen on oluline limbilise süsteemi arengus, äsjased uuringud näitavad Lsamp geeni ülesregulatsiooni mandeltuumas ja ajuveejuhaümbrises hallaines vähese eksploratiivse aktiivsusega rottidel. See on kooskõlas käesoleva uuringuga, kus samuti on suurenenud ärevus seotud Lsamp geeni suurenenud ekspressiooniga mandelkehas.

Järeldused

1. Isaste Wistar rottide eksponeerimine kassilõhnale tekitas rottidel suurenenud ärevust nii ekspositsiooni ajal kui ka järgnenud tõstetud null-puuri katses. Kassilõhnajärgsed geenieksprессiooni muutused roti ajus olid kõige suuremad ärevusega (mandelkeha — proopiomelanokortiin) ja motivatsioonidega (mesolimbiline ala — CCK, proopiomelanokortiin, μ-opioidiretseptor)

seotud ajupiirkondades. Kaasnev morfiini uudistamisaktiivsust vähendava toime kadumine kassilõhnale eksponeerimise järel annab alust arvata, et proopiomelanokortiin muudetakse edasi ß-endorfiiniks.

2. Emastel CCK_2 puudulikkusega hiirtel esines suurenenud eksploratiivne aktiivsus tõstetud pluss-puuris võrreldes „metsikut tüüpi“ pesakonnakaaslastega, sellega kaasnesid μ-opioidiretseptori geeni suurenenud ekspressioon otsmikukoores ja keskajus ning CCK_1 retseptori geeni suurenenud ekspressioon otsmikukoores ja keskajus.
3. Ekspositsioon kassilõhnale pärsib uudistamiskäitumist CCK_2 retseptori puudlikkusega hiirtel, kuid mitte nende „metsikut tüüpi” pesakonnakaaslastel tõstetud pluss-puuri mudelis. Viimastel on ka vähem muutusi geeniekspressioonis — preproCCK geeni allaregulatsioon mesolimbilises alas ning melanokortiin-3 retseptori geeni ülesregulatsioon keskajus. CCK_2 retseptori puudulikkusega hiirtel esinesid kiskjalõhnale eksponeerimise järgselt ka olulised muutused geeniekspressioonis. Proopiomelanokortiini geeni märkimisväärne ülesregulatsioon oimusagaras, keskajus ja mesolimbilises alas ning melanokortiin-3 retseptori geeni ülesregulatsioon oimusagaras ja otsmikukoores tõenäoliselt aitavad kaasa kiskjalõhna ekspositsioonist tuleneva ärevuse tekkele. δ-opioidiretseptori ja pre-pro-enkefaliini geenide ülesregulatsioon oimusagaras on tõenäoliselt kompensatoorsed vastused suurenenud ärevusele.
4. cDNA diferentsiaalanalüüs tuvastas roti mandeltuumas terve rea geene, mis olid kiskjalõhnale eksponeerimise järgselt ülesreguleeritud. Nende hulgas oli nii närviülekandega seotud kui ka närvi- ja gliiarakkude üldfunktsioone tagavaid geene. Suurim ekspressiooni tõus oli Gamm1 geenil, mis on seotud naha pigmentatsiooniga. Lisaks sellele valiti edasisteks uuringuteks Wfs1 geen, mis on seotud meeleoluhäirete ja diabeediga, ning Lsamp geen, mis vastutab limbilise süsteemi arengu eest.

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Scientific work

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Publications

1. Sulev Kõks, Hendrik Luuk, Aleksei Nelovkov, Tarmo Areda, Eero Vasar. A screen for genes induced in the amygdaloid area during cat odor exposure. *Genes, Brain & Behavior* (2004); 3(2):80–9.

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3. Tarmo Areda, Sirli Raud, Mari-Anne Philips, Jürgen Innos, Toshimitsu Matsui, Sulev Kõks, Eero Vasar, Alar Karis, Toomas Asser. Cat odour exposure decreases exploratory activity and alters neuropeptide gene expression in CCK(2) receptor deficient mice, but not in their wild-type littermates. *Behavioural Brain Research* (2006); 169(2):212–9.

4. Aleksei Nelovkov, Tarmo Areda, Jürgen Innos, Sulev Kõks, Eero Vasar. Rats displaying distinct exploratory activity also have different expression patterns of gamma-aminobutyric acid- and cholecystokinin-related genes in brain regions. *Brain Research* (2006); 1100(1):21–31.

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Teadustöö on keskendunud uute molekulaarsete sihtmärkide selgitamisele ärevushäirete raviks. Lisaks on vaatluse all koletsüstokiniini ja endogeensete opioidide interaktsioon ärevuse regulatsioonis, eriti katseloomadel kassihaisu poolt põhjustatud ärevuse korral.

Publikatsioonid

1. Sulev Kõks, Hendrik Luuk, Aleksei Nelovkov, Tarmo Areda, Eero Vasar. A screen for genes induced in the amygdaloid area during cat odor exposure. *Genes, Brain & Behavior* (2004); 3(2):80–9.

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4. Aleksei Nelovkov, Tarmo Areda, Jürgen Innos, Sulev Kõks, Eero Vasar. Rats displaying distinct exploratory activity also have different expression patterns of gamma-aminobutyric acid- and cholecystokinin-related genes in brain regions. *Brain Research* (2006); 1100(1):21–31.