DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
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BEHAVIOURAL AND NEUROGENETIC STUDY OF MOLECULAR MECHANISMS INVOLVED IN REGULATION OF EXPLORATORY BEHAVIOUR IN RODENTS

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ABBREVIATIONS

5-HT	5-hydroxytryptamine or serotonin
ANOVA	analysis of variance
B_{max}	apparent number of binding sites
BLA	basolateral nucleus of the amygdala
BOC-CCK-4	butoxy-carbonyl tetrapeptide of CCK-4
CaM	calmodulin
CaMK	Ca^{2+}/calmodulin-dependent protein kinase (CaMKII, CaMKIV)
CCK	cholecystokinin
CCK-8S	cholecystokinin-8 sulphated
cDNA	complementary DNA synthesised from mature mRNA
cDNA-RDA	cDNA representational difference analysis
CEA	central nucleus of the amygdala
CNS	central nervous system
CREB	cAMP-response-element-binding protein
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
DIG	digoxigenin
DP	differential product (DP1, DP3)
ECL	gastrin-enterochromaffin-like (cells)
ERK2	extracellular signal-related kinase
FMRP	FragileX mental retardation protein
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase (GAD65, GAD67)
HPA	hypothalamic-pituitary-adrenal (axis)
HPRT	hypoxanthineguanine phosphoribosyl transferase
K_d	apparent dissociation constant
KEGG	Kyoto Encyclopedia of Genes and Genomes
LsAMP	limbic system-associated membrane protein
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NPY	neuropeptide Y
PAG	periaqueductal grey
PCR	polymerase chain reaction
PFC	prefrontal cortex
POMC	pro-opiomelanocortin
qRT-PCR	quantitative real-time PCR
PFC	prefrontal cortex
RT-PCR	reverse transcription PCR
SEM	standard error of mean
SSRIs	selective serotonin reuptake inhibitors

I. INTRODUCTION

Generally, the responses of animal to its environment and interactions between animals are mediated by behaviour. One of the behavioural forms, the exploratory behaviour, can be formulated as exploration evoked by novel stimuli and consists of behavioural acts and postures that permit the collection of information about new objects and unfamiliar parts of the environment (Crusio, 2001). The motivation to explore novelty is an innate instinct that drives animals to learn about their environment. Neophobia is an innate instinct that drives animals away from potential dangers. Thus, a conflict between approach and escape associated with animals' reactions to novelty underlies exploratory behaviour (Ennaceur et al., 2006). Later it was suggested that the level of exploratory activity is related to the level of anxiety and fear, and exploratory activity of animals correlates with their anxiety (Montgomery, Monkman, 1955; Ennaceur et al., 2006).

During evolution anxiety has been formed as a necessary warning signal of a dangerous or difficult situation. Without anxiety, animals and humans would have no way of anticipating difficulties ahead and preparing for them. But simply the presence of anxiety does not constitute a disorder. Anxiety becomes a disorder when the symptoms become chronic and interfere with people's daily lives and their ability to function. Numerous studies have been performed with the aim to understand the neurobiology of anxiety and fear. Animal models allow to map neural pathways and circuits involved in the regulation of anxiety-related behaviour. At the same time, information has been collected about the major neurotransmitter systems, regulating anxiety. As a matter of fact, drugs modifying the functioning of the GABA- and 5-HT-ergic system help us to some extent control the severity of anxiety-related conditions (Argyropoulos et al., 2000; Bourin, Lambert, 2002; DeVane, Franklin, 2005).

In the current study an attempt was made to extend our knowledge about the molecular mechanisms of anxiety. Today this kind of basic research can be performed only on laboratory animals. At present, the elevated plus-maze is the simplest animal model of anxiety for studying the molecular mechanisms related to anxiety. It allows a semi-quantitative measuring of the level of anxiety, but at the same time excludes the influence of general locomotor activity (Lister, 1987; 1990; Rodgers, 1997). This model has been widely used in testing the action of anxiolytic and anxiogenic drugs that confirms its advantage for screening of drugs affecting anxiety (Gyertyan, 1992; Belzung, Griebel, 2001). Neural circuits underlying anxiety-related behaviour include the frontal cortex, amygdala and periaqueductal grey. These anxiety pathways were first described by Papez and MacLean, and the role of these brain structures (frontal cortex, amygdala and periaqueductal grey) in the regulation of anxiety and fear related behaviour has later been confirmed by numerous studies

(Coplan, Lydiard, 1998; LeDoux, 1998; Lang et al., 1998; Miller et al., 2005; Sah et al., 2003; Kim et al., 2006).

The present study is also aiming to extend our knowledge about the interaction of CCK- and GABA-ergic systems in the brain structures. The effects of diazepam, widely used anxiolytic drug, modulating the function of $GABA_A$ receptors, were also explored in CCK_2 receptor deficient mice in order to confirm the relevance of CCK and GABA interaction established in gene expression studies. Last but not least, in the final part of the study, brain samples obtained from the PAG were used to establish new molecular targets involved in the regulation of anxiety. The cDNA representational difference analysis (cDNA-RDA) was applied for this purpose, because of its high sensitivity in detecting the smallest differences even for rare mRNA transcripts (Diatchenko et al., 1996).

II. REVIEW OF LITERATURE

1. Neurobiology of anxiety

Anxiety is a mental state that is elicited in anticipation of a threat or a potential threat. It is accompanied by a characteristic set of behavioural and physiological responses including avoidance, vigilance and arousal, which evolved to protect the individual from danger (Gross, Hen, 2004). Anxiety can be viewed as an appropriate, adaptive response to impending danger that is integral to an organism's preparations to either cope with or avoid a potential environmental threat (Cryan, Kaupmann, 2005).

Fear and anxiety are respectively defined as the behavioural and autonomic response of a subject to real or potential threats that may impair its homeostasis (Belzung, Griebel, 2001; Millan, 2003). Transient anxiety proportional to the challenge encountered elicits an appropriate response and is of fundamental importance as a survival strategy for all higher animals. Anxious states are controlled by a highly complex system of both inhibitory and facilitatory mechanisms. The purpose of homeostatic controls is to: 1) maintain an appropriate degree of emotionality (avoid pervasive anxiety) under non-threatening circumstances; 2) efficiently respond to potential threats with a (transient) "fear" proportional to the danger encountered; 3) permit adaptive behavioural responses, such as escape or avoidance; and 4) rapidly restore a "normal" emotional status once the threat has passed.

Thus, the response to threat may include physiological (increase in heart rate, blood pressure etc.), as well as behavioural (inhibition of ongoing behaviours, scanning, avoidance of the source of danger, etc.) parameters (Belzung, Griebel, 2001). However, all these responses are normal physiological reactions to conditioned or unconditioned stressors, and this kind of anxiety could be called 'normal'. But when this response is excessive or maladaptive, it involves 'pathological' anxiety, which leads to the development of anxiety disorders. 'Pathological' anxiety might be considered as an excess of 'normal' anxiety. The relationship between normality and pathology is a qualitative, rather than a quantitative variation when passing from one state to the other (Belzung, Griebel, 2001).

Most of the animal models of anxiety involve exposure of subjects to external (e.g. cues earlier paired with foot-shock, bright light, predator) or internal (e.g. drug states) stimuli that are assumed to be capable of inducing anxiety in animals. Since none of these models involve pathological anxiety-related behaviours, Lister has described them as animal models of 'state' anxiety (Lister, 1990; Belzung, Griebel, 2001). In such procedures, subjects experience anxiety at a particular moment in time and it is increased by the presence of an anxiogenic stimulus. The opposite models of 'pathological' anxiety refer to 'trait' anxiety tests. Unlike 'state' anxiety, 'trait' anxiety does

not vary from moment to moment and is considered to be an enduring feature of an individual. These models either use rodents that were selected for emotional reactivity or employ receptor knockout mice, which exhibit phenotypic changes indicative of increased anxiety (Belzung, Griebel, 2001). Thus, "state" anxiety is "normal" acute response to threat, lasting a few times and being fear induced, in comparison to "trait" anxiety, which is personality-based, sustaining a long time, and determine the level of response to fear (Millan, 2003).

It seems that animal model of 'trait' anxiety better corresponds to the anxiety disorders existing in humans. Anxiety disorders last a long time and involve various neurotransmitter systems Changes take place at different levels, and some of them can have a compensatory meaning. That is why the use of 'anxious' animal strains, which show constant high levels of fearfulness, and serve as an example of "trait" anxiety, may provide better models of anxiety-related disorders than 'state' or single-gene deletion models of anxiety (Belzung, Griebel, 2001).

1.1. Brain structures involved in the regulation of anxiety

The first attempt to provide a neuroanatomically-circumscribed physiological foundation for the expression of emotion and fear was made by Papez in 1937 year. His "Papez circuits" embrace a projection from the hippocampus to the mammilary bodies which are linked in turn to the anterior nucleus of the thalamus: the anterior thalamus is connected to the cingulate cortex which closes the circuit in transmitting information back to the hippocampus. This work formed the basis of nascent limbic system matured in the work of MacLean (1949, 1952), who rectified the original omission of the amygdala and elaborated a network encompassing the frontal cortex and other subcortical nuclei, such as the septum and nucleus accumbens (Barili et al., 1998; Pralong et al., 2002). The septum was subsequently incorporated into a septo-hippocampal "behavioural inhibition system" subject to modulation by cortical input (Gray, 1987; Gray, McNaughton, 2000). Then the role of the paraventricular nuclei and supramamilary nucleus was mentioned in integrating of adrenocortical response to stress and fear conditioning (Herman et al., 2002; Carrasco, van de Kar, 2003; Millan, 2003). Moreover, the participation of the periaqueductal grey and inferior colliculus was confirmed in the regulation of anxious behaviour (Graeff et al., 1993; Macedo et al., 2002; Millan, 2003). Thus, in a series of studies a representation was developed about these and other structures involved in the regulation of anxiety and fear response. A brief characterisation of them will follow.

The prefrontal cortex (PFC), one of the main brain structures participating in anxiety-related behaviour, is commonly divided into three main divisions: the medial PFC (containing the anterior cingulate and paracingulate cortices), the

orbital PFC, and the dorsolateral PFC (Miller et al., 2005). The medial PFC is related to functions relevant to anxiety and fear, including attention to the emotional states of the self and others, guidance of response selection by emotional states, and suppression of anxiety-related behavioural responses as situations change. The orbital PFC functions relevant to anxiety and fear include modulation of behavioural and visceral responses associated with anxiety-related situations as situations change and modulation of emotional responses by correcting associations when they become inappropriate. The dorsolateral PFC is involved in working memory, response preparation and response selection (Coplan, Lydiard, 1998; Miller et al., 2005; Finn et al., 2003).

The amygdala is another important structure involved in the regulation of anxiety and fear. In recent years, this brain structure has been most carefully investigated. The amygdaloid complex, or amygdala, is a group of more than 10 nuclei that are located in the midtemporal lobe of the brain. These nuclei can be distinguished both on cytoarchitectonic and connectional grounds (Sah et al., 2003; Kim et al., 2006). Anatomical tract tracing studies have shown that these nuclei have extensive intranuclear and internuclear connections. The afferent and efferent connections of the amygdala have also been mapped in detail, showing that the amygdaloid complex has extensive connections with cortical and subcortical regions (Sah et al., 2003). Indeed, the amygdala possesses an extensive pattern of reciprocal connections with cortical, limbic, monoaminergic and other structures implicated in emotional, cognitive, autonomic and endocrine responses to stress. Output pathways are delivered primarily from the central nucleus and bed nucleus of the stria terminalis, whereas the basolateral amygdaloid complex is principally responsible for the receipt and filtering of cortical and subcortical sensory inputs. Synaptic plasticity in the amygdala has been convincingly implicated in the induction, processing and extinction of conditioned fear, in the generation of anticipatory anxiety and in the coordination of the global response to threat (Millan, 2003).

The third important structure is the periaqueductal grey (PAG), a grey substance localised around the aqueduct in the midbrain. The PAG is involved in coordinating defensive and aversive response to fear and stress. It is responsible for a stereotyped, reflexive, autonomic and behavioural "fight or flight" response to unconditioned fear (Lang et al., 1998; Coplan, Lydiard, 1998; Millan, 2003). The PAG has its own functional organisation. The ventral PAG is the fear-"freezing" path, whereas the dorsal grey is a critical part of the "fight or flight" action circuit (Lang et al., 1998).

The role of the hippocampus, which bi-directionally communicates with the amygdala and other corticolimbic structures, in the regulation of anxiety-related behaviour is controversial (Millan, 2003). Being connected to the amygdala by reciprocal pathways, the hippocampus seems to be involved in contextual and trace fear conditioning, and its ventral subregion is implicated in anxiety-related behaviours (Bannerman et al., 2004; Kim et al., 2006). A significant role belongs to the locus coeruleus (Coplan, Lydiard, 1998), which is localised in the

brain stem and is a critical component of efferent response systems, implicated in the orchestration of the fundamental "alarm reaction". Some studies indicate that the cerebellar vermis may be an important part of the fear conditioning circuit and modulates fear-related behaviors (Kim et al., 2006). The rats with vermal lesions exhibit less freezing to a cat predator and fewer signs of fear in an open field (Supple et al., 1987; Kim et al., 2006).

A simplified organisation of the neural circuits underlying anxiety, the so-called "anxiety circuit", can be represented as follows (Figure 1). Information about threat passes from the sense organs to the thalamus and from there to the sensory cortex (including orbitofrontal, medial prefrontal, posterior cingulated and enthorinal cortex), and then from cortex to the sensory specific nuclei of thalamus or directly to the amygdala (Miller et al., 2005; Finn et al., 2003). The lateral nucleus of the amygdala receives input signals from the thalamus, transmitting them to the amygdala's central nucleus. There are three important connections efferent to the amygdala: a) a projection from the central amygdala to the lateral hypothalamic area that mediates the autonomic emotional response; b) projections to the midbrain central (periaqueductal) grey region, which mediates coping behaviours; and c) a direct projection to the nucleus reticularis pontis caudalis, which modulates the startle circuit (Lang et al., 1998; Miller et al., 2005). The autonomic response means the activation of the sympathetic nervous system and neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis, leading to changes in blood pressure, heartbeat and respiration rate. The PAG mediates simple automatic responses to fear. The amygdala has also reciprocal projections to the locus coeruleus, which in turn is connected with the PAG. By feedback principle, the PAG via locus coeruleus or directly can regulate the activity of the amygdala, and by this way, the activity of other structures involved in anxiety and fear related conditions (Coplan, Lydiard, 1998; LeDoux, 1998).

Numerous studies in animals have confirmed a role of the frontal cortex, amygdala and PAG in mediating of anxiety and fear. Lesions of the amygdala decrease fear to novel objects in monkeys (Prather et al., 2001). Electric or chemical stimulation of the amygdala increase the level of anxiety-related behaviours (Adamec, Shallow, 2000). At the same time, lesions of the PAG alter fear and anxiety produced by the stimulation of the amygdala (Behbehani, 1995). Stimulation of the PAG also modulates anxiety response in rodents, as well as in cats (Adamec, 2001). Intra-amygdala microinjections of GABA, benzodiazepines, CRH antagonists, opiate agonists, neuropeptide Y, dopamine antagonists and glutamate antagonists decrease measures of anxiety and fear in animals (Davis, Whalen, 2001; Menard, Treit, 1999). Microinjection of various compounds, such as CCK-8S, BIBP3226 (an NPY Y1 receptor antagonist), CRH, midazolam ($GABA_A$ receptor agonist), FG7142 ($GABA_A$ receptor full inverse agonist), into the PAG also modifies anxiety-related behaviours (Zanoveli et al., 2004; Kask et al., 1998; Martins et al., 1997; Russo et al., 1993; Menard, Treit, 1999). Infusions of $GABA_A$ receptor agonists into the medial

prefrontal cortex increase exploratory activity of the rodents and decrease the level of anxiety (Shan et al., 2004).

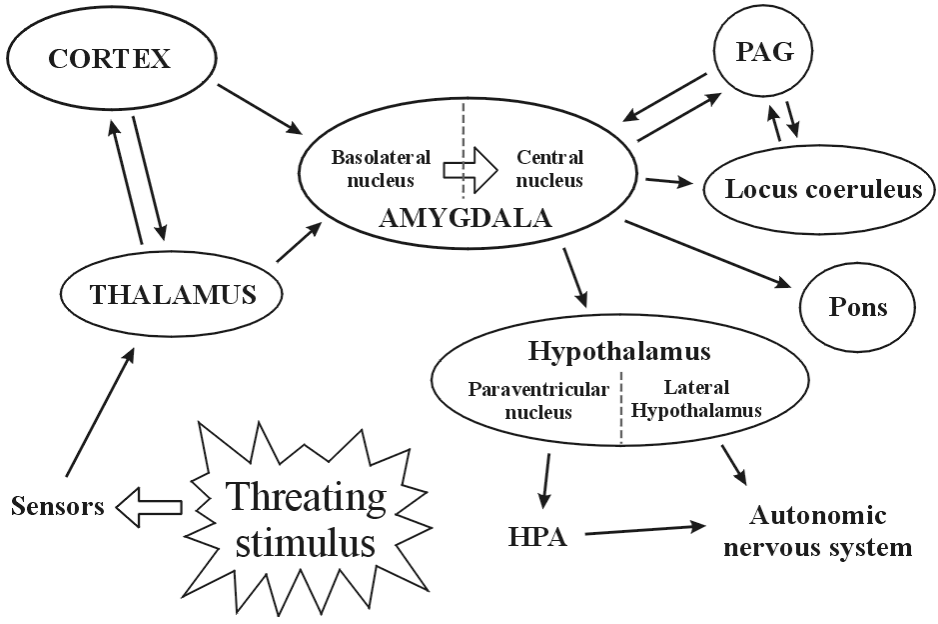

Figure 1. Anxiety circuitry in the brain (based on Lang et al., 1998; Finn et al., 2003; Miller et al., 2005)

1.2. Main neurotransmitter systems involved in the regulation of anxiety

Neurotransmitter systems involved in anxiety include γ-aminobutyric acid (GABA), serotonin (5-hydroxytryptamine (5-HT)), norepinephrine, dopamine, and neuropeptides, such as corticotrophin-releasing hormone (CRH), cholecystokinin (CCK), and neuropeptide Y (NPY) (Wood, Toth, 2001). Some of these systems, such as GABA, serotonin, norepinephrine, dopamine, have better been investigated, while studies of neuropeptide Y, corticotrophin-releasing hormon, melanocortins, vasopressin and substance P have a relatively brief history (Holmes et al., 2003; Millan, 2003). Many of these systems have been identified as sites of action for drugs that have proved to be effective in animal models of anxiety disorders. However, in clinical practice only benzodiazepines, $GABA_A$ receptor agonists, and selective serotonin reuptake inhibitors (SSRIs) have been used (DeVane, Franklin, 2005; Argyropoulos et al., 2000; Bourin, Lambert, 2002).

1.2.1 GABA system

GABA is the main inhibitory neurotransmitter in the brain. It is synthesised from glutamate in neuronal cytosol by two glutamate decarboxylases (GADs) (Soghomonian, Martin, 1998). These two isoforms, GAD65 and GAD67, have different molecular weight, 65 and 67 kDa respectively. GABA is synthesised from glutamate mostly by GAD67 (Asada et al., 1996; 1997). Sythesised GABA is degraded in mitochondria either in neurones or astrocytes by GABA-transaminase, which produces succinic semialdehyde. The succinic semialdehyde is then converted to succinate, a tricarboxylic acid cycle intermediate. Glutamine is produced in astrocytes and exported to neurones (Soghomonian, Martin, 1998).

Secreted GABA acts via its receptors. There are two major classes of GABA receptors: ionotropic $GABA_A$ (including $GABA_C$) receptors and metabotropic $GABA_B$ receptors (Cryan, Kaupmann, 2005). The $GABA_A$ receptor consists of five protein subunits, arranged like a rosette around a central pore, crossing the cell membrane (Nutt, Malizia, 2001). Although altogether nineteen $GABA_A$ receptor subunits (α1–6, β1–4, γ1–4, δ, ε, θ, π and ρ1–3) have been cloned from the mammalian CNS, only some of them, α1-3, β2-3, and γ2, arranged pseudo-symmetrically around the ion channel in the sequence γ−β−α−β−α, commonly form the receptor pore (Figure 2) (Farrant, Nusser, 2005). Thus, the most distributed receptor's variants are α1β2γ2, α3β3γ2 and α2β3γ2 (Whiting, 2003). Subunit composition dictates not only the properties of the receptors, but also their cell surface distribution and dynamic regulation (Nutt, Malizia, 2001; Bowery, Smart, 2006). Receptor activation leads to chloride ion influx into the cell that causes the hyperpolarisation of membranes and results in the inhibition of neurons. $GABA_A$ receptors that are localised in postsynaptic membranes inhibit the action of other neurotransmitter systems in the brain.

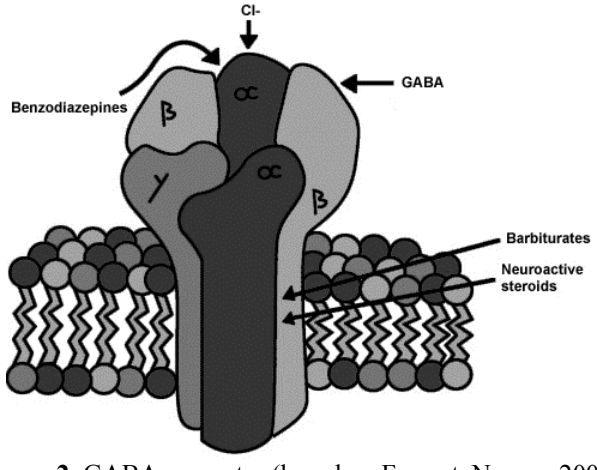

Figure 2. $GABA_A$ receptor (based on Farrant, Nusser, 2005)

Numerous compounds can modify the activity of $GABA_A$ receptors (Lydiard, 2003; Millan, 2003). Benzodiazepines are the most commonly used medications with a very wide spectrum of actions. Diazepam and alprazolam act as full agonists, while β-carboline FG 7142 is full inverse agonist of $GABA_A$ receptor (Millan, 2003). Flumazenil blocks the effect of both agonists and inverse agonists and acts as a receptor antagonist (Nutt, Malizia, 2001). Other compounds also modulate the activity of $GABA_A$ receptor. For example, muscimol acts as an agonist of these receptors. Neurosteroids, acting on different receptor subunits, can evoke either anxiolytic or anxiogenic effect (Lydiard, 2003).

Studies with transgenic mice and with selective receptor agonists and antagonists have revealed a different functional role of $GABA_A$ receptor subunits. It has been shown that amnesic, sedative and anticonvulsant effect of benzodiazepines is associated with α1 receptor subunit. At the same time, α2 subunit is the most specific site to anxiolytic and myorelaxant effects of benzodiazepines. The α3 subunit is responsible for the hypnotic effect of GABA agonists, and to a lesser extent for anxiolytic and myorelaxant action. The α5 subunit is associated with amnestic effect of benzodiazepines (Millan, 2003). The β subunit seems to play an important role in regulating channel properties and appears to be responsible for the ion selectivity, and is also needed for the action of barbiturates (Ymer et al., 1989; Jensen et al., 2002). The γ subunits are necessary for benzodiazepine binding (Pritchett et al., 1989). The γ2 subunit enhances the efficacy of GABA (Lorez et al., 2000) and is also needed for the synaptic clustering of $GABA_A$ receptors (Essrich et al., 1998). Thus, the anxiolytic effect of $GABA_A$ receptor is associated with α2, and probably, with α3 and α5 subunits, while the role of other receptor subunits in anxiety is not clear.

$GABA_B$ receptor is a heterodimer consisting of two subunits, $GABA_{B1}$ and $GABA_{B2}$ (Figure 3). These two subunits ($GABA_{B1}$ and $GABA_{B2}$) have 7 transmembrane domains and are coupled via their intracellular C-termini. The $GABA_{B1}$ is needed for GABA binding, while $GABA_{B2}$ is responsible for coupling with G-protein. Different isoforms of $GABA_{B1}$ (1a–1f) have been reported, although only 1a, 1b and 1c appear to act as functional subunits (Bowery, Smart, 2006). Although there is some evidence of differential association of $GABA_{B1a}$ and $GABA_{B1b}$ with pre-synaptic and post-synaptic structures, respectively, no conclusive picture has emerged to date. It seems more likely that, depending on the brain region, $GABA_{B1a}$ and $GABA_{B1b}$ participate in the formation of both pre- and post-synaptic receptors through hetero-dimerization with $GABA_{B2}$ (Cryan, Kaupmann, 2005).

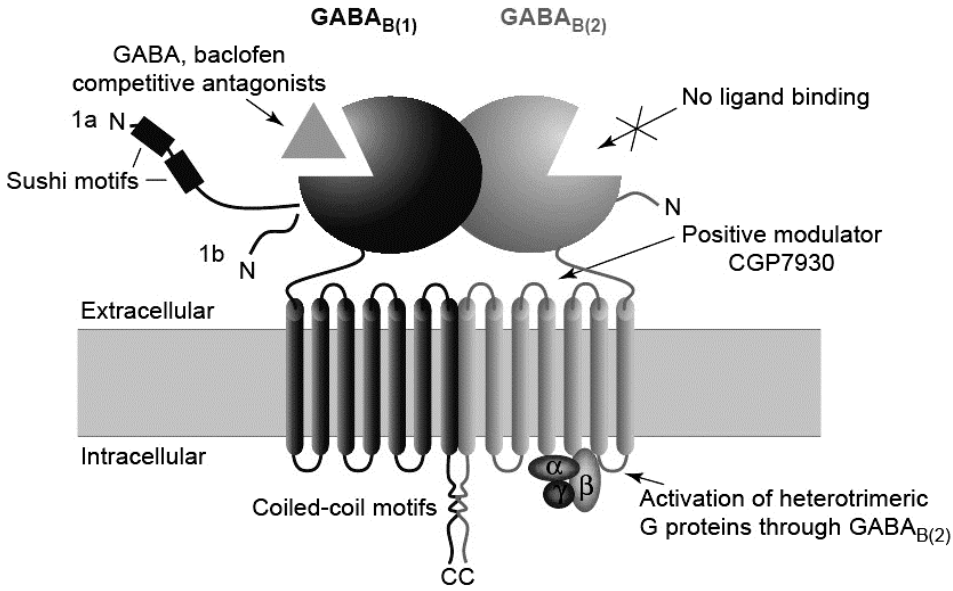

Figure 3. $GABA_B$ receptor (from Bowery, Smart, 2006)

Pre-synaptic $GABA_B$ receptors modulate neurotransmitter release by depressing Ca^{2+} influx via voltage-activated Ca^{2+} channels. Postsynaptic $GABA_B$ receptors are coupled mainly to inwardly rectifying K^+ channels and mediate slow inhibitory postsynaptic potentials. Either calcium current suppression or potassium conductance increase leads to neuronal hyperpolarisation (Cryan, Kaupmann, 2005).

Data about the specific role of $GABA_B$ receptors in GABA-mediated neurotransmission are limited and variable. Mombereau and colleagues demonstrated anxiolytic-like effect of baclofen (β-ρ-chlorophenyl-GABA), the $GABA_B$ receptor agonist (Mombereau et al., 2004a). $GABA_B$ receptor positive modulator GS39783 has been shown to be active in several animal models of anxiety, including the elevated plus-maze and the light-dark box exploration (Cryan et al., 2004). The $GABA_B$ receptor antagonist SGS742 (CGP36742) displays pronounced cognition enhancing effects, significantly improved attention and memory (Froestl et al., 2004).

$GABA_B$ receptor subunit knock-out studies have been more promising. $GABA_{B1}$-deficient mice display increased anxiety in light-dark box exploration and elevated zero-maze tests, and have a panic-like response on an elevated zero-maze (Mombereau et al., 2004b). Genetic deletion of $GABA_{B2}$ receptor subunit induced a similar effect as observed in $GABA_{B1}^{-/-}$ mice in validated models of anxiety. All these facts demonstrate a possible role of $GABA_B$ receptor in anxiety-related behaviour. However, the mechanisms responsible for the influence of $GABA_B$ receptors on anxiety-related behaviour are not well

understood, and additional studies are needed for understanding the role of $GABA_B$ receptor activation in anxiety and fear.

1.2.2. Cholecystokinin (CCK) system

In 1975 Vanderhaeghen and colleagues identified widespread distribution of gastrin-like immunoreactivity in several brain structures. Some years later it was established that gastrin-like immunoreactivity mostly consists of cholecystokinin octapeptide (CCK-8) (Van Dijk et al., 1984; Rotzinger, Vaccarino, 2003).

Pre-pro-CCK is a 115-amino-acid-length peptide, the processing of which gives a number of smaller fragments with CCK-like bioactivity (Figure 4). The largest fragments, 58- and 33-aminoacid-length, are ligands for CCK_A receptor,

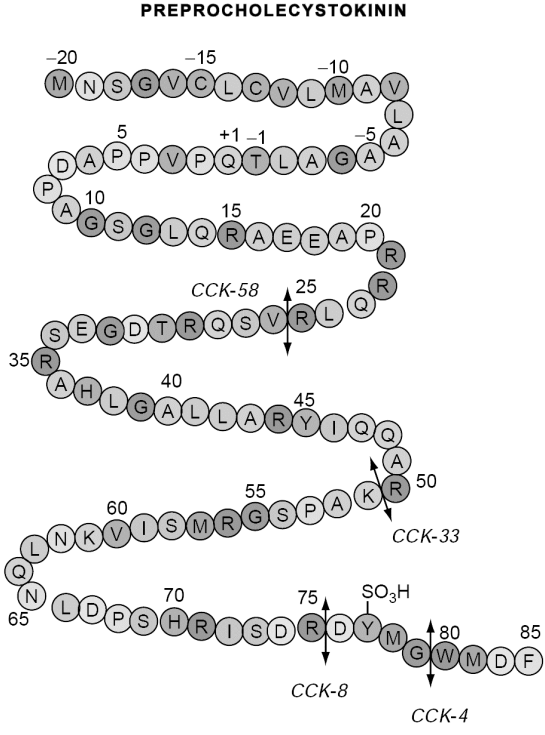

Figure 4. Preprocholecystokinin molecule

while smaller fragments, with 8, 5 and 4 amino acid in length (correspondingly CCK-8, CCK-5 and CCK-4), are ligands for CCK_B receptors (Hernandez-Gomez et al., 2002; Rotzinger, Vaccarino, 2003; Schäfer et al., 1994). In the brain, CCK-8 in sulphated form is the main physiological ligand of CCK receptors activating both CCK_A and CCK_B receptors in equal extent (Hernandez-Gomez et al., 2002). CCK peptides are abundant throughout the brain, with the highest levels in the cerebral cortex, hippocampus, amygdala and lower levels in the thalamus, hypothalamus, and PAG (Beinfeld et al., 1981; Vanderhaeghen, Schiffmann, 1992; Lindefors et al., 1993; Noble et al., 1999; Wang et al., 2005).

CCK acts via two G-protein coupled receptors, CCK_1 (also called CCK_A) and CCK_2 (also called CCK_B) (Table 1). Their stimulation leads to the activation of intracellular signal transduction pathways and, by this way, to an increase in neuronal activity. The concentration of CCK_1 receptors in the brain is significantly lower in comparison to CCK_2 receptor depending of brain structure (Noble et al., 1999; Rotzinger, Vaccarino, 2003). Besides physiological agonists, a number of selective agonists and antagonists for CCK receptors have been synthesised. (Noble, Roques, 2002). It is interesting, that most of the CCK receptor antagonists are derivates of benzodiazepines. The biological role of CCK receptors is heterogeneous. CCK_2 receptors regulate the interrelations of the dopaminergic and opioid system and by this way can be involved in the control of drug abuse, nociception and depression (Noble, Roques, 2002). Locomotor activity can also be regulated through CCK_2 receptors. In learning and memory functions, CCK_1 and CCK_2 receptors have different roles. Generally, CCK_1 receptors mediate mnemonic effects and CCK_2 receptor mediate amnesic effects in learning and memory tasks, but these data are still controversial (Lemaire et al., 1992; Sebret et al., 1999; Dauge et al., 2001b; Noble, Roques, 2002). The main role of CCK_1 receptors in the brain is the control of food intake via the central regulation of appetite (Noble, Roques, 2002).

One of the most significant tasks of the CCK-ergic system is the regulation of anxiety. It has been demonstrated that CCK-8 and caerulein, nonselective CCK agonists, injected either systemically or into the amygdala or periaqueductal grey, induce an anxiogenic-like effect in both rats and mice in the elevated plus-maze (Harro, 1990; Vasar et al., 1992, 1994; Belcheva et al., 1994; Rotzinger, Vaccarino, 2003; Netto, Guimaraes, 2004). Selective CCK_2 agonists such as butoxycarbonyl tetrapeptide of CCK (BOC-CCK-4) and pentagastrin increase anxiety in the elevated plus-maze, while CCK_2 antagonists CI-988 and L-365,260 produce anxiolytic-like effects in this test in rodents (Singh et al., 1991a; 1991b; Rataud, 1991; Rotzinger, Vaccarino, 2003). Knockout mice studies also confirmed the role of CCK_2 receptor in anxiety. Female mice lacking CCK_2 receptors are less anxious than their wild-type littermates (Raud et al., 2005). Thus, these data demonstrate a direct anxiogenic effect of CCK mediated through CCK_2 receptors.

Table 1 Characterisation of subtypes of CCK receptors

Receptor	**CCK_1** CCK_A /Alimentary / Peripheral	**CCK_2** CCK_B / Brain / Central
Structure – human	428 – amino acid sequence (P32238 7TM)	447 – amino acid sequence (P32239 7TM)
Gene and location **Human:** **Mouse:**	*CCK_1* Chromosome 4 Chromosome 5	*CCK_2* Chromosome 11 Chromosome 7
Splice variants	No	Long form, short form, Δ form
Genetically induced disruption of gene in mice	Kopin et al., 1999	Nagata et al., 1996
Distribution	Gall bladder, pancreas, pylorus, intestine, spinal cord, vagus nerve, limited brain areas (nucleus tractus solitarius, area postrema, nucleus interpedun-cularis, posteromedial part of nucleus accumbens)	Throughout the brain (with the highest densities in the cerebral cortex, nucleus caudatus, anterolateral part of nucleus ac-cumbens), vagus nerve, stomach, pancreas
Endogenous ligands according to their affinity to specific receptor	CCK-8s >> gastrin, des-CCK-8 > CCK-4	CCK-8s ≥ gastrin, des-CCK-8, CCK-4
Agonists	Caerulein (amphibian CCK ana-logue); A71623; GW5823; JMV-180	Caerulein; CCK-4; Boc-CCK-4; BC 197; BC 264; des-CCK-8; gastrin; RB400
Antagonists	Proglumide; Lorglumide; Deva-zepide; Lintitript (SR 27897); T0632; IQM95333; PD140548	Proglumide; L-365260; L-740093; LY 288513; CI-988; YM022; GV150013 ; RP73870; LY262691
Intracellular activation	$G_{q/11}/G_s$	$G_{q/11}/G_s$
Functional role	Mediates CCK actions on gall bladder contraction, secretion of pancreatic enzymes, gastric emptying, inhibits feeding and respiration, potentiates dopa-mine-mediated behaviours and dopamine release in shell of nucleus accumbens	Mediates CCK actions on increases in neuronal firing rates, nociception, anxiety, respiration, inhibits dopamine-mediated beha-viours and dopamine release, regulates insulin release in pancreas

The anxiogenic effect of CCK was demonstrated not only on rodents. Similar results were received in human and primate studies. Intravenous injection of CCK-4 and pentagastrin produces panic attacks in humans, especially in patients with panic disorders (de Montigny, 1989; Dauge, Lena, 1998). Primates' studies with the administration of CCK_2 receptor agonist and antagonist also supported the anxiogenic role of CCK_2 receptors (Powell, Barrett, 1991; Palmour et al., 1992).

CCK is colocalised on cell bodies and terminals with many other neurotransmitters such as gamma-aminobutyric acid, dopamine, serotonin and opiates and is involved in the modulation of their function (Rotzinger, Vaccarino, 2003; Derrien et al., 1993; Biro et al., 1997; Noble, Roques, 2002). However, the interaction between the GABA-ergic and CCK-ergic systems remains the most interesting and intriguing question.

Bradwejn and de Montigny demonstrated that benzodiazepines at clinically relevant doses blocked CCK-induced neural excitation in the hippocampus (Bradwejn, de Montigny, 1984). The functional interaction between CCK and GABA was also described in the work of Yaksh and colleagues, where the ability of GABA to induce a dose-dependent decrease in the resting release of CCK was demonstrated (Yaksh et al., 1987). Singh and collegues revealed the ability of CCK receptor antagonists to block FG 7142-induced anxiety (Singh et al., 1992). Then the suppression of the anxiogenic effects of CCK by benzodiazepine antagonist flumazenil was demonstrated (Chopin, Briley, 1993). It has been shown that GABA release in the brain is influenced by CCK and vice versa (Rezayat et al., 2005). The recent studies confirm the complexity of mechanisms underlying the GABA and CCK interaction, where GABA release in the nucleus accumbens can be mediated via CCK action on the CCK_2 receptors localised on GABA-ergic neurones (Rezayat et al., 2005; Kombian et al., 2005). Human studies also support the hypothesis of a possible interaction between the GABA and the CCK system. Alprazolam, a GABA agonist, can reduce CCK-4-induced panic attacks, while GABA-transaminase inhibitor vigabatrin exerts anxiolytic effects in CCK-4-induced panic in healthy volunteers (Zwanzger et al., 2001; 2003).

2. Animal models of anxiety

According to McKinney, animal models are "experimental preparations developed in one species for the purpose of studying phenomena occurring in another species" (McKinney, 1984). Kaplan added that a model might be valid if it has the same structure as the human behaviour or pathology (Kaplan, 1973). Other authors have proposed additional criteria. According to them, an animal model should have predictive validity (pharmacological correlation), face validity (isomorphism) and construct validity (homology and similarity of

underlying neurobiological mechanisms) to be suitable for research (Belzung, Griebel, 2001).

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 implies that the anxiety response observed in the animal model should be identical to the behavioural and physiological responses observed in humans. This indicates that the expression of a given emotion is supposed to be similar across species. Construct validity 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 (Belzung, Griebel, 2001).

2.1. Overview of anxiety models

There are different classifications of animal models of anxiety. According to type of behaviour these can be classified as tests with spontaneous behaviour or as tests with evoked behaviour (Finn et al., 2003). The other authors propose classifications according to the normality of anxiety-related reactions, classifying them into models of "normal" anxiety (which are related to "state" anxiety tests) and into models of "pathological" anxiety (which are often referred to as "trait" anxiety tests) (Belzung, Griebel, 2001). One of the possible classifications of animal models of anxiety is presented in Table 2, where the animal models are considered as conditioned or unconditioned (ethological). The first group involves animals' conditioned responses to stressful and often painful events (e.g. exposure to electric footshock); the second includes ethologically based paradigms and involves animals' spontaneous or natural reactions (e.g. flight, avoidance, freezing) to stress stimuli that do not explicitly involve pain or discomfort (e.g. exposure to a novel highly illuminated test chamber or to a predator).

Conditioned animal models of anxiety are based on classical conditioned Pavlovian reflexes. In these models previously innocuous stimulus that has been associated, through repeated pairings, with an innately aversive stimulus, induces anxiety and fear related behavioural reaction, the degree of which can be measured (Davis, 1990). Conditioning models require considerable training of subjects (rodents, pigeons, monkeys), food or water deprivation and/or the use of an aversive stimulus (Rodgers, Dalvi, 1997).

Table 2. Animal models of anxiety

Conditioned models	Unconditioned models
Geller-Seifter conflict	Elevated plus-maze (and zero-maze)
Vogel conflict	Open field
Conflict tests: pigeons, primates	Light/dark exploration
Four-plate test	Free exploration
Conditioned emotional response	Holeboard
Conditioned taste aversion	Staircase test
Fear potentiated startle	Social interaction
Defensive burying	Social competition
Active/passive avoidance	Ultrasonic vocalization (pain or separation)
Learned helplessness	Human threat (primates)
Electrical brain stimulation	Fear/anxiety-defence test batteries

Additionally, conditioned tests are amenable to experimental manipulation to a degree that is impossible with unconditioned (or ethological) tests (Flint, 2003). In general, these tests were widely used for the investigation of anxiolytic drugs. One of the most used tests was Vogel conflict test (Vogel et al. 1971; Millan, 2003). This test is based on the suppression of punished responses. Water-deprived rodents are exposed to the conflict between licking the spout of a bottle with water and receiving a mild shock on the tongue. Anxiolytic drugs exert anti-conflict effects and increase the number of punished licks, an effect consistently seen with drugs that potentiate the action of GABA such as the benzodiazepines.

The logical extension of conditioned models of fear is represented by unconditioned tests. These tests are based on a 'spontaneous' or 'innate' behavioural patterns responding to certain environmental stimuli (object or subject), which arises without any conditioning and which are difficult to eliminate by training. The stimulus can be represented by some new object, as new cage or maze, by new cage neighbour (cage-mate), or by even predator. Most popular among these animal models are the ones based on exploratory 'approach-avoidance' tasks.

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.

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.

2.2. Elevated plus-maze

One of the most widely used experimental models for the study of anxiety is the elevated plus-maze (Salum et al., 2000). This model is derived from the work of Montgomery (1955) on the relation between fear and exploratory drive in rats, based on the premise that environmental novelty evokes both fear and curiosity, thereby creating a typical approach-avoidance conflict. Using a Y-maze, Montgomery found that the intensity of this conflict evoked when the rat was exposed to an open alley was greater than when it was exposed to enclosed alleys. Handley and Mithani (1984) were the first to use an X-maze to investigate the effect of anxiolytic and anxiogenic drugs in this model and concluded that it may provide a valid model of 'fear-motivated' behaviour. Following these studies, Pellow, Chopin, File and Briley (1985) performed an extensive investigation validating the plus-maze as a behavioural, physiological and pharmacological anxiety model. Lister (1987) confirmed this validation using mice, exposing the animals to a holeboard for 5 min immediately before the test. As a result, he could get independent measures of locomotion, exploration and anxiety enabling the discussion of specific drug effects. Adapted for rats and mice, the elevated plus-maze has become increasingly popular in the studies investigating anxiety and fear related behaviour (Lister, 1990).

Classically, the X-shaped plus-maze for rats consists of two open arms 45×10 cm, two closed arms $45 \times 10 \times 40$ cm, and a central platform 10×10 cm (Figure 5). Two closed arms are opposite each other and have sidewalls and an end wall that is 40 cm high. The maze is usually elevated 50–70 cm above the floor (Harro, 1993).

During an experiment the animal is placed at the centre of the maze, facing one of the open or closed arms, depending on the laboratory procedure. The animal begins active rearing and peering around the platform, and then chooses one of the arms, either open or closed. Usually the first choice is an enclosed arm, because of the new environment is initially too aversive (fearful) to rodents. After some time the animal starts exploring the central platform and other arms of maze, and exploratory activity of rodent depends of its level of anxiety (Rodgers, 1997).

Figure 5. The elevated plus-maze

By measuring the number of open arm entries, number of closed arm entries, and time spent in open arms, observer can determine the level of exploratory activity. Animals with increased level of anxiety – i.e more "anxious" animals – have reduced total number of entries, number of open arm entries and time spent in the open arms (Rodgers, 1997). Head-dip is an act of exploration over the sides of the maze towards the floor. It can be protected (occurring on a closed arm or central platform) or unprotected (occurring on an open arm of plus-maze). Stretched attend posture is a forward elongation of head and shoulders followed by retraction to original position. It also can be protected (occurring on a closed arm or central platform) or unprotected (occurring on a open arm) (Espejo, 1997). Locomotor activity is reflected in number of total arm entries and closed arm entries, while the number of total head-dips and the number of total stretched attend postures are characteristics of exploration (Rodgers, Dalvi, 1997).

2.3. Selection of rats according to their exploratory behaviour

As it has been shown in some studies, rodents according to their behavioural activity in the elevated plus-maze can be divided into subgroups. Rägo and colleagues showed that after measuring of activity of rats in the elevated plus-maze they can be divided into animals with high exploratory activity, or "non-anxious", into animals with low exploratory activity, or "anxious", and into the animals with intermediate activity, or "intermediate" group (Rägo et al., 1988, 1991; Harro et al., 1990). Later studies established differences in binding

parameters of benzodiazepine, cholecystokinin and 5-HT_{2A} receptors between these groups and also the blood levels of growth hormone were different (Harro et al., 1990, Kõks et al., 1997). Moreover, two different Wistar rat lines have been bred, selected for either high (HAB) or low (LAB) anxiety-related behaviour on the elevated plus-maze (Wigger et al., 2001). The behavioural and neuroendocrine parameters of HAB and LAB rats are markedly different. The HABs have been recommended as a novel, psychopathologic animal model of anxiety disorders.

3. Phenotype of CCK_2 receptor deficient mice

CCK_2 receptor deficient mice were generated by Nagata and colleagues by replacing a part of exon 2, exons 3, 4 and 5 of the Cckbr gene (1996). These mice are fertile and without obvious behavioural abnormalities up to the age of 24 months (Nagata et al., 1996). Kopin and colleagues did not establish differences between CCK_2 receptor deficient mice and their wild-type (+/+) littermates in the food intake, weight gain and pancreatic function (1999). Other authors have shown that CCK_2 receptor lacking mice had increased energy expenditure, higher basal metabolic rate, increased body weight, water consumption, elevated body temperature and decreased scotophase locomotor activity (Miyasaka et al., 2002, 2004; Weiland et al., 2004). CCK_2 receptor deficient mice display markedly impaired gastric acid secretion, atrophy of the oxyntic mucosa and hypergastrinaemia, what partially could be explained by reduced parietal cell mass, a reduced proportion of actively secreting parietal cells, and a replacement of ECL cells by histamine-free ECL-like cells (Nagata et al., 1996; Chen et al., 2002).

CCK_2 receptor deficient mice display increased locomotor activity in the open-field test. Some authors have demonstrated that the behavioural activation in CCK_2 receptor deficient mice could be suppressed by treatment with dopamine D_2 antagonists (Dauge et al., 2001a). On the other hand, dopaminergic drug amphetamine causes a stronger hyperlocomotion in genetically modified mice compared to their wild-type littermates (Kõks et al., 2001, 2003). These data suggest an increased sensitivity of dopamine D_2 receptors in CCK_2 receptor deficient mice in comparison to their wild-type littermates. 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 demonstrated that administration of morphine or inhibition of enkephalin metabolism induces a significantly stronger hyperlocomotion in homozygous (–/–) mice compared to wild-type (+/+) littermates (Pommier et al., 2002).

Pommier and colleagues also found that these mice display hyperalgesia in the hotplate test (Pommier et al., 2002). Reduced jumping latency of homozygous (–/–) mice in this test was confirmed by our group (Veraksitš et al., 2003). In another widely used pain test, plantar analgesia test, pain sensitivity of CCK_2 receptor deficient mice was again significantly reduced compared to wild-type littermates, leading authors to the statement that CCK_2 receptor deficient mice have decreased pain sensitivity but reduced pain tolerance (Veraksitš et al., 2003). Recently Kurrikoff and colleagues reported that CCK_2 receptor deficient mice display mechanical hyposensitivity, which can be reversed to the level of wild-type (+/+) animals by the administration of naloxone (Kurrikoff et al., 2004). The finding that mice, lacking CCK_2 receptors, display higher expression levels of lumbar CCK_1, opioid delta and kappa receptor genes could possibly explain the reduced mechanical sensitivity established in genetically modified animals. Moreover, it was demonstrated that CCK_2 receptor deficient mice do not develop mechanical hyperalgesia in the Bennett's neuropathic pain model. Induction of neuropathy resulted in a decrease of lumbar pro-opiomelanocortin (POMC) gene expression in wild-type (+/+) mice, whereas the opposite change was found in CCK_2 receptor deficient mice. These findings confirm the evidence that the genetic invalidation of CCK_2 receptors results in an upregulation of the opioidergic system in mice (Pommier et al., 2002; Kurrikoff et al., 2004).

4. Concluding remarks

Studies of the molecular mechanisms of exploratory behaviour provide a better understanding of the nature of anxiety and stress-related states. According to the existing literature, the elevated plus-maze is a widely used model for the study of exploratory behaviour and anxiety. Moreover, this model allows not only to measure the level of exploratory activity of a single animal, but also to divide rodents into subgroups according to their exploratory activity (Rägo et al., 1988, 1991; Harro et al., 1990; Kõks et al., 1997). These subgroups have differences in the binding parameters of benzodiazepine, cholecystokinin and $5\text{-}HT_{2A}$ receptors, and also in the blood level of growth hormones. On the other hand, Wigger and colleagues have bred two different lines from the original population of Wistar rats, selected for either high (HAB) or low (LAB) anxiety-related behaviour on the elevated plus-maze (Wigger et al., 2001). As studies have demonstrated, the behavioural and neuroendocrine parameters of HAB and LAB rats are markedly different. Taking into account these data, the hypothesis was postulated that animals behaving differently in the elevated plus-maze could have differences in their gene expression patterns in different brain structures. Previously, similar studies have been performed in order to reveal gene expression differences between two independent animal groups. Wang and

colleagues compared the gene expression profiles between two rat lines, hooded PVG and Sprague-Dawley, by means of gene chip technology and established differences in their anxiety level after exposing them to cat odour (Wang et al., 2003). Some years earlier, Farook and colleagues (Farook et al., 2001) demonstrated, using reverse-transcriptase PCR, different CCK_2 receptor gene expression in the cerebral cortex of the same rat lines. Furthermore, using cDNA RDA technology Kõks and colleagues described differences in gene expression patterns in the amygdala of rats exposed to cat odour and rats not exposed to cat odour (Kõks et al., 2004).

A number of studies have established an interaction of GABA- and CCK-ergic systems in the regulation of exploratory behaviour and anxiety (Harro et al., 1993; Shlik et al., 1997; Noble et al., 1999), but the exact molecular mechanisms of this interaction remain to be established. Furthermore, previous studies have been focused either on the cerebral cortex or hippocampus (Yaksh et al, 1987; Acosta, 2001), while the interaction of GABA and CCK system in the PAG and amygdala, two other structures related to the regulation of emotional behaviour, have been poorly explored. These facts determined the choice of the brain structures and neurotransmitter systems for the present study, where the expression of CCK- and GABA-ergic genes in the frontal cortex, amygdala and PAG was investigated.

However, gene expression characteristics do not always reflect the real functional status of neurotransmitter systems. Therefore, in addition to gene expression experiments, the behavioural effects of diazepam, an anxiolytic drug interacting with the $GABA_A$ receptors, were studied in mice lacking CCK_2 receptors, a major target for the action of CCK in the brain. Also the parameters of [^{3}H]-flunitrazepam binding were compared in the brain structures of wild-type and homozygous mice in order to measure the changes in the density of bensodiazepine binding sites due to the genetic invalidation of CCK_2 receptors.

After the validation of the plus-maze selection models in Wistar rats the next step was to reveal new candidate genes involved in the regulation of anxiety. It allows us to extend our knowledge about the intracellular pathways underlying the regulation of exploratory behaviour and anxiety. This study was conducted on the level of the PAG, a brain structure serving a role of the major output for the amygdaloid complex.

III. AIMS OF THE STUDY

The data presented in the review of literature demonstrate the complex nature of mechanisms underlying the regulation of anxiety. Pharmacological, neuro-physiological and -histological studies have revealed the basic brain structures and neurotransmitter systems playing a key role in the mechanisms of anxiety, but the exact molecular mechanism of their interaction remains to be established. As the latest development, the application of molecular genetic methods gives a chance to study the mechanisms anxiety at the molecular level. There is a growing body of evidence demonstrating an increasing impact of gene screening technologies for the study of brain functions, and among these are cDNA representational difference analysis (cDNA-RDA) and real-time qPCR. In the present study, an attempt was made to establish new molecular targets implicated in the regulation of anxiety. Selection of rats according to their exploratory activity was chosen as a model to reveal rats having different levels of exploratory activity and anxiety. The model was further evaluated by the measurement of expression of patterns of GABA and CCK systems shown to interact closely in the regulation of exploratory behaviour and anxiety. The more specific tasks for the current study were as follows:

1. To study differences in the expression of GABA- and CCK-related genes in the brain structures involved in the regulation of anxiety in male Wistar rats displaying different exploratory activity in the elevated plus-maze.
2. To show, by taking into account the results obtained in the gene expression studies, whether the behavioural effects of diazepam, a drug interacting with $GABA_A$ receptors, are affected in mice, lacking CCK_2 receptors.
3. To identify, by means of cDNA representational difference analysis, genes differentially expressed in the PAG of rats displaying different level of exploratory activity in the elevated plus-maze, and subsequently analyse the potential biological meaning of these genes.
4. To study the differences in the expression of the LsAMP gene in the frontal cortex, amygdala and periaqueductal grey in rats displaying different exploratory activity.

IV. MATERIALS AND METHODS

1. Animals

The studies were performed on female CCK_2 receptors deficient mice and male Wistar rats. CCK_2 receptor deficient mice were generated by Nagata and colleagues (Nagata et al., 1996). A part of exon 2 and exon 3–5 of Cckbr gene was replaced 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. 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 C57/Bl6 background to minimise possible genetic influence from the 129Sv strain. Genotyping was carried out as follows. Polymerase chain reaction (PCR) using two pairs of primers, HE2F (TGG AGT TGA CCA TTC GAA TCA C) and LacZrev (GTG CTG CAA GGC GAT TAA GTT G) for detecting of the mutant allele, and HE3F (TAT CAG TGA GTG TGT CCA CTC T) and HE3R (ACA TTT GTT GGA CAC GTT CAC) for detecting of the wild-type allele, was performed using following steps: 96ºC for 10 min (initial denaturation); 96ºC for 50 s, 60ºC for 50 s and 72ºC for 2 min (25 cycles); and 72ºC for 10 min (final amplification); the presence of amplification products was confirmed by electrophoresis. Altogether, 180 homozygous (–/–) CCK_2 receptor deficient, 197 heterozygous (+/–), and 200 wild-type (+/+) three months old female mice were used in the behavioural and radioligand binding studies.

Male Wistar rats (Han/Kuo: WIST) weighing 260–300 g were purchased from the Finnish National Laboratory Animal Center, in Kuopio, Finland. The behavioural experiments were performed 14 days after the arrival of the rats from the breeding company. Altogether 51 rats were used in this study. Forty-three handling naïve rats were subjected to the plus-maze experiment, and eight animals were combined into control group. Control rats were taken randomly from the same cages as animals subjected to the plus-maze exposure. These rats were not tested in the plus-maze, and they were used as the home-cage control group in the gene expression studies.

All animals were kept in the animal house at 21 ± 2°C under a 12 h/12 h light/dark cycle (lights on at 07:00 h). Tap water and food pellets were available *ad libitum*. All animal procedures were approved by the University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24th of November 1986 (86/609/EEC).

2. Drugs

The behavioural effects of diazepam (Sigma), an anxiolytic $GABA_A$ receptor agonist, on the CCK_2 receptors deficient mice were studied in the elevated plus-maze and rotarod test. All injections were performed intraperitoneally (i.p.) 30 min prior to the experiments. For the studying of the anxiolytic action of the drug in the elevated plus-maze diazepam doses of 0.5, 1 and 3 mg/kg were used. For the rotarod test, diazepam was used in doses of 0.5 and 3 mg/kg. Before administration diazepam was suspended in physiological saline (0.9% sodium chloride solution) with the help of a few drops of Tween-80 (Sigma). Vehicle, a few drops of Tween 80 in physiological saline, was also administered intraperitoneally 30 min before the experiment.

3. Behavioural studies

3.1. Elevated plus-maze test

The method was initially suggested by Handley and Mithani for the measurement of exploratory activity (Handley, Mithani, 1984; Pellow et al., 1985; Rodgers, 1997). Male Wistar rats were exposed to the elevated plus-maze with the aim to select them into groups with different exploratory activity. The elevated plus-maze used for the selection of rats consisted of two opposite open arms (50 × 10 cm) without side walls and two enclosed arms (50 × 10 × 40 cm) with side walls and an end wall, extending from a central square (10 × 10 cm), and was elevated to the height of 50 cm. The open arms were divided by lines into equal sections. The lines were also present between the open arms and central square, and between the central square and closed arms. Taking into account a fact that in previous studies changes in the GABA and CCK system were observed only in stressed animals, the plus-maze experiment was conducted by using more aversive conditions (Pratt, Brett, 1995; Kõks et al., 1997; 2000). Therefore, the animals were isolated for 15 min before the study, and the experiment was performed in a brightly lit room. Illumination was around 500 lux at the level of open arms.

Behavioural experiments were performed between $12^{\underline{00}}$ and $18^{\underline{00}}$. The plus-maze study was carried out by an experienced person. Since the separation of animals into subgroups was performed after the completion of the experiment, it can be assumed that this person was blind as to whether the animals belonged to the high or low exploratory activity group. During a 5-min observation session the following measures were taken: (1) time spent in exploring the central square and open arms of the plus-maze, (2) the number of attempts to enter the central square from the closed arm, (3) the number of head-dipping and stretched attend postures, (4) the number of line crossings, and (5) the number

of closed and open arm entries. Subsequently, the ratio between open and total arm entries was calculated. At the beginning of the experiment the animal was placed on the centre of the plus-maze facing the closed arm. An arm entry was counted only when all four limbs of a rat were within a given arm. A line crossing was taken when the animal crossed the line with both forelimbs. Time spent on open arms, number of open arm entries, and the ratio between the open and total arm entries are conventional measures of anxiety in the plus-maze (Pellow et al., 1985; Lister, 1987; Rodgers, 1997). The numbers of head-dipping and stretched attend postures are ethological measures of anxiety (Rodgers, 1997). Due to the low number of open arm entries in the current study, head dipping was not divided into protected or unprotected, but the total number of head dipping was counted. An attempt to enter the central square from the closed arm was counted when an animal crossed with two forelimbs or head the line between the closed arm and central square, and after that returned back to the closed arm. This reflects the risk assessment behaviour of rats. Rats have a strong desire to explore a new unknown area, but as this area looks too aversive for them to move further, they decide to return back into the closed arm that feels safer to them. The number of closed arm entries reflects the locomotor activity of rodents (Rodgers, 1997). Selection of rats into low and high exploratory activity groups was performed after the completion of the plus-maze experiment using the frequency of open and closed arm entries as the initial criteria for dividing the animals.

Female CCK_2 receptor deficient mice were exposed to the elevated plus-maze in order to study the effect of diazepam on the exploratory activity of animals. The plus-maze consists of two opposite open (17.5 × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14-cm-high sidewalls and an endwall. The arms extended from a common central square (5 × 5 cm) and were angled at 90º to each other, making the shape of a plus sign. To determine locomotor activity, the open arms were divided by lines into three equal sections. The entire plus-maze apparatus was elevated to a height of 30 cm and placed in a brightly lit room (illumination level ~750 lux). In order to encourage open arm exploration, a slightly raised edge (0.25 cm) was put around the perimeter of the open arm, providing a grip for the animals. Similarly, as mentioned in the case of rats, more aversive conditions were preferred.

The mice were brought into the experimental room 1 h before the experiment. All behavioural procedures were performed between $11^{\underline{00}}$ and $19^{\underline{00}}$ hours. Standard 5-min test duration was employed. The behaviour of mice was video-recorded, and the videotapes were subsequently blind-scored by a trained observer. The following measures were taken by the observer: 1) time spent on the central square and open arms of the plus-maze; 2) number of closed and open arm entries; 3) number of line crossings; 4) ratio between the open and total arm entries. The wild-type (+/+) mice were always used in parallel with their genetically modified littermates with the aim to exclude possible daily fluctuations in the exploratory behaviour of animals.

3.2. Rotarod-test

Rotarod test was performed to study the effect of diazepam on the motor coordination of the female CCK_2 receptors deficient mice. The rotarod test was first described by Dunham and Miya to test neurological deficits in rats and mice, and is widely used for assessing the motor coordination and balance of rodents (Dunham, Miya, 1957; Crawley, Paylor, 1997; Monville et al., 2006). This test measures the ability of rodents to maintain balance on a rotating rod. In the present study, rotarod test was performed as follows. A 1-min training session was carried out 5 min before the first measurement, after which the mice were placed on a horizontal rubber-coated metal rod (8 cm in diameter) rotating at constant speed of 9 rpm, and motor performance (time until the first fall) was registered during a 2-min session on three consecutive days. The effect of diazepam in doses of 0.5 and 3 mg/kg was studied on the fourth day. Diazepam and vehicle were administered intraperitoneally 30 min before the experiment. The rotarod and elevated plus-maze tests were performed using separate groups of CCK_2 receptor deficient mice.

4. Gene expression studies

4.1. Dissection of brain structures

Rats were killed by decapitation immediately after the plus-maze exposure. The assessment of exploratory behaviour and decapitation of rats were performed in separate rooms and by different people in order to avoid the influence of the decapitation procedure on the plus-maze experiment. Brains were rapidly removed from the skull, and three brain structures, the frontal cortex, amygdala and PAG, were dissected and frozen in liquid nitrogen. Dissection of the brain was performed according to the rat brain atlas of Swanson (Swanson, 1998). The frontal and prefrontal corteci were dissected firstly by cutting a 4-mm-thick slice from the anterior part of the brain. Then, after the dissection of the brain stem and the 4-mm-thick slice involving the entire cerebral aqueduct, the PAG was punched out by a round shape puncher with a 3.5-mm inner diameter. This puncher ensures that the PAG and its surrounding tissues are dissected entirely, and the samples contain identical material. The amygdala was dissected by a round-shape puncher, from the central coronal slice of brain, containing ventral hippocampus, temporal lobes and both amygdala. Our tissue samples contained the basolateral, central and medial nuclei of amygdala. All procedures were performed under a dissection microscope (Nikon SMZ 1500).

4.2. mRNA isolation and cDNA synthesis

From the brain tissue samples of the frontal cortex, amygdala and PAG, total RNA was extracted using QIAGEN RNeasy Midi Kit (Qiagen, Hilden, Germany). Total RNA from different animals of each group was pooled to get four different pools per group (2–3 animals per pool). Thirty-six different pools of total RNA (three brain structures, three groups of animals, four pools) were received. mRNA was purified from the total RNA using Oligotex mRNA Extracting Kit (Qiagen, Hilden, Germany). Then the first strand of cDNA was synthesised using two different kits.

For the cDNA representational difference analysis (RDA), both strands of cDNA were synthesised by SuperScript II Choice System for cDNA Synthesis (SuperScript II Reverse Transcriptase, Invitrogen, Gibco BRL, Paisley, UK), according to the manufacturer's protocol.

For the quantitative real-time PCR, the first strand cDNA was synthesised using RevertAid M-MuLV Reverse Transcriptase from First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Synthesis reactions were performed according to the manufacturer's protocol for first strand cDNA Synthesis (Fermentas).

4.3. Quantitative reverse-transcriptase PCR

Quantitative reverse-transcriptase PCR was performed in a pilot study with the aim to investigate the differential expression of the limbic-system associated membrane protein (LsAMP) gene in the PAG of animals with different exploratory activity in the elevated plus-maze. After first-strand cDNA synthesis by SuperScript II Choice System for cDNA Synthesis the PCR reaction was performed from 10% of the first-strand reaction mix. Specific primers for the LsAMP gene and the cyclophilin A gene (reference gene to control the amount of RNA) were used (Table 3). Amplification products were subjected to 1.5% agarose gel electrophoresis, where clearly distinguishable bands were detected.

Optical densities of fragments were compared by the densitometric analysis using Quantity One Software (GS 710 Calibrated Imaging Densitometer, BioRad). The experiment was repeated five times with similar results.

Table 3. Forward and reverse primers of investigated genes used in the quantitative real-time and reverse-transcriptase PCR

Gene	Forward primer	Reverse primer
$GABA_A$ receptor, α_1 subunit	5' – gac gga cat ctt tgt cac cag t – 3'	5' – cca gat ttt act ggc cat cag g – 3'
$GABA_A$ receptor, α_2 subunit	5' – cac aga gga tgg cac tct gct – 3'	5' – ttc agc tct cac ggt caa cct – 3'
$GABA_{B1A}$ receptor	5' – gtc gtg gtt tcc ttt cct tca t – 3'	5' – aga gac acc aca gtg tga aag g – 3'
$GABA_{B1B}$ receptor	5' – ccg ttt cct gtc tca gaa act c – 3'	5' – act ggc ttc tcc cta tgt ggt a – 3'
$GABA_{B2}$ receptor	5' – tat gac acc gag tgt gac aat g – 3'	5' – tct tct tat ccg caa gaa cag g – 3'
GAD-65	5' – aca agc tat gcg ctc tgc tct a – 3'	5' – ctt gca gaa atg cga gag tg – 3'
GAD-67	5' – ctg gaa ccc tca caa gat gat g – 3'	5' – gcc aga act tga aga tgt cca c – 3'
GABA-T	5' – cag aga ggt ttc tcc aaa gag g – 3'	5' – act cct cca ggg gat att tca g – 3'
Pre-pro-CCK	5' – tgt aga agc tgt gga ccc tat g – 3'	5' – atg tag tcc cgg tca ctt atc c – 3'
CCK_1 receptor	5' – gtg ctg att cga aac aag agg – 3'	5' – aga tgg cta cca ggt tga agg – 3'
CCK_2 receptor	5' – acc tag gac tcc act ttg atg g – 3'	5' – gtg tgg tta gcg ttg tca tct c – 3'
LsAMP	5' – gac gac aag ctt ccc tca aa – 3'	5' – aaa gga cta ggc tgg cat tg – 3'
Plectin	5' – cag atg aac gag acc gtg tg – 3'	5' – tct gaa cgt ttt gca gct tg – 3'
Heat stable antigen CD24	5' – atc ccc gat gtg ttg ttt tg – 3'	5' – atg ctt tgc cgt tgt gac tt – 3'
5E5 antigen	5' – ctc cca cca aac aga tga cc – 3'	5' – att cag act cgg aca ctg agg – 3'
rELO1 fatty acid elongase 1	5' – gct caa cat ctg gtg gtt tg – 3'	5' – tgg tct gga tga ttg tca gc – 3'
ERM-binding phosphoprotein 50	5' – aga aag gcc cca atg gtt at – 3'	5' – ctt cat cac ctc cag cct tg – 3'
Spindlin	5' – gca ctt gaa gtc ctc cct ga – 3'	5' – caa gac agg gtc ttt ctc ata gg – 3'

Table 3. Continuation

Gene	Forward primer	Reverse primer
Extracellular signal-related kinase (ERK2)	5' – cta cgg cat ggt ttg ttc tg – 3'	5' – cat ctg ctc aat ggt tgg tg – 3'
Calmodulin (RCM3)	5' – aag tag atg ccg acg gta atg – 3'	5' – ctc caa ggt ttg tca tca cg – 3'
Ribosomal protein S7	5' – tgc ggg aac tca aca tca c – 3'	5' – ctt ggg cag aat cct cct ct – 3'
Myelin-associated glycoprotein (MAG)	5' – gag gac ggc atc tat gct tg – 3'	5' – tgc ttc tcc ttg aag atg gtg – 3'
Cyclophilin A	5' – ggt caa ccc cac cgt gtt ctt cga cat – 3'	5' – gga caa gat gcc agg acc tgt atg ct – 3'
HPRT	5' – gcc cca aaa tgg tta agg tt – 3'	5' – tcc act ttc gct gat gac ac – 3'

4.4. Quantitative real-time PCR

Quantitative real-time PCR (qPCR, ABI Prism 7000 SDS, Applied Biosystems) was applied to quantify the differential expression of the LsAMP, GABA- and CCK-related genes, and some genes revealed by cDNA RDA. After the first strand cDNA synthesis qPCR reactions were performed in a final volume of 20 µl, using 0.5–50 ng of the first strand cDNA as template. In the experiments SYBR Green I (SYBR Green I qPCR™ Core Kit, Eurogentec) based real-time PCR was used. The primer sequences used in reactions are presented in Table 3. Prior to the quantification, primers were tested by common PCR followed by agarose gel electrophoresis, where single distinct bands were observed. PCR was set up using the following steps: 50°C for 2 min; 95°C for 10 min; 95°C for 15 s and 60°C for 1 min, repeated for 45 cycles. Melting curve analyses were performed throughout the quantification to check for possible presence of primer dimers. Rat hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used 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 (Jiralerspong, Patel, 1996; de Kok et al., 2005). Relative gene expression levels were calculated by using comparative $\Delta\Delta C_T$ method (Livak, Schmittgen, 2001). The mRNA level in the home-cage control group was defined as 1 and the change of mRNA amounts in other groups was shown as fold-increase. Reactions were run on a 96-plate in 4 parallel samples for each gene of interest and house-keeper gene, and every reaction for each gene of interest was repeated 4 times on different plates to minimise the fluctuations coming from the qPCR reactions.

4.5. cDNA Representational Difference Analysis (cDNA-RDA)

cDNA-RDA was performed to find the genes differentially expressed in the PAG of Wistar rats having different exploratory activity in the elevated plus-maze. After cDNA synthesis using SuperScript II Choice System for cDNA Synthesis (Gibco BRL, Paisley, UK), the RDA was performed according to the protocol of Hubank and Schatz with some modifications (Hubank, Schatz, 1999; Pastorian et al., 2000). Synthesised double-stranded cDNA was digested with DpnII to obtain linker-compatible ends. Then restricted cDNA was ligated to the R-12/24-mer linkers (Table 4) and amplified by PCR, using R-24-mer, as the primer. The PCR product was cut by DpnII to create a "driver". A small amount of the "driver" DNA was purified from the cut R-linkers and ligated to the J-12/24-mers (Table 4) to create a "tester".

Table 4. Oligo-12/24-mers used in cDNA RDA

Oligomer	Sequence
R-Bgl-12-oligomer	5'– gat ctg cgg tga –3'
R-Bgl-24-oligomer	5'– agc act ctc cag cct ctc acc gca –3'
J-Bgl-12-oligomer	5'– gat ctg ttc atg –3'
J-Bgl-24-oligomer	5'– acc gac gtc gac tat cca tga aca –3'
N-Bgl-12-oligomer	5'– gat ctt ccc tcg –3'
N-Bgl-24-oligomer	5'– agg caa ctg tgc tat ccg agg gaa –3'

Then the "driver" from one DNA population and the "tester" from the opposite DNA population were mixed in a ratio of 1:100 and hybridised following amplification by PCR using J-24-mer as the primer to result the exponential enrichment of the "tester":"tester" hybrids in the first difference product (DP1). Then the DP1 was cut by DpnII, purified, ligated to the N-12/24-mers (Table 4), and the second round of hybridisation with the same "driver" was performed. Altogether, three rounds of hybridisation were performed with hybridisation ratios of 1:100 (for the first difference product (DP1)), 1:800 (for the second difference product (DP2)), and 1:40 000 (for the third difference product (DP3)). All procedures were performed twice whereas in the first part the "driver" was DNA from animals with high exploratory activity and the "tester" was DNA from animals with low exploratory activity; in the second part the opposite scheme was used. The DP3 was digested finally with DpnII to obtain Bam HI compatible ends. Subtracted library was fractionated by agarose gel electrophoresis. Different bands were cut in 1.5% low-melting agarose gels and cDNA was eluted by QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). Fractions were cloned into the BamHI site of vector pGEM-7Zf. The plasmids were purified from 2-ml cultures of the white colonies by alkaline lysis protocol. Three hundred nanograms of each plasmid DNA was used to perform cycle sequencing on ABI310 sequencer (Applied Biosystems, Foster City, CA,

USA) with M13 forward primer according to the manufacturer's instructions. Sequence alignments were performed with the UK-HGMP software NIX (http://www.hgmp.mrc.ac.uk/).

4.6. Dot-blot analysis of clones

Dot-blot analysis was applied with the aim to confirm the results of the cDNA RDA performed on cDNA from the PAG. After sequencing and alignment, only clones containing different inserts were used for dot-blot analyses. Plasmid DNA with chosen inserts was amplified by PCR using M13 forward and reverse primers, and about 2.5 ml of PCR mix was denatured in 0.4 M NaOH and 10 mM EDTA (10 min at 100°C). Thereafter DNA was dotted onto a Hybond N+ nylon membrane, followed by UV cross-linking. DIG High Prime DNA Labelling and Detection Starter Kit I (Roche, Mannheim, Germany) was used for this experiment. An equal amount of synthesised and DpnII digested cDNA was used for the synthesis of DIG-labelled probes (digoxigenin(DIG)-labelled sondes). Each set of clones included genes from different animal groups was denatured (as described above) and dotted onto different identical membranes. Each membrane was hybridised with a different labelled probe, in a separate hybridisation tube and in identical conditions. Thus, two hybridisations of membranes containing the same set of DNA clones with different DIG-labelled probe were performed: one membrane was hybridised with starting cDNA from the group with low exploratory activity and another was hybridised with starting cDNA from the group with high exploratory activity. Dot-blots were scanned and analysed with Quantity One Software (GS 710 Calibrated Imaging Densitometer, Bio-Rad, München, Germany). Comparisons of identical dots (same clones) but hybridised with different probes were performed. To correct for the grey value, a small area in between dots was measured as a local reference. Each grey value of the measured areas was corrected for this local reference. The resulting optical densities of dots were divided by optical density of the house-keeper gene (cyclophilin A) to eliminate possible fluctuations between membranes. Dot-blot analysis was performed three times and only dots with similar results were taken into account. Results were expressed as fold changes of respective dots.

5. Radioligand binding studies

In the radioligand binding studies, mice that had not been exposed to behavioural testing, were used. After decapitation, the brains were rapidly dissected on ice, and cooled down in liquid nitrogen. The cerebral cortex (including frontal and parietal cortices), hippocampus, and cerebellum were dissected and

stored in liquid nitrogen at –80°C until sample preparation (Franklin, Paxinos, 1997). The brain structures from six mice were pooled. The radioligand binding studies were performed according to the method of Kõks and colleagues (Kõks et al., 1997). [^{3}H]-flunitrazepam (specific activity 96 Ci/mmol, Amersham Radiochemicals) was used for the labelling of benzodiazepine receptors. The parameters of benzodiazepine receptors were determined in the presence of 0.5–16 nM [^{3}H]-flunitrazepam at 4° C for 60 min. Diazepam (Sigma, 10 mM) was added to determine non-specific binding at benzodiazepine receptors. The brain tissue was homogenised in 20 volumes of ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) using a Potter-S glass-teflon homogeniser (1,000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48,000 x g for 20 min) and resuspension. After the last centrifugation, crude brain membranes were suspended in the incubation buffer: 50 mM Tris-HCl (pH 7.4 at 4°C). The protein content was measured according to the method of Bradford (1976). The saturation curves of [^{3}H]-flunitrazepam binding were analysed using GraphPad Prism (Version 3.00) for Windows software. The experiments were repeated four times.

6. Statistical analysis

Results of the behavioural, radioligand binding and gene expression studies are expressed as mean values ± SEM. The data of behavioural studies were analysed by means of one-way analysis of variance (ANOVA) for rats and by means of two-way analysis of variance (ANOVA) for mice behavioral experiments. *Post hoc* comparisons were performed using Tukey HSD test. The results of the plus-maze exploration in rats were also analysed by means of Pearson r correlation coefficient (simple linear correlation test) in order to establish correlations between the different measures of the plus-maze. The data of obtained from the gene expression studies in rats were analysed by means of one-way analysis of variance (ANOVA). *Post hoc* comparisons were performed using Tukey HSD and Scheffe test. For radioligand binding studies, the Student's t-test was applied. The data were analysed using *Statistica 5.0 for Windows* software.

V. RESULTS

1. Behavioural experiments

1.1. Exploratory behaviour in the elevated plus-maze

1.1.1. Behavioural selection of Wistar rats according to their exploratory behaviour

According to the exploratory activity in the elevated plus-maze, three groups of rats were selected from the population of 43 animals (Table 5). The initial selection was performed by taking into account the frequency of open and closed arm entries performed by rats. Animals making one or two open arm visits were selected into the high exploratory activity group (9 rats). Rats, making only one closed arm entry, were selected into the low exploratory group (10 rats). Animals, not making open arm entries, but performing two and more closed arm entries (mean value 4.0 ± 0.5) formed the intermediate group (24 rats).

Table 5. The selection of rats according to their exploratory behavior in the elevated plus-maze (mean ± SEM).

Behavioural parameters	**Low exploratory activity, n=10**	**Intermediate group, n=24**	**High exploratory activity, n=9**
Latency to enter central square (s)	77 ± 27	51± 8	29 ± 6
Number of attempts to enter from closed arm into central square	7.9 ± 1.0	6.2 ± 0.7	4.0 ± 0.8*
Number of line crossings	4 ± 1	14 ± 2*	24 ± 2*,**
Time spent on open arm (s)	0 ± 0	0 ± 0	9 ± 2*,**
Time spent in exploring of open parts (s)	26 ± 6	84 ± 7*	142 ± 6*,**
Number of open arm entries	0 ± 0	0 ± 0	1.2 ± 0.2*,**
Number of closed arm entries	1 ± 0	4 ± 0.5*	6.8 ± 0.7*,**
Ratio between open and total arm entries (x 100)	0 ± 0	0 ± 0	15 ± 2*,**
Number of head-dipping	1.1 ± 0.5	4.9 ± 0.7*	9.9 ± 0.9*,**
Number of stretched attend postures	0.9 ± 0.3	0.8 ± 0.2	0.4 ± 0.2

* $P < 0.05$, high exploratory activity and intermediate group in comparison with rats displaying low exploratory activity (Tukey HSD test after significant one-way ANOVA);
** $P < 0.05$, high exploratory activity rats if compared to the intermediate group.

The initial selection criteria were supported by time spent in exploring the open parts (open arms and central square). Indeed, according to this parameter, there was a 6-fold difference between the high (142 ± 5 s) and low (26 ± 6 s) exploratory activity groups. The intermediate group (84 ± 7 s) remained exactly between these two groups and this parameter was almost equal to the corresponding value for all selected animals (81 ± 6 s).

Statistical analysis confirmed significant differences between the groups (Table 5). Rats with high exploratory activity spent longer time in exploring the open arms ($F_{2,40} = 32.4$, $P < 0.01$) and the central square ($F_{2,40} = 36.8$, $P < 0.01$), visited the open ($F_{2,40} = 36.7$, $P < 0.01$) and closed ($F_{2,40} = 20.1$, $P < 0.01$) arms more frequently and performed more head-dippings ($F_{2,40} = 21.8$, $P < 0.01$) and line crossings ($F_{2,40} = 26.7$, $P < 0.01$) compared to the other groups. These animals had also a significantly higher ratio between open and total arm entries ($F_{2,40} = 226.9$, $P < 0.01$). The intermediate group spent more time on the central square, performed more closed arm entries, head-dippings and line crossings compared to the group with low exploratory activity ($P < 0.05$, Tukey HSD test). Rats with low exploratory activity performed more attempts ($F_{2,40} = 3.52$, $P < 0.05$) to enter the central square from the closed arm compared to the high exploratory activity group.

There were significant correlations between several parameters of the plus-maze exploration. The number of open arm entries was highly correlated with the open arm exploration time ($r = 0.86$, $P < 0.0001$) and the ratio between open and closed arm entries ($r = 0.93$, $P < 0.0001$), a classical measure of anxiety in rodents (Pellow et al., 1985). The number of open arm entries also demonstrated a significant correlation with the time spent in the exploration of the central square ($r = 0.60$, $P < 0.0001$), line crossings ($r = 0.65$, $P < 0.0001$), number of closed arm entries ($r = 0.59$, $P < 0.0001$) and number of head dippings ($r = 0.59$, $P < 0.0001$).

1.1.2. Comparison of exploratory behaviour of wild-type and CCK_2 receptor deficient mice

Comparison of exploratory activity of the CCK_2 receptor deficient mice relative to their wild-type (+/+) littermates established several differences. Homozygous (–/–) mice visited the open arms more frequently than wildtype (+/+) mice ($F_{2,85} = 4.50$, $P < 0.05$) (Figure 6). Also, homozygous (–/–) mice spent a significantly longer time on the open arms and central square of a plus-maze (open arms: $F_{2,85} = 5.30$, $P < 0.01$; central square: $F_{2,85} = 3.77$, $P < 0.05$). However, changes in the other parameters of the plus-maze exploration did not reach a statistically significant level: the number of line crossings ($F_{2,85} = 2.24$, $P = 0.11$), and ratio between the open and total entries ($F_{2,85} = 2.69$, $P = 0.07$). Frequency of the closed arm entries in homozygous (–/–) mice did not differ from the respective value of their wild-type (+/+) littermates, but was different from the number of

closed arm entries of heterozygous (+/–) animals ($F_{2,85}$ = 4.66, P < 0.05) (Figure 6).

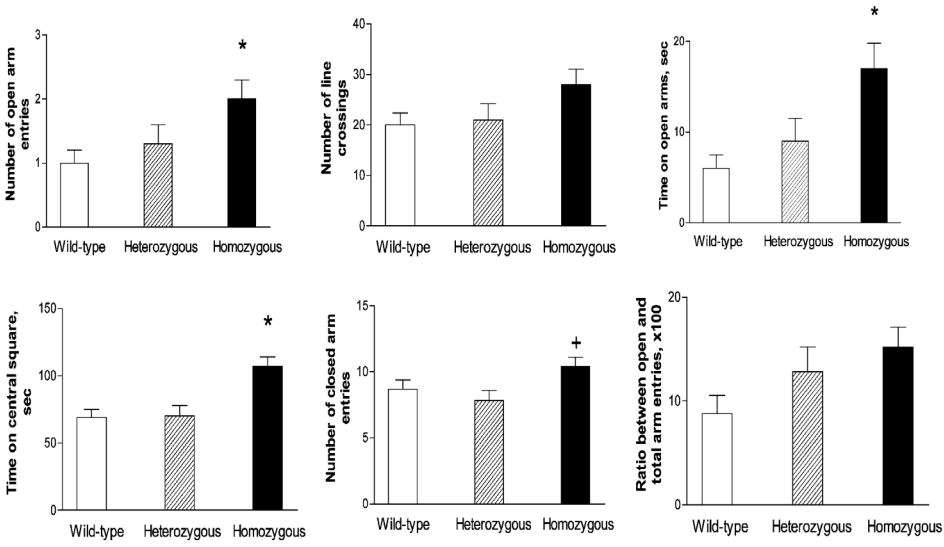

Figure 6. The exploratory activity of CCK_2 receptor deficient mice in the elevated plus-maze.

The number of animals in each group was between 28 and 30. White bars – wild-type; striped bars – heterozygous; black bars – homozygous.
* P < 0.05, compared to wild-type mice, Tukey HSD test after significant one-way ANOVA;
+ P < 0.05, compared to heterozygous mice.

1.2. Effect of diazepam on the behaviour of wild-type and CCK_2 receptor deficient mice

The effect of diazepam in the plus-maze was studied in a separate group of mice. It should be noted that the baseline exploratory activity of heterozygous (+/–) mice was higher in this experiment compared to the above-described experiment. However, differences in the exploratory activity of wild-type (+/+) and homozygous (–/–) mice remained at the same level in these two separate studies (Figure 6, 7). The administration of diazepam (0.5–3 mg/kg) caused a dose-dependent anxiolytic-like effect in wild-type (+/+) mice. Diazepam increased the number of open arm entries, time spent on the open arms, and ratio between the open and total arm entries (Figure 7). Two-way ANOVA was applied in order to compare the behavioural effects of diazepam in wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice (Figure 7). The number of open arm entries was differently affected by diazepam in wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice (genotype $F_{2,391}$ = 1.16, P = 0.32,

treatment $F_{3,391} = 11.84$, $P < 0.01$; genotype × treatment $F_{6,391} = 2.19$, $P < 0.05$). The following *post hoc* analysis (Tukey HSD test) established that the lowest dose of diazepam (0.5 mg/kg) induced a statistically significant increase ($P < 0.05$) of open arm entries in wild-type (+/+) mice. In homozygous (–/–) mice, a significant change was found after the administration of diazepam at a dose of 1 mg/kg ($P < 0.01$), whereas in heterozygous (+/–) animals the effect of a benzodiazepine agonist was not significant ($P = 0.36$). The highest dose (3 mg/kg) caused an increase in open arm entries in wild-type (+/+) mice ($P < 0.05$), but not in genetically modified animals. Frequency of open arm visits in heterozygous (+/–) and homozygous (–/–) mice after the administration of diazepam (3 mg/kg) did not differ from that of vehicle-treated animals (Figure 7).

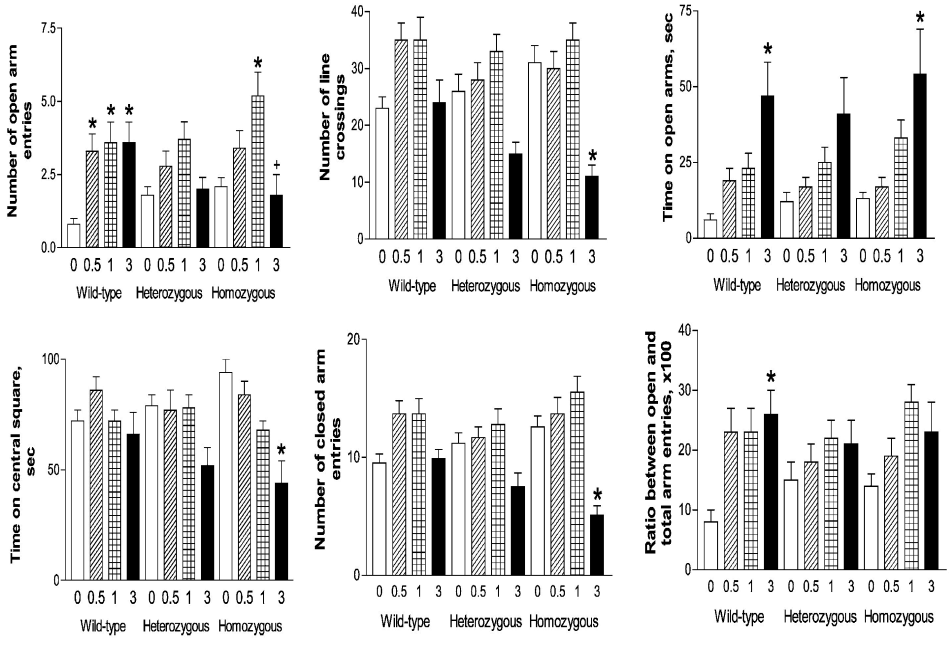

Figure 7 The effect of diazepam (0.5–3 mg/kg) on the exploratory activity of CCK_2 receptor deficient mice in the elevated plus-maze.

The number of animals in each group was between 30 and 37. White bars – vehicle; striped bars – diazepam 0.5 mg/kg; hatched bars – diazepam 1 mg/kg; black bars – diazepam 3 mg/kg.

* $P < 0.05$, compared to the respective vehicle-treated group, Tukey HSD test after significant two-way ANOVA;

\+ $P < 0.01$, compared to the effect of diazepam (1 mg/kg) in homozygous mice.

Subsequent *post hoc* analysis established a statistically significant difference ($P < 0.01$) between the actions of two doses (1 and 3 mg/kg) of diazepam in homozygous (–/–) mice. Two-way ANOVA also demonstrated that diazepam affected differently the number of line crossings in these groups of mice (genotype $F_{2,391} = 1.96$, $P = 0.14$, treatment $F_{3,391} = 21.06$, $P < 0.01$; genotype × treatment $F_{6,391} = 2.22$, $P < 0.05$). Diazepam at lower doses (0.5 and 1 mg/kg) tended to increase the number of line crossings in wild-type (+/+) mice, but this effect was not statistically significant (Figure 7). The highest dose (3 mg/kg) of diazepam reduced this behavioural measure in heterozygous (+/–) and homozygous (–/–) mice, but only in homozygous (–/–) animals was it significant (Tukey HSD test: $P = 0.21$ for heterozygous (+/–) and $P < 0.01$ for homozygous (–/–) animals). The statistical analysis did not reveal any differences between the genotypes if time spent on the open arms was studied (genotype $F_{2,391} = 0.85$, $P = 0.43$, treatment $F_{3,391} = 15.31$, $P < 0.01$; genotype × treatment $F_{6,391} = 0.32$, $P = 0.92$). Nevertheless, the *post hoc* analysis demonstrated that the action of diazepam (3 mg/kg) was statistically significant in wild-type (+/+) ($P < 0.01$) and homozygous (–/–) ($P < 0.01$) animals, but not in heterozygous (+/–) ($P = 0.14$) mice (Figure 7). Diazepam (3 mg/kg) did not change time spent on the central square and the number of closed arm entries in the wild-type (+/+) mice if compared to vehicle-treated animals. However, diazepam significantly reduced these parameters of exploratory behaviour in genetically modified mice (time spent on the central square: genotype $F_{2,391} = 0.18$, $P = 0.84$, treatment $F_{3,391} = 11.57$, $P < 0.01$; genotype × treatment $F_{6,391} = 2.22$, $P < 0.05$, number of closed arm entries: genotype $F_{2,391} = 0{,}64$, $P = 0.53$, treatment $F_{3,391} = 26.24$, $P < 0.01$; genotype × treatment $F_{6,391} = 2.62$, $P < 0.05$). Again, *post hoc* analysis established that these effects of diazepam were significant in the homozygous (–/–) mice ($P < 0.01$ for time spent on the central square and $P < 0.01$ for the number of closed arm entries), but not in heterozygous (+/–) animals (respective values: $P = 0.15$ and $P = 0.38$). The application of two-way ANOVA did not demonstrate any difference between the genotypes if the action of diazepam (0.5–3 mg/kg) was studied on the ratio between the open and total arm entries (genotype $F_{2,391} = 0.61$, $P = 0.54$, treatment $F_{3,391} = 9.04$, $P < 0.01$; genotype × treatment $F_{6,391} = 0.86$, $P = 0.52$). The following *post hoc* analysis demonstrated that only in wild-type mice (+/+), but not in heterozygous (+/–) and homozygous (–/–) animals, did diazepam (3 mg/kg) induce a statistically significant ($P < 0.05$) increase in this parameter of plus-maze exploration (Figure 7).

The performance of wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice in the rotarod test did not differ on days 1, 2 and 3 (Figure 8). Treatment with diazepam (0.5 and 3 mg/kg) on the fourth day caused a dose-dependent impairment of motor coordination in all genotypes (genotype $F_{2,77} = 4.94$, $P < 0.01$; treatment $F_{2,77} = 38.04$, $P < 0.01$; genotype × treatment $F_{4,77} = 0.99$, $P > 0.25$) (Figure 9). However, further *post hoc* analysis established that diazepam (0.5 and 3 mg/kg) caused a significantly greater impairment (Tukey

HSD test: $P < 0.05$) of motor coordination in homozygous (–/–) mice compared to their wild-type (+/+) littermates (Figure 9).

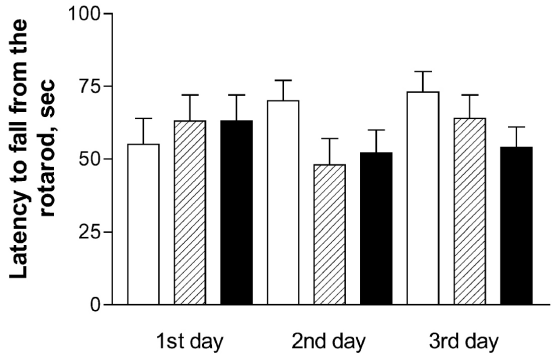

Figure 8. The performance of CCK_2 receptor deficient mice in the rotarod test.

The number of animals in each group was as follows: 30 wild-type, 28 heterozygous, and 28 homozygous animals. The study was repeated on 3 consecutive days. White bars – wild-type; striped bars – heterozygous; black bars – homozygous.

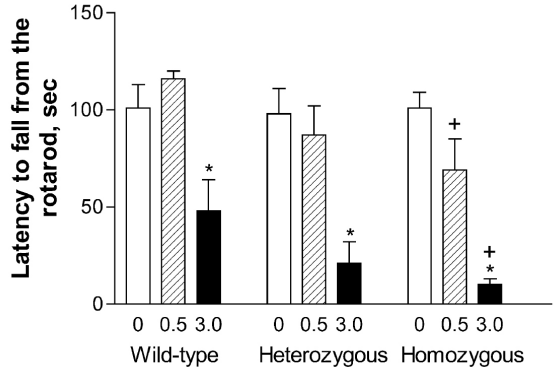

Figure 9. The effect of diazepam (0.5 and 3 mg/kg) on the performance of CCK_2 receptor deficient mice in the rotarod test.

The number of animals in each group was between 8 and 11. White bars – vehicle; striped bars – diazepam 0.5 mg/kg; black bars – diazepam 3 mg/kg.

* $P < 0.05$, compared to the respective vehicle-treated group, Tukey HSD test after significant one-way ANOVA;

\+ $P < 0.05$, compared to diazepam-treated wild-type mice.

2. Radioligand [^{3}H]-flunitrazepam binding studies in wild-type and CCK_2 receptor deficient mice

The density of [^{3}H]-flunitrazepam binding sites (B_{max}) in the cerebellum was increased in homozygous (–/–) mice compared to their wild-type (+/+) littermates (Figure 10). No such difference was established between wild-type (+/+) and homozygous (–/–) mice in the cerebral cortex and hippocampus. The density of benzodiazepine binding sites tended to be higher in the cerebral cortex and hippocampus of heterozygous (+/–) mice relative to wild-type (+/+) animals. However, these differences were not statistically significant. The affinity of benzodiazepine binding sites (K_d) in the cerebral cortex, hippocampus and cerebellum did not differ in wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice (Figure 10).

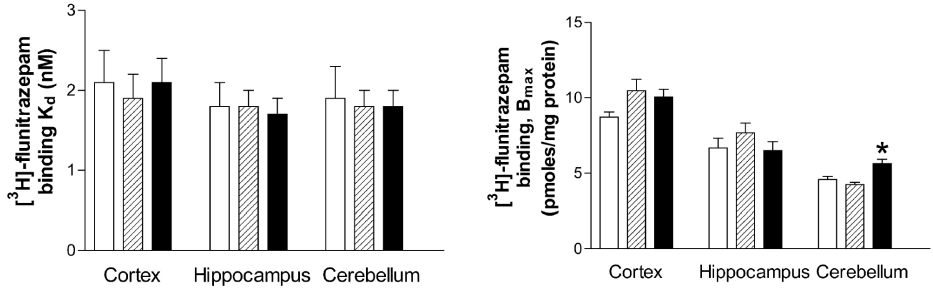

Figure 10. The parameters of [^{3}H]-flunitrazepam binding in the brain structures of CCK_2 receptor deficient mice.

The number of animals in each group was 24, the brains of six mice were pooled, and the mean is a result of four experiments. White bars – wild-type; striped bars – heterozygous; black bars – homozygous.

* $P < 0.05$, compared with wild-type mice, Student's t-test.

3. Quantitative gene expression measurement

3.1. Changes in LsAMP gene expression in the PAG revealed by RT-PCR

Using reverse-transcriptase PCR followed by densitometric analysis of gel electrophoresis, the relative optical densities of LsAMP bands compared to respective cyclophilin A bands were measured and calculated (Figure 11). The performed statistical analysis revealed a 1.6-fold higher expression of LsAMP transcripts in animals with low exploratory activity in comparison to animals with high activity (Figure 11). The rats with high exploratory activity also had a 1.4-fold lower expression of LSAMP gene compared to control animals.

However, the control rats and the "intermediate" group did not differ significantly by their LsAMP gene expression level (Paper II).

Taking into the account this fact, and that the main aim of the study was to reveal gene expression differences between groups with high and low exploratory activity, the "intermediate" group was not included into later gene expression studies.

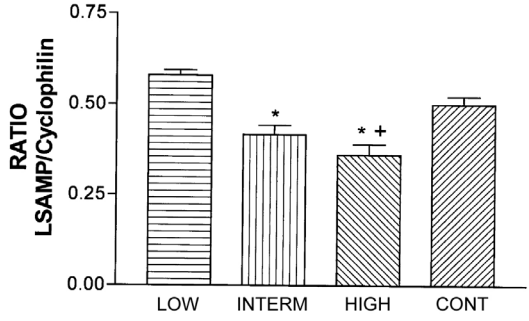

Figure 11. Statistical analysis of differences in LsAMP/cyclophilin A ratio (mean ± SEM).

* $P < 0.01$, rats with low exploratory activity compared to rats with high exploratory activity and "intermediate" groups (Scheffe test after significant one-way ANOVA ($F_{3,16} = 16.85$, $P < 0.01$));
+ $P < 0.01$, animals with high exploratory activity compared to control animals. LOW – animals with low exploratory activity, INTERM – "intermediate" animals, HIGH – animals with high exploratory activity, CONT – control group.

3.2. Changes of gene expression in the frontal cortex, amygdala and PAG revealed by real-time qPCR

After revealing changes in the LsAMP gene expression in the PAG of the animals displaying different exploratory activity in the elevated plus-maze using reverse-transcriptase PCR as was described above, we decided to confirm our finding by more sensitive real-time qPCR method. We compared the expression level of the LsAMP gene in rats with different exploratory activity in the elevated plus-maze not only in the PAG, but also in other brain structures like frontal cortex and amygdala. Moreover, the expression of GABA- and CCK-ergic genes was investigated (Paper III).

3.2.1. Expression of LsAMP gene

The expression of the LsAMP gene was increased in all three brain structures, but these changes were statistically significant only in the amygdala (one-way ANOVA, $F_{2,9} = 96.4$, $P < 0.001$) and PAG ($F_{2,9} = 5.47$, $P < 0.05$) (Figure 12). In the amygdala of low exploratory rats, the expression of the LsAMP gene was 1.6-fold higher compared to the home-cage control group ($P < 0.05$) and 2.4-fold higher compared to high exploratory activity rats ($P < 0.05$). In the PAG, only the difference (2.1-fold) between low exploratory activity and home-cage control rats was statistically significant.

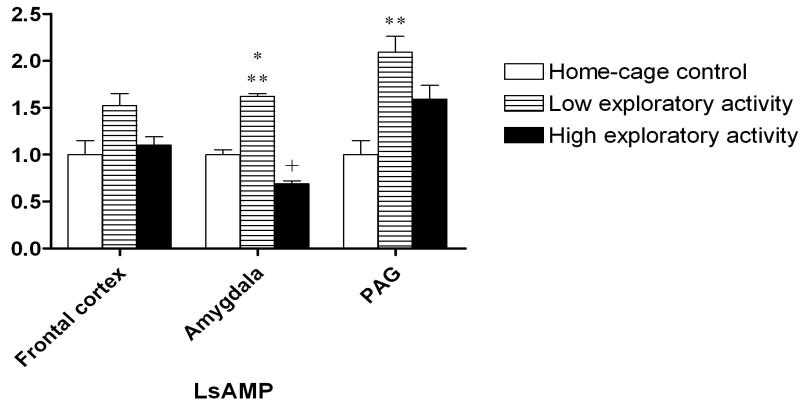

Figure 12. The expression patterns of the LsAMP gene in low and high exploratory activity rats.

* $P < 0.05$, low exploratory activity animals in comparison with high exploratory activity group (Tukey HSD test after significant one-way ANOVA);
** $P < 0.05$, low exploratory activity animals compared to the home-cage control group;
\+ $P < 0.05$, high exploratory activity rats in comparison with the home-cage control group.

3.2.2. Expression of GABA-related genes

The analysis of GABA-related gene expression patterns revealed distinct changes in low and high exploratory activity rats in all three brain structures. In the frontal cortex, the reduction of GABA-related gene expression dominated in both groups compared to home-cage control animals (Figure 13). In the PAG, both the elevation and decline of gene expression were established in low and high exploratory activity rats compared to home-cage control animals (Figure 13). By contrast, in the amygdala, a strong increase in the expression of GABA-related genes was evident in low exploratory activity animals compared to high exploratory activity and home-cage control groups (Figure 13).

In the frontal cortex, one-way ANOVA established that the changes of the following genes were significant: $GABA_A$ receptor α_1 subunit ($F_{2,9} = 6.49$, $P < 0.05$) and α_2 subunit ($F_{2,9} = 20.5$, $P < 0.001$), $GABA_{B1a}$ receptor ($F_{2,9} = 11.9$, $P < 0.01$), $GABA_{B1b}$ receptor ($F_{2,9} = 17.2$, $P < 0.001$), $GABA_{B2}$ receptor ($F_{2,9} = 7.10$, $P < 0.05$), glutamate decarboxylase-67 (GAD-67) ($F_{2,9} = 6.43$, $P < 0.05$) and GABA-transaminase ($F_{2,9} = 26.0$, $P < 0.001$). *Post hoc* analysis by means of Tukey HSD test demonstrated that the expression of $GABA_A$ receptor α_2 subunit, $GABA_{B2}$ receptor, GAD-67 and GABA-transaminase genes was significantly higher in low exploratory activity rats compared to high exploratory activity animals. The comparison of low exploratory activity rats with home-cage control group demonstrated that the expression of $GABA_{B1a}$ and $GABA_{B1b}$ receptor genes was significantly decreased in animals with reduced exploratory behavior. $GABA_A$ receptor α_1 and α_2 subunits, $GABA_{B1b}$ receptor, $GABA_{B2}$ receptor, and GABA-transaminase gene expression were evidently reduced in high exploratory activity rats compared to the control group not exposed to the plus-maze (Figure 13).

In the amygdala, one-way ANOVA established that the changes in the expression of all studied GABA-related genes were statistically significant: $GABA_A$ receptor α_1 subunit ($F_{2,9} = 455.2$ $P < 0.0001$) and α_2 subunit ($F_{2,9} = 59.4$, $P < 0.001$), $GABA_{B1a}$ receptor ($F_{2,9} = 190.4$, $P < 0.0001$), $GABA_{B1b}$ receptor ($F_{2,9} = 560.5$, $P < 0.0001$), $GABA_{B2}$ receptor ($F_{2,9} = 136.3$, $P < 0.0001$), GAD-65 ($F_{2,9} = 174.0$, $P < 0.0001$), GAD-67 ($F_{2,9} = 181.9$, $P < 0.0001$) and GABA-transaminase ($F_{2,9} = 119.0$, $P < 0.0001$). The expression of all GABA-related genes was 2-fold to 5-fold higher in low exploratory activity rats compared to high exploratory activity animals. The only exception was the $GABA_{B1a}$ receptor gene which mRNA levels were similar in low and high exploratory activity animals. The expression of GABA-related genes was also significantly higher in low exploratory activity rats compared with rats not exposed to the elevated plus-maze. Again, the exception was the $GABA_{B1a}$ receptor gene the level of which was almost 2-fold reduced in low exploratory activity rats. The expression of the same gene was also significantly reduced in high exploratory activity rats compared to the home-cage control group. By contrast, differences in the expression of other GABA-related genes did not reach a statistically significant level if animals displaying high exploratory activity were compared to rats not exposed to the plus-maze (Figure 13).

In the PAG, one-way ANOVA demonstrated that changes in the expression of the following GABA-related genes were significant: $GABA_A$ receptor α_1 subunit ($F_{2,9} = 133.0$, $P < 0.0001$) and α_2 subunit ($F_{2,9} = 685.4$, $P < 0.0001$), $GABA_{B1a}$ receptor ($F_{2,9} = 9.36$, $P < 0.01$), $GABA_{B1b}$ receptor ($F_{2,9} = 78.4$, $P < 0.0001$), $GABA_{B2}$ receptor ($F_{2,9} = 104.3$, $P < 0.0001$), GAD-67 ($F_{2,9} = 33.8$, $P < 0.001$) and GABA-transaminase ($F_{2,9} = 21.7$, $P < 0.001$). *Post hoc* analysis revealed that the expression of $GABA_A$ receptor α_1 subunit, $GABA_A$ receptor α_2

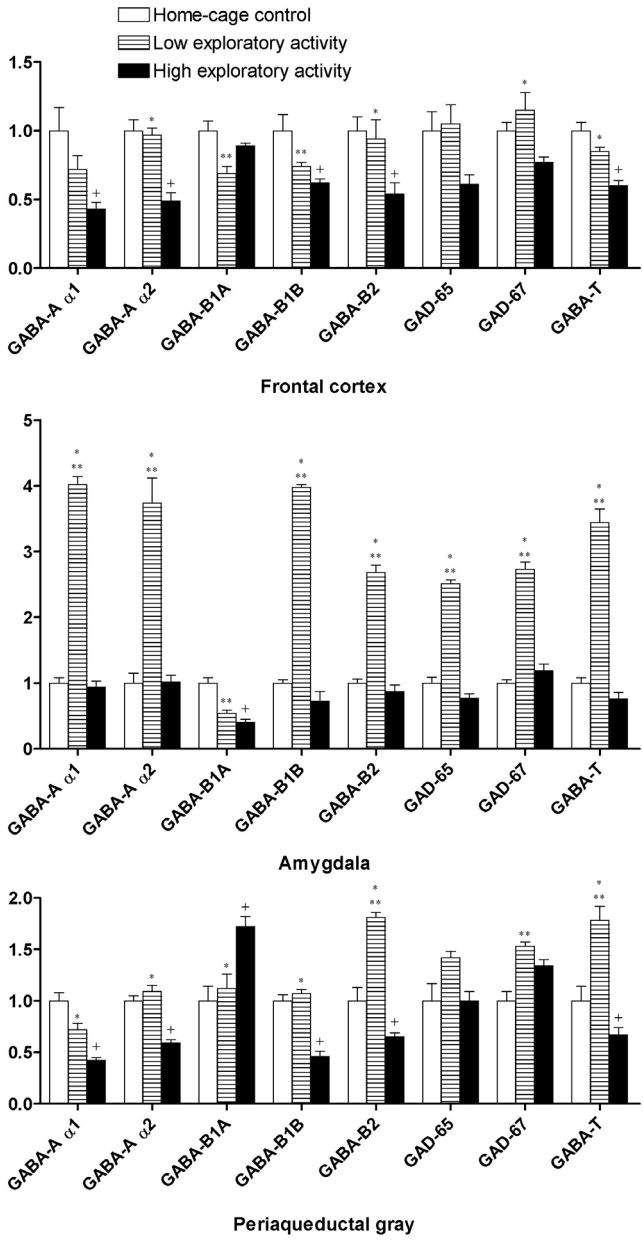

Figure 13 Differences in the expression of GABA-related genes in the frontal cortex, amygdala and PAG (mean values ± SEM).

* P < 0.05, low exploratory activity animals in comparison with the high exploratory activity group (Tukey HSD test after significant one-way ANOVA);
** P < 0.05, low exploratory activity animals compared to the home-cage control group;
+ P < 0.05, high exploratory activity rats in comparison with the home-cage control group.

subunit, $GABA_{B1b}$ receptor, $GABA_{B2}$ receptor and GABA-transaminase genes was significantly higher in low exploratory activity rats compared to high exploratory activity animals. Only the expression of the $GABA_{B1a}$ receptor gene was significantly lower in low exploratory activity rats. The comparison of low exploratory activity rats with the home-cage control group demonstrated that the expression of the $GABA_{B2}$ receptor, GAD-67 and GABA-transaminase genes was significantly higher in animals with decreased exploratory behavior.

As in the case of the frontal cortex, the expression of $GABA_A$ receptor α_1 and α_2 subunits, $GABA_{B1b}$ receptor, $GABA_{B2}$ receptor and GABA-transaminase genes was evidently reduced in high exploratory activity rats compared to the control group not exposed to the plus-maze. Moreover, the expression of $GABA_{B1a}$ receptor gene was significantly increased in high exploratory activity rats compared to the home-cage control group (Figure 13).

3.2.3. Expression of CCK-related genes

As in the case of GABA-related genes, the study of gene expression patterns of CCK-related genes revealed different changes in low and high exploratory activity rats. Again, these changes were dependent on the brain structure studied. The most significant changes between the groups were established, as in the case of GABA-related genes, in the amygdala. One-way ANOVA demonstrated that in the frontal cortex only the difference in the expression of the CCK_1 receptor ($F_{2,9} = 19.6$, $P < 0.01$) gene was statistically significant (Figure 14). In the amygdala and PAG, changes in most of the CCK-related genes were significant: pre-pro-CCK ($F_{2,9} = 42.2$, $P < 0.0001$ [amygdala]; CCK_1 receptor ($F_{2,9} = 161.9$, $P < 0.0001$ [amygdala]; $F_{2,9} = 10.9$, $P < 0.01$ [PAG]) and CCK_2 receptor ($F_{2,9} = 95.2$, $P < 0.0001$ [amygdala]; $F_{2,9} = 17.2$, $P < 0.01$ [PAG]) (Figure 14).

The expression level of the CCK_1 receptor gene in the frontal cortex of high exploratory activity rats was 2-fold lower compared to low exploratory activity animals and the home-cage control group (Figure 14). In the amygdala, the level of pre-pro-CCK mRNA in high and low exploratory activity rats was 2-fold reduced compared to rats not exposed to the plus-maze (Figure 14). The expression of CCK_1 and CCK_2 receptor genes was significantly increased in the amygdala of low exploratory activity rats compared to high exploratory activity animals and the home-cage control group. The comparison of high exploratory activity rats and home-cage control animals established a reduced expression of CCK_1 and CCK_2 receptor genes in the amygdala of animals displaying increased exploratory behavior (Figure 14).

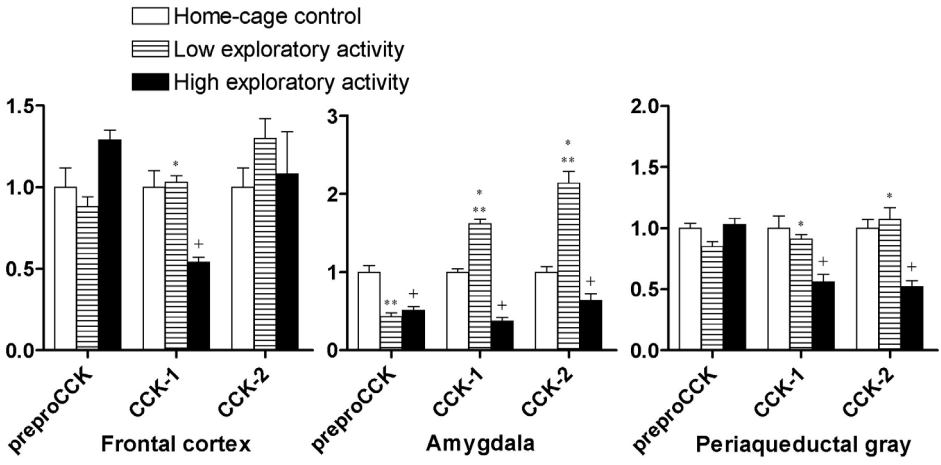

Figure 14. Differences in the expression of CCK-related genes in the frontal cortex, amygdala and PAG (mean values ± SEM).

* $P < 0.05$, low exploratory activity animals in comparison with the high exploratory activity group (Tukey HSD test after significant one-way ANOVA);
** $P < 0.05$, low exploratory activity animals compared to the home-cage control group;
+ $P < 0.05$, high exploratory activity rats in comparison with the home-cage control group.

In the PAG, the expression of the CCK_1 and CCK_2 receptor genes was almost 2-fold decreased in high exploratory activity rats compared to low exploratory activity group and animals not exposed to the plus-maze (Figure 14).

In order to compare the obtained values with the information existing in the literature, we also compared the expression levels of CCK-related genes in these three brain structures. The expression of pre-pro-CCK was 2.6-fold higher in the frontal cortex compared to the amygdala. On the other hand, the level of pre-pro-CCK mRNA in the PAG was more than 10-fold lower than in the amygdala. The expression level of the CCK_1 receptor gene was similar in the amygdala and PAG but nearly 10-fold higher than in the frontal cortex. As in the case of pre-pro-CCK, the expression level of the CCK_2 receptor gene was the highest in the frontal cortex, namely, 1.76-fold higher than in the amygdala and 4-fold higher than in the PAG. The ratio between CCK_2 and CCK_1 receptor mRNA was 181 ± 42 in the frontal cortex, 19 ± 1 in the amygdala and 8 ± 1 in the PAG. Altogether these results are in good accordance with the existing data concerning the levels of pre-pro-CCK, CCK_1 and CCK_2 receptors in different brain structures (Noble et al., 1999).

Table 6. Transcripts over-expressed in the PAG of rats displaying low exploratory activity in the elevated plus-maze.

Genes	Bank Access Number	Fold increase
Heat stable antigen CD24	U49062	4,18
LASP-1	AF242187	3,41
Mus musculus C21orf70 protein (C21orf70)	AF391115	3,35
Mus musculus spindlin (Spin)	U48972	2,27
Guanosine monophosphate reductase	AF090867	2,11
Plectin	X59601	2,11
Mouse kinesin family protein KIF1a	D29951	2,03
Limbic system-associated membrane protein	U31554	1,70
5E5 antigen (sirtuin 2)	D37934	1,70
Thioredoxin-related transmembrane protein	Q9H3N1	1,70
ERM-binding phosphoprotein 50	AF154336	1,70
Cyclic nucleotide phosphodiesterase (CaM-PDE)	M94537	1,69
rELO1 fatty acid elongase 1	AB071985	1,69
Extracellular signal-related kinase (ERK2)	M64300	1,69
Mus musculus ventrhoid transmembrane protein (VRHO gene)	AJ313479	1,65
Hepatic multiple inositol polyphosphate phosphatase (MIPP1)	AF012714	1,65
Mus musculus xCT cystine / glutamate transporter	AB022345	1,36
Mus musculus nischarin	AF315344	1,35
EH-domain containing protein 2 (Ehd2)	AF494093	1,35
M.musculus splicing factor U2AF (65 kD)	X64587	1,27
GABA-B receptor 2 (GABA-BR2)	AF074482	1,27
XLalphas protein (XLas gene) and ALEX protein	X84047	1,24
Vasopressin V1a receptor/V1aR	S83363	1,21
Alpha 1a/d adrenergic receptor	L31771	1,14

Results of dot-blot analysis, corrected semiquantitative differences between groups.

4. Gene expression screening

4.1. cDNA Representational Difference Analysis (cDNA-RDA) followed by dot-blot analysis of clones in the PAG

The cDNA-RDA was aimed to find genes differentially expressed in the PAG of animals with different exploratory activity. Altogether two parallel RDA experiments were performed, where cDNA from groups with “low” and “high” exploratory activity were compared. After cloning of the third difference product we were able to isolate 96 clones for each cDNA population (altogether 192). Clones were sequenced and analysed by means of NIX program. Database search revealed several genes with different functions. There were transcription factors, enzymes, receptors, different regulatory proteins and recently cloned genes with unknown function.

Performed dot-blot analysis with 69 selected clones of interest confirmed our findings and showed at least 50 differentially expressed genes. 24 genes were over-expressed in animals with low exploratory activity in the elevated plus-maze and 26 genes were over-expressed in animals with high exploratory activity. However, the different expression of several genes was not confirmed by dot-blot analysis. This can be explained by possible presence of false-positive results in the RDA-analysis (Pastorian et al., 2000). Semiquantitative analysis of differences between different dots was performed and data were presented as fold changes of respective dots (Table 6, 7). The fold increase/decrease of expression was very various and depended on the concrete gene (from 1.1- to 4.2-fold).

Table 7. Transcripts over-expressed in the PAG of rats displaying high exploratory activity in the elevated plus-maze.

Genes	Bank Access Number	Fold increase
Mus musculus ldlBp (LDLB)	AF109377	2,36
G protein gamma subunit (gamma7 subunit)	L23219	2,36
Ribosomal protein S7	X53377	2,20
Calmodulin (RCM3)	M17069	2,05
Similar to stress-associated endoplasmic reticulum protein 1; ribosome associated membrane protein 4	BC029067	1,97
Mus musculus Rev-ErbA-alpha protein	AF291821	1,77
Alpha-c large chain of the protein complex AP-2 associated with clathrin	X53773	1,77
Thyroid hormone receptor-associated protein complex component TRAP95	Q9Y2X0	1,77
1B236/myelin-associated glycoprotein (MAG)	M22357	1,77
Furosemide-sensitive K-Cl cotransporter (KCC1)	U55815	1,75
ATP-dependent RNA helicase A (Nuclear DNA helicase II)	O70133	1,54
General control of amino acid synthesis protein 5-like 2	Q92830	1,52
ZnBP gene for zinc binding protein	X64053	1,49
rRNA promoter binding protein	U77931	1,47
Aldehyde reductase	D10854	1,43
Mouse stromal cell-derived factor 2-like protein 1 precursor	Q9ESP1	1,38
M. musculus interferon regulatory factor 3 (IRF-3)	P70671	1,33
Mus musculus testican-3 protein	AJ278998	1,33
Neural membrane protein 35	AF044201	1,31
Mus musculus ataxin-1 ubiquitin-like interacting protein	BC017686	1,30
Translin	AF262356	1,18
Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-60)	P11598	1,18
Mus musculus sequence specific single-stranded DNA-binding protein 2	AY037837	1,18
2,3-cyclic nucleotide 3-phosphodiesterase (CNPII)	L16532	1,08
Formin 1 isoforms I/II/III (Limb deformity protein)	Q05860	1,08
Voltage-dependent anion channel 3 (VDAC3)	AF268469	1,07

Results of dot-blot analysis, corrected semiquantitative differenties between groups.

4.2. Confirmation of changes in the expression of several genes revealed by cDNA-RDA in the PAG

Real-time qPCR was performed with the aim to confirm the results of the dot-blot analysis and increase the accuracy of findings. qPCR revealed a significant increase in the expression of 5E5 antigen (1.61-fold, $F_{2,9} = 5.44$, $P < 0.05$), CD24 heat stable antigen (1.75-fold, $F_{2,9} = 29.74$, $P < 0.001$), ERM-binding protein 50 (2.03-fold, $F_{2,9} = 239.42$, $P < 0.001$), plectin (2.11-fold, $F_{2,9} = 30.66$, $P < 0.001$), rELO fatty acid elongase I (1.30-fold, $F_{2,9} = 52.98$, $P < 0.001$), spindlin (2.25-fold, $F_{2,9} = 52.58$, $P < 0.001$) and extracellular signal-related kinase 2 (ERK2) (3.59-fold, $F_{2,9} = 54.79$, $P < 0.001$) gene in animals with low exploratory activity in comparison with high exploratory activity rats (Figure 15, 16). On the other hand, qPCR confirmed the decrease in the expression of calmoduline (2.12-fold, $F_{2,9} = 224.76$, $P < 0.001$), ribosomal subunit S7 (2.02-fold, $F_{2,9} = 277.56$, $P < 0.001$) and myelin-associated glycoprotein (MAG) (1.50-fold, $F_{2,9} = 27.19$, $P < 0.001$) gene in the same behavioural group in comparison with another.

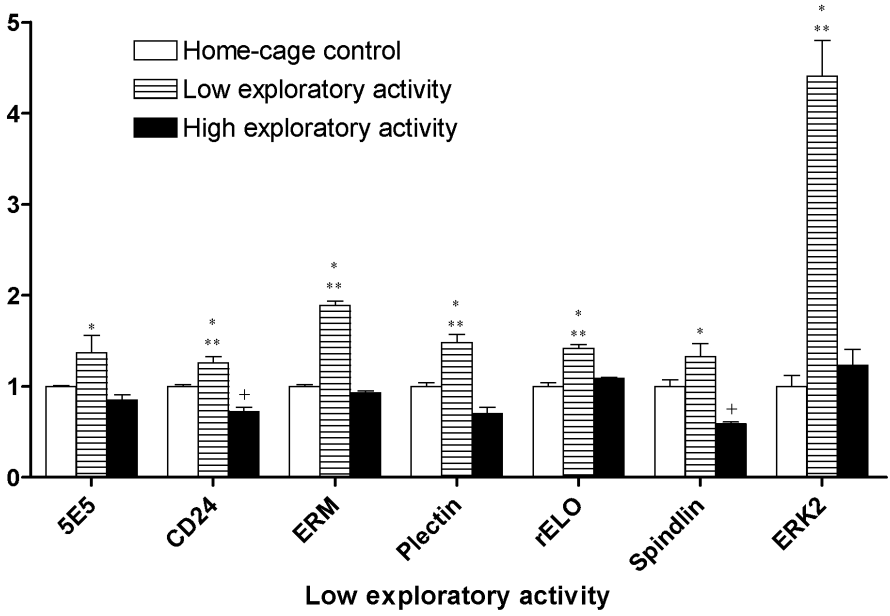

Figure 15 Differences in the expression of CCK-related genes in the frontal cortex, amygdala and PAG (mean values ± SEM).

* $P < 0.05$, low exploratory activity animals in comparison with the high exploratory activity group (Tukey HSD test after significant one-way ANOVA);
** $P < 0.05$, low exploratory activity animals compared to the home-cage control group;
+ $P < 0.05$, high exploratory activity rats in comparison with the home-cage control group.

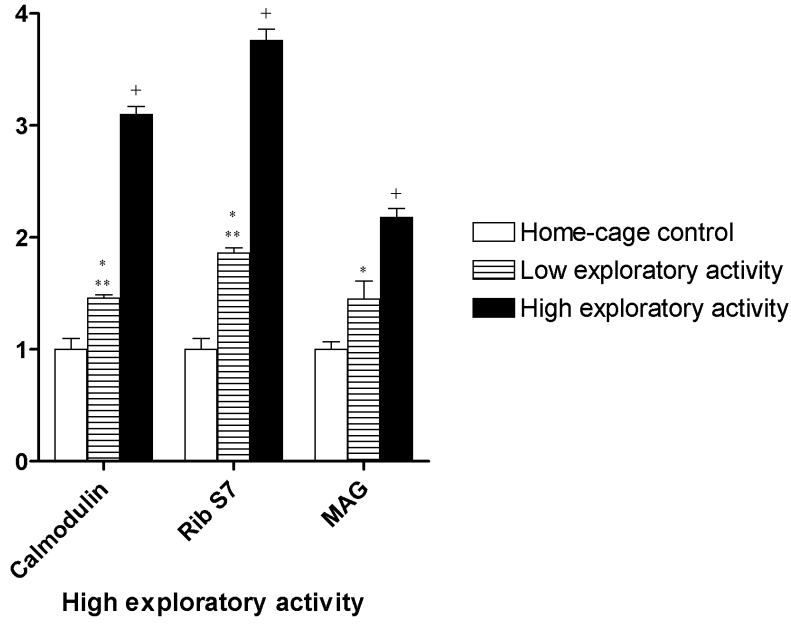

Figure 16. Differences in the expression of genes over-expressed in the PAG of rats with high exploratory activity (mean ± SEM).

* P < 0.05, low exploratory activity animals in comparison with high exploratory activity group (Scheffe test after significant one-way ANOVA);
** P < 0.05, low exploratory activity animals in comparison with home-cage control group;
+ P <0.05, high exploratory activity rats in comparison with home-cage control group.

Moreover, the expression of CD24 heat stable antigen, ERM, plectin, rELO fatty acid elongase I, and ERK2 was increased in low activity group, and the expression of CD24 heat stable antigen and spindlin gene was decreased in high activity group in comparison with control animals. On the other hand, the expression of calmodulin and ribosomal subunit S7 was in equal manner increased in animals with high exploratory activity and decreased in animals with low exploratory activity in comparison with control group, but in comparison with control animals the expression of MAG was increased in only animals with high exploratory activity. All data were confirmed statistically (Figure 15, 16).

VI. DISCUSSION

1. Selection of rats according to their exploratory behaviour in the elevated plus-maze

The results of the present work are in agreement with previous studies showing that male Wistar rats have different exploratory activity in the elevated plus-maze (Harro et al., 1990; Kõks et al., 1997). The reason for these behavioural differences is not clear, but could be attributed to the existence of a social hierarchy among the rats (Raab et al., 1986; Kozorovitskiy, Gould, 2004). Rats having a lower ranking in the hierarchy are submissive and display passive coping strategies in stressful conditions, whereas animals having a higher position display active coping strategies. Landgraf and Wigger (2002) performed an extensive breeding of Wistar rats starting from the low and high exploratory activity animals. After selective breeding for many generations they received two rat lines with different anxiety levels. As a matter of fact, Landgarf and Wigger (2002) demonstrated that low anxiety rats display active coping strategies, whereas in high anxiety rats passive coping strategies dominate. Therefore, variations in exploratory behaviour probably reflect the stress coping differences in the population of Wistar rats. We were able to select three subgroups of rats with significantly different exploratory behaviour from the group of 43 animals. Animals making one or two open arm visits were selected into the high exploratory activity group (9 rats). Rats making only one closed arm entry, were selected into the low exploratory group (10 rats). Animals not making open arm entries, but performing two and more closed arm entries remained in the intermediate group (24 rats).

Subsequent statistical analysis demonstrated that low and high exploratory activity animals differed by parameters reflecting anxiety in the plus-maze (number of open arm entries, time spent on the open arms and the ratio between open and total arm entries), but also by parameters reflecting locomotor activity (number of line crossings and closed arm entries). In correlation analysis, the frequency of open arm visits significantly correlated with measures reflecting locomotor activity (number of closed arm entries and line crossings). Therefore, one can conclude that the established behavioural differences between high and low exploratory activity groups are due to suppressed locomotor activity in low exploratory activity animals. However, this seems not to be the only reason for reduced exploratory activity, because low exploratory activity animals performed almost twice as many attempts to enter from the closed arm into the central square of the plus-maze compared to high exploratory activity rats. Accordingly, it is likely that the suppressed activity of low exploratory activity rats results from a strong aversion of animals to the open parts of the plus-maze.

2. Changes in the expression profile of CCK and GABA related genes in the frontal cortex, amygdala and PAG of rats displaying different exploratory activity

It has been shown that CCK- and GABA-ergic systems are two neurotransmitter systems having a close interaction in the brain. CCK is localised within GABA-ergic neurons in the cerebral cortex, hippocampus and basolateral nucleus of amygdala (Hendry et al., 1984; Kosaka et al., 1985; McDonald, Pearson, 1989; Cope et al., 2002). Besides neuroanatomical evidence there are studies showing functional interactions between these two systems. The initial suggestion that CCK interacts with GABA in the regulation of anxiety came from the experiments performed by Bradwejn and de Montigny (1984). They demonstrated that benzodiazepine receptor agonists could attenuate CCK-induced excitation of rat hippocampal neurons. CCK has been shown to increase the release of GABA in the cerebral cortex and hippocampus, and this effect is mediated via CCK_2 receptors (Perez de la Mora et al., 1993; Miller et al., 1997; Ferraro et al., 1999). Administration of CCK_2 receptor antagonists reverses the signs of diazepam withdrawal in rodents (Singh et al., 1992; Rasmussen et al., 1993). Recently we found that female mice, lacking CCK_2 receptors, display increased activity of GABA-ergic system in the brain (Raud et al., 2005).

Gene expression experiments revealed significant differences in the expression of GABA- and CCK-related genes between the selected groups in the frontal cortex. In high exploratory activity rats the expression of all GABA-related genes and CCK_1 receptors was reduced compared to the home-cage control group. In low exploratory rats also some reduction of GABA-related genes was established compared to control group animals. However, the reduction of GABA-related genes was significantly stronger in high exploratory activity rats compared to low exploratory activity animals. The profile of the CCK_2 receptor gene expression was similar to that of established in the radio-ligand binding studies (Kõks et al., 1997). There was a tendency toward increased expression in low exploratory activity rats compared to the home-cage control and high exploratory activity groups, but this change was not statistically significant. It has to be noted that the increase in the expression of the CCK_2 receptor gene was in the same range as was established after cat odour exposure in the study of Wang and colleagues (2003).

In the amygdala, we found significant changes in the expression of CCK- and GABA-related genes in animals displaying different exploratory activity. In low exploratory activity rats, the expression of most of GABA- and CCK-related genes was elevated in the amygdala compared to the home-cage control group. The only exceptions were $GABA_{B1A}$ receptor and pre-pro-CCK genes. The expression of these genes was reduced in both low and high exploratory rats compared to the control group. Therefore, it is likely that these changes are

induced by the exposure of rats to the elevated plus-maze. In the light of this finding it is important to note that virtually 100% of large CCK containing neurons in the basolateral and lateral nuclei of amygdala contain $GABA_{B1}$ receptor immunoreactivity (McDonald et al., 2004). The increase in the expression of α1 and α2 subunits of $GABA_A$ receptors is in agreement with the finding of Chacur and colleagues (1999) that the exposure of rats to the plus-maze induces an immediate increase of benzodiazepine receptors in the hippocampus and amygdala. In high exploratory activity rats several genes (pre-pro-CCK, CCK_1, CCK_2 and $GABA_{B1A}$ receptors) were down-regulated compared to the home-cage control group, whereas the expression of other genes had not changed. In respect of CCK_2 receptors, there is a contradiction with the study of Wunderlich and colleagues (2002). They described a reduced number of CCK_2 receptors in the amygdala of anxious rats (Wunderlich et al., 2002). However, one has to take into account that in this study only single radioligand concentration was used. The chosen concentration of ^{125}I-CCK8 (unsulfated) (50 pM) was almost 10 times below the dissociation constant of CCK_2 receptors established in saturation experiments (Van Dijk et al., 1984; Clark et al., 1986; Hama, Ebadi, 1987). Therefore, the single point binding study does not allow us to detect whether the reduced binding of CCK_2 receptors is related to a reduced number or affinity of receptors. Reduced affinity could be the case as the authors stated that the reduced number of CCK_2 receptors could be a compensatory response to the increased release of CCK due to emotional stress (Wunderlich et al., 2002).

In the PAG, the expression of several GABA-related genes was increased in low exploratory activity animals compared to the home-cage control group. Both $GABA_A$ and $GABA_B$ receptors have been implicated in the regulation of anxiety in the PAG (Bueno et al., 2005). Particularly, the expression of the $GABA_{B2}$ receptor gene was increased in the PAG of low exploratory activity rats. Indeed, mice lacking $GABA_{B2}$ receptors display increased anxiety compared to their wild-type littermates (Mombereau et al., 2005). By contrast, in high exploratory activity group the expression of several GABA-related genes was reduced compared to control group and low exploratory activity rats. The expression of both CCK receptors was also down-regulated in high exploratory activity rats. This finding is of interest, because several studies have demonstrated that CCK_2 receptors are major targets for anxio- and panicogenic action of CCK in the PAG (Netto et al., 2004; Zanoveli et al., 2004). The only exception in high exploratory activity rats was the $GABA_{B1A}$ receptor gene, because the expression of this gene was significantly elevated compared to low exploratory activity rats and the home-cage control group.

Changes in gene expression are not always reflected in the level of neurotransmitters and their receptors, but current study established a distinct expression profile of GABA- and CCK-related genes in various brain structures of rats displaying different exploratory activity in the plus-maze. Increased expression of GABA-related genes and especially α1 and α2 subunits of

$GABA_A$ receptors in the amygdala may explain higher sensitivity of low exploratory activity animals to the anxiolytic effect of diazepam. Liebsch and colleagues (1998) have demonstrated a significantly stronger anxiolytic effect of diazepam in animals bred for reduced exploratory activity in the plus-maze compared to high exploratory activity rats. Bert and colleagues (2001) established a similar correlation between low basal exploratory activity and increased anxiolytic action of diazepam using Fischer344 and Wistar rats. It has to be stressed that the established changes in gene expression are dependent on the brain structure studied. The largest differences in gene expression in the amygdala probably reflect the fact that the established variations in exploratory behaviour are due to different anxiety in low and high exploratory activity rats rather than due to different locomotor activity.

In conclusion, the exposure of rats to the elevated plus-maze revealed not only significant differences in the behaviour of rats, but also distinct gene expression patterns in low and high exploratory activity rats. Overall, the established changes in low and high exploratory activity rats are in opposite directions if compared to the home-cage control group. In low exploratory activity rats an increased expression of CCK receptors is evident in the amygdala. Recently Chen and colleagues (2006) demonstrated that the genetically induced over-expression of CCK_2 receptors significantly increased anxiety in mice. This change in behaviour of mice was antagonised by the treatment of animals with diazepam (Chen et al., 2006). In high exploratory activity rats, a significant down-regulation of CCK_2 receptors is evident in the amygdala and PAG. This finding indicates a reduced CCK-ergic tone in these rats and also reflects reduced anxiety. Alterations in the expression of GABA-related genes in low and high exploratory activity rats can be taken as compensatory responses to the increased and reduced anxiety, respectively.

3. Effect of diazepam on the exploratory behaviour of wild-type and CCK_2 receptor deficient mice

In order to confirm the functional validity of findings obtained from the gene expression studies the behavioural effects of diazepam, anxiolytic agonist of $GABA_A$, were studied in genetically modified mice, lacking CCK_2 receptors, a major target of CCK action in the brain. The elevated plus-maze studies demonstrated that the effect of diazepam on the exploratory activity of mice differed significantly between the genotypes. It is obvious that the effect of an anxiolytic drug depends on the baseline exploratory activity of animals. In the elevated plus-maze, the baseline activity of homozygous mice was higher compared to wild-type mice. The administration of diazepam (0.5 mg/kg) significantly increased open arm entries in wild-type mice in the elevated plus-maze, whereas in order to get a similar increase in homozygous animals we had

to inject diazepam at the dose of 1 mg/kg. A further increase in the dose of diazepam leads to an inhibition of locomotor activity in mice and, therefore, the suppression of locomotor activity masks the anxiolytic action of the drug. This inhibitory effect was very clearly demonstrated by the highest dose of diazepam (3 mg/kg), inducing a strong suppression of exploratory activity in the elevated plus-maze in mice, lacking CCK_2 receptors. Moreover, the administration of diazepam (3 mg/kg) also caused a significantly greater impairment of motor coordination in the rota-rod test in homozygous (–/–) mice compared to their wild-type (+/+) littermates.

Radioligand binding studies revealed an increased binding of benzodiazepine receptors in the cerebellum, but not in the hippocampus and cerebral cortex of CCK_2 receptor deficient mice. The cerebellum has a key role in the regulation of motor coordination (Mason, Sotelo, 1997). Diazepam-induced ataxia in rodents is most probably related to the stimulation of $GABA_A$ receptors located in the cerebellum (Korpi et al., 1999). Accordingly, an increased density of benzodiazepine receptors in the cerebellum could be a reason for the increased impairment of motor co-ordination and suppression of locomotor activity established in homozygous (–/–) mice after the administration of diazepam (3 mg/kg). Raud and colleagues (2005) studied the expression levels of selected $GABA_A$ receptor subunit (α1, α2, and γ2) genes, playing a role in the action of anxiolytic drugs in the frontal cortex, hippocampus and cerebellum. These studies revealed a 1.6-fold increase of α2 subunit of $GABA_A$ receptors in the frontal cortex of homozygous mice. This subunit mediates the anxiolytic action of diazepam and the genetic invalidation of this gene abolishes this effect of diazepam (Löw et al., 2000; Möhler et al., 2002). Also some increase in the expression of γ2 subunit of $GABA_A$ receptors (1.24-fold) was found in the frontal cortex, but this was not statistically significant. Still, it is interesting to note that heterozygous (+/–) γ2 subunit deficient mice display increased anxiety in the elevated plus-maze (Crestani et al., 1999). Davidson and Irwin (1999) suggest that the frontal cortex promotes adaptive goals in the face of strong competition from behavioural alternatives that are linked to immediate emotional consequences. Moreover, stressful manipulations with mice and rats increase the release of CCK and the number of CCK_2 receptors in the frontal cortex (Shlik et al., 1997; Becker et al., 2001). Also, an increase of CCK_2 receptor mRNA in the frontal cortex was established in response to the exposure of rats to a cat (Farook et al., 2001). CCK is localised only within GABA-ergic neurons in the cerebral cortex (Hendry et al., 1984) and, therefore, CCK strongly modulates the activity of these neurons (Ferraro et al., 1999). The lack of CCK_2 receptors leads to a situation where the balancing influence from the side of CCK is lost for GABA-ergic neurons. This could be a reason why the expression of the α2 subunit of $GABA_A$ receptors is increased in the frontal cortex of mice, lacking CCK_2 receptors. Despite some discrepancies between the gene expression and binding data, there is clear evidence about the increased function of GABA-ergic system in the brain of CCK_2 receptor deficient mice.

Altogether the present work and study performed by Raud and colleagues (2005) confirm the increased tone of the GABA-ergic system in mice lacking CCK_2 receptors. Moreover, these studies confirm the validity of findings obtained from the studies performed with rats displaying different exploratory activity and differences in the expression of GABA and CCK related genes in the brain structures related to the regulation of emotional behaviour.

4. Analysis of genes revealed by cDNA RDA in the PAG of rats displaying different exploratory activity

cDNA RDA followed by dot-blot analysis of clones was performed with the aim to extend our knowledge about the molecular mechanisms underlying anxiety. Therefore, we tried to identify genes differently expressed in animals with low or high exploratory activity in the elevated plus-maze. Present study established significant differences in the gene expression pattern in the PAG of rats displaying different exploratory activity in the elevated plus-maze. Genes with a very wide spectrum of functions were revealed in this study. Among them were enzymes, transcription factors, receptors and genes with unknown function. To determine the potential biological meaning of the revealed genes, their analysis was performed using the web-based on-line DAVID Bioinformatics Resource (The Database for Annotation, Visualization and Integrated Discovery, DAVID 2006) (Dennis et al., 2003, http://niaid.abcc.ncifcrf.gov). List of the Genebank Accession numbers of differentially expressed genes were uploaded to the site and then analysed using Functional Annotation Tool. Functional Annotation Tool in DAVID website, converts gene list to associated biology based on gene annotation enrichment analysis. During analysis, list of gene identifiers are annotated and summarised according to shared categorical data for Gene Ontology. Functional categories over-represented in a gene list relative to the representation within the transcriptome/proteome of a given species are identified by Fisher's exact test. Usually p-value equal to or smaller than 0.05 is considered strongly enriched in the annotation categories.

DAVID database search analysis identified different functional classes of analysed genes. The profile of rats with low exploratory activity gave best match to second-messenger-mediated signaling category in Gene Ontology (GO) annotation. This match was statistically significant with p-value 6.47E-06. Other matches were GO categories with similar function (cAMP-mediated signaling, intracellular signaling cascade). This finding shows that rats with low exploratory behavior in elevated plus-maze have increased neurotransmission and signaling. Prevalent change is related to intracellular signaling, but we were able to see more specific pathways. Namely, GO annotation gave good match to the term "$GABA_{B2}$ receptor activity" with p-value 0.005. This finding is in a good agreement with our real-time qPCR study where more than two-fold

elevation of $GABA_{B2}$ receptor gene in the PAG of low exploratory rats compared to high exploratory group was revealed. In addition, up-regulation of vasopressin V1a and alpha 1a/d adrenergic receptors was found. Therefore, exposure of rats to the elevated plus-maze possibly activates $GABA_B$ receptor systems, along with vasopressin and adrenergic systems. All these three receptors, $GABA_{B2}$, vasopressin V1a and alpha 1a/d adrenergic, are potential candidates for the anxious-type behavior the in elevated plus maze.

Annotation of genes up-regulated in rats with high exploratory activity indicated significant activation of genes from GO category "behavioral fear response" ($p = 0.009$). On the other hand, some genes matched to "direct protein sequencing" category, indicating possible protein synthesis activation. Interestingly, "fear response" genes were over-expressed in rats displaying high exploratory activity ("non-anxious" response), while rats with low exploratory activity did not show expression of this type of genes. This possibly means that genes called "fear response genes" are up-regulated in a stressful situation when animals have to cope with the situation. Animals, unable to overcome the aversive circumstances, do not express this set of genes. Are these genes needed for coping of situation or do they reflect certain inheritable trait, remains unclear.

Taken together, differential cloning and functional annotation of these genes indicated that rats with a different response in the elevated plus-maze also have functionally different genes activated – in low exploratory activity animals $GABA_{B2}$ receptor signaling is activated, whereas in high exploratory activity rats "behavioral fear response" genes are activated.

In addition to functional annotation of entire gene lists, we analysed descriptions of genes individually. This approach should give additional support to described findings. Calmodulin (CaM) is a major Ca^{2+}-binding protein playing a key role in the calcium signalling pathway. It was shown that membrane depolarisation and the resulting Ca^{2+} influx through voltage-sensitive calcium channels lead to the activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMKII, CaMKIV) and subsequently phosphorylation of cAMP-responsive element binding protein (CREB) (Shirasaki et al, 2006; Shum et al, 2005; Du et al., 2004). Moreover, Shum and colleagues revealed a direct role of CaMKIV dependent pathway in the regulation of anxiety-related behaviour (2005). They had reported that $CaMKIV^{-/-}$ mice exhibit decreased anxiety-like behaviours in the elevated plus-maze (Shum et al., 2005). Du and colleagues also demonstrates a regulatory role of CaMKII in the pathophysiology of mood and anxiety disorders (2004).

The extracellular signal-regulated protein kinase (ERK2) is a member of mitogen-activated protein kinase (MAPK) family that play an important role in transducing extracellular signals to the nucleus. On activation, the ERKs are translocated to the nucleus, where they phosphorylate transcription factors, leading to changes in gene expression (English et al., 1999; Selcher et al., 2006). Rats exposed to swim stress displayed increased activity of ERK2 in the

neocortex, prefrontal cortex and striatum of Wistar rats. Also the activity of CREB was increased in the prefrontal and neocortex areas demonstrating an interaction of these enzymes (Shen et al, 2004).

Both ERK2 and calmodulin lead to the activation of cAMP-responsive element-binding protein (CREB). The gene transcription factor cAMP-responsive element-binding protein (CREB) binds to a CRE site within the regulatory region of numerous genes, and regulates their transcription (Carlezon Jr et al., 2005). The target genes could be corticotropin releasing factor (CRF), neuropeptide Y (NPY), cFos, brain-derived neurotrophic factor (BDNF), opioid peptides, glutamate receptor 1 subunit, the genes have been associated with the regulation of anxiety (Carlezon Jr et al., 2005). Increasing number of evidences indicates that CREB function can regulate anxiety-like behaviour in rats. For example, disruption of the CREB function within the nucleus accumbens produces anxiety-like effects (Barrot et al., 2005), whereas induction of the CREB function within the amygdala produces similar behavioural effects (Wallace et al., 2004; Carlezon Jr et al., 2005). CREB deficient mice show increased anxiety in the elevated plus-maze (Graves et al., 2002; Hebda-Bauer et al., 2004). Moreover, Adamec and colleagues indicated an increased expression and immunoreactivity of CREB in the PAG of rats displaying increased anxiety-like behaviour after exposure to a predator (Adamec et al., 2003). The exact mechanisms of so different behavioural reactions are not clear, but it can be related to fact that in different brain structures CREB activates the transcription of different genes (Carlezon Jr et al., 2005). These data demonstrate that calmodulin and ERK2 playing a role in intracellular signal transduction pathways can be candidate genes involved in the regulation of anxiety.

There are few data about the role of other genes identified in this study in the regulation of behaviour and anxiety states. Expression of neural membrane protein 35 was similarly 1.6-fold up-regulated in Sprague-Dawley rats displaying lower anxiety-like behaviour if compared to PVG hooded rat strain after live cat exposure (Wang et al., 2003). Also, our findings to a certain extent correlate with the finding of Kõks and colleagues (Kõks et al., 2004). Comparing cDNA of rats exposed to cat odour with cDNA of rats from the control-group, they revealed several genes over-expressed in animals not exposed to stress. These were voltage-dependent anion channel 3, 2,3-cyclic nucleotide 3-phosphodiesterase (CNPII), testican-3 protein, ZnBP gene for zinc binding protein, Rev-ErbA-alpha protein, G-protein gamma 7 subunit, neural membrane protein 35. The same genes were over-expressed in animals with high exploratory activity in the elevated plus-maze.

There is also quite intriguing evidence about 1B236/myelin-associated glycoprotein (MAG). Narita and colleagues showed that animals displaying increased anxiety due to an exposure to the light-dark exploration test have a drastic reduction in myelin-associated glycoprotein (MAG)-like immunoreactivity (MAG-IR) in the dorsal raphe nucleus, amygdala and hypothalamus (Narita et al., 2005). This finding is in agreement with our study, because the

expression of this gene was significantly lower in the PAG of rats displaying low exploratory activity.

Taken together, these data demonstrate that differences in exploratory activity in the elevated plus-maze are related not only to differences in neurotransmitters and their receptors, but also to apparent differences in intracellular signal transduction, $GABA_B$ receptor activity, and "behavioral fear response" system. There is a growing body of evidences that intracellular processes are involved in the regulation of behavioural responses. Indeed, the possible role of ERK2, CREB, calmodulin and CAMK was already mentioned. There is also evidence about the role of adenylyl cyclase type VIII, protein kinase Cγ (PKCγ), tyrosine kinase (fyn), C-terminal dystrophins, intracellular glucocorticoid receptor and FMRP in the regulation of anxiety (Wood, Toth, 2001). All these data support the role of intracellular processes in the regulation of anxiety.

5. Characterisation of LsAMP gene expression in the frontal cortex, amygdala and PAG of rats displaying different exploratory activity

In our pilot study, by means of reverse-transcriptase PCR followed by electrophoresis and bands density analysis, we described a 1.6-fold increase in the expression of the limbic system associated membrane protein (LsAMP) gene in the PAG of animals displaying low exploratory activity in the plus-maze compared to high exploratory activity group. Thereafter, by using more sensitive real-time qPCR, we confirmed that the expression of LsAMP gene is somewhat increased in the PAG of animals with low exploratory activity, however these results did not reach statistical significance. Moreover, we found that the expression of this gene is increased 1.4-fold in the frontal cortex and 2.4-fold in the amygdala in low activity animals compared to high activity rats. 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. This study confirms earlier findings showing that the expression of the LsAMP gene is increased in the amygdala of rats under the influence of emotional stress. In his study Kõks and colleagues demonstrated an increased expression of the LsAMP gene in rats exposed to cat odour in comparison with control animals (Kõks et al., 2004).

However, the role of LsAMP in the regulation of emotional behaviour is not clear and needs to be established in further studies.

6. Concluding remarks and future prospects

The present study demonstrates that despite similar genetic background, male Wistar rats display significant differences in exploratory activity. These differences are most probably related to the existence of a social hierarchy among the male rats living in the same cage. Moreover, animals having different exploratory behaviour also display differences in the gene expression patterns in the brain structures. This is in good accordance with the study of Wigger and colleagues demonstrating significant differences in the gene expression of high and low anxiety rats (Wigger et al., 2004). Therefore, one could conclude that the comparison of gene expression profiles in low and high exploratory rats may be used for the establishment of new target genes involved in the regulation of anxiety.

The validity of this suggestion was further extended by showing that low and high exploratory rats display different expression of CCK- and GABA-related genes in the brain structures. CCK and GABA systems are shown to have the opposite role in the regulation of exploratory behaviour and anxiety (Harro et al., 1993). Recently, Chen and colleagues (2006) demonstrated that genetically induced over-expression of CCK_2 receptors significantly increased anxiety in mice and this increase was suppressed by pre-treatment with diazepam, facilitating GABA-ergic neurotransmission in the brain. Altogether, animals with an increased level of CCK_2 receptors display higher level of anxiety compared to rats with a lower level of CCK_2 receptors. The increased tone of the CCK-ergic system in the brain is responsible for the increased anxiety established in low exploratory rats. Liebsch and colleagues (1998) and Bert and colleagues (2001) demonstrated that in the elevated plus-maze the anxiolytic effect of diazepam is stronger in animals bred for reduced exploratory activity than in high exploratory activity rats. The increased sensitivity to diazepam in the plus-maze model of anxiety can be explained by an increased expression of $GABA_A$ receptor subunits in animals with low exploratory activity established in our work. The brain structure dependent alterations in the expression of GABA-related genes in low and high exploratory activity rats can be taken as compensatory responses to the increased and reduced anxiety, respectively.

The present study also shows that the targeted mutation of the CCK_2 receptor gene induces alterations in the function of the GABA-ergic system. The anatomical finding that CCK is co-localised with GABA in the neurons of the cerebral cortex and hippocampus (Hendry et al., 1984; Kosaka et al., 1985; Cope et al., 2002) and the neurochemical evidence that CCK can induce the release of GABA in the cerebral cortex and hippocampus (Perez de la Mora et

al., 1993; Miller et al., 1997; Ferraro et al., 1999) have indicated that these two neurotransmitter systems are in close interaction in certain structures of the brain. In this work, we established that the genetic invalidation of CCK_2 receptors increased the exploratory activity of mice. The changes in the action of diazepam, a $GABA_A$ receptor agonist, on the animal behaviour suggest that the activity of the GABA-ergic system is affected by the genetic invalidation of CCK_2 receptors. Alterations in the activity of the GABA-ergic system, established in pharmacological studies, are confirmed by the data from radio-ligand binding studies with [^{3}H]-flunitrazepam. An increased density of benzodiazepine receptors in the cerebellum (results of the present study) and an increased expression of $GABA_A$ receptor subunit α2 gene (Raud et al., 2005) are clear indications of an increased function of the GABA-ergic system in the brain. Altogether, the data of behavioural, pharmacological and neurochemical studies reflect an increased tone of GABA-ergic system in mice, lacking CCK_2 receptors. Altogether, the present study is in favour of an antagonistic inter-action of the CCK and GABA systems in the regulation of anxiety. Moreover, this is giving us the strong indication that the low and high exploratory activity rats can be used for revealing new target genes involved in the regulation of anxiety. As a matter of fact we found an increased expression of the LsAMP gene in the frontal cortex, amygdala and PAG of animals with low exploratory activity. 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 to that. Kõks and colleagues (2004) also described elevated expression of this gene in animals exposed to cat odour. Thus, the findings of Kõks and colleagues and the data of the present work demonstrate a possible role of the LsAMP gene in the regulation of exploratory behaviour and anxiety. Currently we are developing transgenic mice lacking LsAMP. The validation of the behavioural phenotype of LsAMP gene deficient mice is out of scope of the present study.

Besides the LsAMP gene we tried to establish other new genes differently expressed in low and high exploratory activity rats. The cDNA RDA study showed that not only neurotransmitter systems but also several intracellular processes are involved in the regulation of exploratory behaviour. The present study revealed the participation of intracellular signal transduction, $GABA_B$ receptor activity, and "behavioral fear response" system in the regulation of anxiety-related behaviour. We established significant differences of the ERK2 and calmodulin genes in high and low exploratory activity rats. Both genes are involved in intracellular signal transduction. Taking into account the increased tone of CCK system in the brain of low exploratory activity rats it is possible that these pathways are activated via CCK_2 receptors. This suggestion is probably supported by the finding that CCK activates via its receptors MAPK signalling pathway (Williams et al., 2002).

VII. CONCLUSIONS

1. According to their exploratory activity in the elevated plus-maze rodents can be devided into subgroups with low and high exploratory activity. Animals from these subgroups have differences not only in their blood hormone levels and receptor density, but also in their gene expression profile. Low exploratory rats have an increased expression of CCK_1 and CCK_2 receptors in various brain structures compared to high exploratory rats. It is apparent that low exploratory rats have an increased tone of the CCK system especially in the amygdala, a key brain structure in the regulation of anxiety. Animals with low exploratory activity have an increased expression of the GABA-ergic system related genes in the brain structures. This can be interpreted as a compensatory change to the increased activity of the CCK system and elevated level of anxiety.
2. CCK_2 receptor deficient mice, a model of reduced tone of CCK system, demonstrate a decreased level of anxiety in the elevated plus-maze model in comparison with their wild-type littermates. Simultaneously the anxiolytic effect of diazepam is somewhat weaker in mice, lacking CCK_2 receptors. Moreover, the genetic invalidation of CCK_2 receptors elevates the density of benzodiazepine binding sites in the cerebellum explaining the increased effect of diazepam on motor coordination in mice, lacking CCK_2 receptors. Altogether these findings demonstrate that the genetic invalidation of CCK_2 receptors leads to an increased tone of the GABA system in the brain.
3. Animals with different exploratory activity in the elevated plus-maze have also differences in the expression of several genes in the intracellular signal transduction, $GABA_B$ receptor activity, and "behavioral fear response" system in the PAG. In particular, these changes affect ERK2, calmodulin, $GABA_B$, vasopressin V1a, alpha 1a/1d adrenergic receptors and some other genes involved in intracellular processes.
4. Animals with low exploratory activity in the elevated plus-maze display increased expression of the LsAMP gene in the amygdala and PAG. This can be interpreted as a compensatory change to the increased anxiety. These findings are in good agreement with the previous studies demonstrating the increased expression of LsAMP gene in the amygdala of rats, displaying the increased anxiety. LsAMP and genes involved in several intracellular processes are potential targets for the development of new anxiolytic drugs.

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SUMMARY IN ESTONIAN

Eksploratiivse käitumise regulatsioonis osalevate molekulaarsete mehhanismide käitumuslik ja neurogeneetiline uurimus närilistel

Sissejuhatus

Eksploratiivne ehk uudistav käitumine on üks käitumise vormidest, mis haarab endasse uue keskkonna uurimisega seotud käitumuslikud aktid, eesmärgiga saada vajalikku informatsiooni uutest objektidest ja ümbritsevast keskkonnast. Eksploratiivse käitumise aluseks on kaasasündinud ajed, mis panevad loomi uurima ümbritsevat keskkonda. Nende ajedele töötavad vastu mehhanismid, mis sunnivad loomi uutesse objektidesse ja keskkonda ettevaatlikult suhtuma. Seega on eksploratiivse käitumise aluseks kaks vastandlikku jõudu ja eksploratiivse käitumise aktiivsus peegeldab loomadel esineva ärevuse taset.

Huvi ärevuse mehhanismide vastu on seotud asjaoluga, et ärevushäired on kaasaegses ühiskonnas väga laialt levinud ning seotud kõrgenenud haigestumuse ja suremusega, aga ka suurte majanduslike kulutustega. Oma olemuselt on ärevus normaalne nähtus, mis võimaldab inimesel ja loomadel muutuvas maailmas paremini kohaneda. Ülemäärane ärevuspõhjustab aga tõsiseid kannatusi ning töö ja igapäevaeluga toimetuleku häireid. Ehkki ärevuse ja teiste emotsioonide mehhanisme on uuritud aastakümneid, ei ole kaasaegne ärevuse medikamentoosne ravi siiski alati efektiivne ja omab palju kõrvaltoimeid. Seetõttu on oluline tuvastada uusi molekulaarseid sihtmärke, mis osalevad ärevuse regulatsioonis. Taolisi uuringuid on võimalik läbi viia vaid katseloomadel ning seepärast tuleb rakendada asjakohaseid ärevuse mudeleid.

Üheks ärevuse loomamudeliks on tõstetud pluss-puur. See on lihtne meetod, mis lubab ärevust mitte ainult kvalitatiivselt määrata, vaid ka mõõta erinevusi selle tasemes. See mudel lubab jaotada katseloomad (hiired ja rotid) vastavalt eksploratiivse aktiivsuse ehk ärevuse tasemele erinevateks gruppideks. Käesolevas töös otsustati rakendada katseloomade selekteerimise mudelit eksploratiivse käitumise regulatsioonis osalevate molekulaarsete mehhanismide uurimiseks.

Uurimuse põhieesmärgid

Käesoleva töö peamiseks eesmärgiks oli selgitada uusi molekulaarseid sihtmärke ärevuse regulatsioonis. Ärevuse uurimiseks valiti tõstetud pluss-puuri mudel, mille abil jaotati isased Wistar liini rotid vastavalt nende eksploratiivsele aktiivsusele erinevatesse gruppidesse. Seejärel rakendati nende gruppide geenidekspressiooni mustrite erinevuste väjaselgitamiseks molekulaargeneetilisi meetodeid. Uuringu täpsemad eesmärgid olid järgmised:

1. Uurida mõnede koletsüstokiniini-(CCK)- ja γ-aminovõihappe-(GABA)-ergiliste süsteemidega seotud geenide ekspressiooni erinevust madala ja kõrge eksploratiivse aktiivsusega Wistar liini rottidel kolmes ajustruktuuris: frontaalses ajukoores, mandelkehas ja veejuha ümbritsevas hallaines.
2. Näidata diasepaami, $GABA_A$ retseptorite agonisti, käitumuslike efektide muutust CCK_2 retseptori puudulikkusega emastel hiirtel võrreldes metsikut tüüpi loomadega, eesmärgiga tuvastada muutusi GABA-ergilises süsteemis.
3. Leida cDNA diferentsiaalanalüüsi abil erineva eksploratiivse aktiivsusega isaste Wistar liini rottide veejuha ümbritsevas hallaines erineva ekspressiooniga geene, analüüsida neid ja valida neist asjakohased edasisteks uuringuteks. Veejuha ümbritseva hallaine kasuks otsustati seoses selle ajustruktuuri rolliga ärevuse neuraalsetes mehhanismides ning samuti käesoleva töö esimeste katsete alusel, mis näitasid suuri erinevusi GABA ja CCK geenide ekspressioonis antud ajustruktuuris.
4. Võrrelda limbilise süsteemiga seotud membraanvalgu (LsAMP) geeni ekspressiooni madala ja kõrge eksploratiivse aktiivsusega isaste Wistar liini rottide kolmes ajustruktuuris: frontaalkoores, amügdalas ja veejuha ümbritsevas hallaines. cDNA diferentsiaalanalüüs näitas, et LsAMP geeni ekspressioon oli madala eksploratiivse aktiivsusega katseloomade veejuha ümbritsevas hallaines suurenenud.

Katseloomad ja meetodid

Katseloomadeks olid isased Wistar liini rotid ning emased 129Sv/C57Bl6 taustaga transgeensed CCK_2 retseptori puudulikkusega hiired ja nende metsikut tüüpi pesakonnakaaslased.

Rotte testiti tõstetud pluss-puuri mudelis ning jaotati nad vastavalt eksploratiivse käitumise aktiivsusele kolmeks:madala, keskmise ja kõrge eksploratiivse aktiivsusega grupiks. Keskmise aktiivsusega katseloomad jäeti geenieksressiooni uuringutest välja. Loomade ajustruktuuridest eraldati mRNA ja sünteesiti selle alusel cDNA. Kvantitatiivse reaalaja polümeraasi ahelreaktsiooni (qPCR) abil hinnati LsAMP, GABA ja CCK süsteemidega seotud geenide ekspressiooni kolmes eelnimetatud ajustruktuuris.

CCK_2 retseptori puudulikkusega hiiri testiti samuti tõstetud pluss-puuri mudelis, hindamaks nende ärevuse taset ja diasepaami toimet. Motoorse koordinatsiooni hindamiseks kasutati rotarodi testi, mis näitab katseloomade võimet püsida pöörleval silindril. Selle testiga selgitati diasepaami mõju motoorsele koordinatsioonile. Radioligandi sidumiskatsetes mõõdeti bensodiasepiini retseptorite afiinsust ja nende tihedust suurajukoores, hipokampuses ja väikeajus. Bensodiasepiini retseptorite märgistamiseks kasutati [^{3}H]-flunitrasepaami ja mittespetsiifilise sidumise määramiseks bensodiasepiini retseptorite agonisti diasepaami.

Uute sihtmärkgeenide selgitamiseks isaste Wistar liini rottide veejuha ümbritsevas hallaines kasutati cDNA diferentsiaalanalüüsi (cDNA-RDA). Muutunud ekspressiooniga kloonid kvantiteeriti dot-blot analüüsil ning seejärel kinnitati 10 geeni ekspressiooni erinevust qPCR-i abil.

Tulemused

Tõstetud pluss-puuri katse kinnitas varasemaid andmeid, et loomad käituvad selles mudelis erinevalt ning et tänu sellele on võimalik jaotada nad kõrge ja madala eksploratiivse aktiivsusega gruppideks. qPCR-i rakendamine näitas, et madala eksploratiivse aktiivsusega loomadel esineb CCK_1 ja CCK_2 retseptorite üle-ekspressioon kolmes ajustruktuuris: frontaalkoores, amügdalas ja veejuha ümbritsevas hallaines. Saadud andmed on kooskõlas varasemate uuringutega, kus CCK retseptorite kõrgenenud tase oli seotud katseloomade suurenenud ärevusega. Lisaks CCK retseptoritele olid üle-ekspresseeritud ka $GABA_A$ retseptorite alaühikute α1 ja α2, $GABA_{B2}$, $GABA_{B1B}$ ja mõned teised GABA geenid amügdalas ja veejuha ümbritsevas hallaines. Ilmselt on need muutused GABA-ergilises süsteemis kompensaatorseks vastuseks suurenenud CCK-ergilisele aktiivsusele madala eksploratiivse aktiivsusega loomadel. Madala eksploratiivse aktiivsusega rottidel oli üle-ekspresseeritud ka LsAMP geen ja see erinevus oli kõige suurem amügdalas. LsAMP geen on oluline limbilise süsteemi arengus ning äsjased uuringud näitavad LsAMP geeni kõrgenenud taset rottide amügdalas peale eksponeerimist kassilõhnale.

Katsed CCK_2 retseptori puudulikkusega hiirtega näitasid, et nende loomade eksploratiivne aktiivsus oli võrreldes metsikut tüüpi pesakonnakaaslastega oluliselt suurenenud. Farmakoloogilised uuringud näitasid, et bensodiasepiini retseptorite agonisti diasepaami anksiolüütiline toime avaldub metsikut tüüpi hiirtel tugevamini kui geneetiliselt modifitseeritud pesakaaslastel. Samas avaldab diasepaam CCK_2 retseptori puudulikkusega hiirte puhul oluliselt tugevamat motoorikat pärssivat toimet kui metsikut tüüpi liigikaaslaste puhul. Radioligandi sidumiskatsed ja geenieksprессiooni uuringud kinnitasid farmakoloogiliste uuringute andmeid, mille järgi CCK_2 retseptori geneetiline väljalülitamine põhjustab olulisi muutusi GABA-ergilises süsteemis. Sidumiskatsetest selgus, et homosügootsetel hiirtel on väikeajus bensodiasepiini retseptorite arv suurenenud. See tulemus aitas meil põhjendada, miks diasepaam pärssis CCK_2 retseptori puudulikkusega hiirtel motoorset aktiivsust tunduvalt enam kui metsikut tüüpi pesakonnakaaslastel. Seega antud andmete põhjal võib väita, et CCK_2 retseptori geeni väljalülitamine põhjustab GABA-ergilise süsteemi toonuse olulist tõusu kesknärvisüsteemis.

Ülaltoodud tulemused olid aluseks edasistele katsetele leida uusi sihtmärke ärevuse regulatsioonis. Selleks viidi läbi cDNA-RDA katse, leidmaks teisi geene, mis on madala eksploratiivse aktiivsusega loomade veejuha ümbritsevas hallaines üle-ekspresseeritud. Selles uuringus tuvastati rohkem kui 50 geeni,

mis on madala eksploratiivse aktiivsusega loomadel ja nende kõrge aktiivsusega liigikaaslastel erinevalt ekspresseeritud. Läbiviidud qPCR kinnitas ERK2 geeni üle-ekspressiooni madala eksploratiivse aktiivsusega loomadel ja kalmoduliini geeni üle-ekspressiooni kõrge eksploratiivse aktiivsusega loomadel. Mõlemad geenid osalevad rakusiseses signaaliülekandes ja mõjutavad CREB geeni funktsiooni. CREB omakorda reguleerib paljude teiste geenide transkriptsiooni. Antud uuring näitas rakusisese signaaliülekande olulist rolli eksploratiivse käitumise mehhanismides.

Järeldused

1. Isased Wistar liini rotid käituvad tõstetud pluss-puuri mudelis erinevalt ning erineva eksploratiivse aktiivsusega loomad omavad olulisi erinevusi ka GABA ja CCK seotud geenide ekspressioonis amügdalas ja veejuha ümbritsevas hallaines. CCK retseptorite ekspressioon on suurem madala eksploratiivse aktiivsusega loomadel, mille alusel võib väita, et suurenenud ärevus on tingitud CCK-ergilise süsteemi suurenenud toonusest nimetatud katseloomade ajus. Muutused GABA-ga seotud geenide ekspressioonis on tõenäoliselt kompensatoorseks vastuseks CCK-ergilise süsteemi suurenenud aktiivsusele ja tõusnud ärevusele.
2. Emaste CCK_2 retseptori puudulikkusega hiirte uudistamisaktiivsus on tõstetud pluss-puuris võrreldes metsikut tüüpi pesakonnakaaslastega suurenenud, samuti avaldab diasepaam neile nõrgemat anksiolüütilist toimet kui metsikut tüüpi hiirtele. Lisaks sellele on CCK_2 retseptori puudulikkusega hiirtel suurenenud bensodiasepiini retseptorite tihedus väikeajus, mis on seostatav diasepaami oluliselt tugevama motoorset koordinatsiooni häiriva toimega nendel hiirtel. Uuringute tulemuste alusel võib väita, et CCK_2 retseptori geeni väljalülitamine kutsub esile GABA-ergilise süsteemi toonuse olulise tõusu ajus.
3. Erineva eksploratiivse käitumisega isastel Wistar liini rottidel on erinev rakusisesese signaaliülekande rajas osalevate, GABAB retseptorite aktiivsust peegeldavate ja "käitumusliku hirmu reaktsiooni" süsteemis osalevate geenide ekspressioon veejuha ümbritsevas hallaines. Eriti tasub esile tõsta muutusi ERK2, kalmoduliini, $GABA_B$, vasopressiini V1a ja alfa 1a/1d adrenergiliste retseptorite geenide ning mõnede teiste rakusiseste protsessidega seotud geenide ekspressioonis.
4. Madala eksploratiivse aktiivsusega loomadel on suurenenud LsAMP geeni ekspressioon amügdalas ja veejuha ümbritsevas hallaines. See muutus on ilmselt kompensatoorne vastus suurenenud ärevusele. Need andmed on heas kooskõlas eelnevate tulemustega, mille kohaselt kõrgema ärevusega loomadel on suurenenud LsAMP geeni ekspressioon amügdalas. LsAMP ja geenid, mis on seotud rakusisese signaaliülikandega, on potentsiaalsed sihtmärgid uute anksiolüütiliste ravimite väljatöötamiseks.

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PUBLICATIONS

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2002–	Tartu Ülikooli arstiteaduskond, infektsioonhaiguste residentuur

Täiendus

Aprill, 2004 kursus "Experimental Design and Statistical Methods in Biomedical Experimentation" Kuopios, Soomes.
Mai, 2003 "Behavioural studies in drug discovery" Kuopios, Soomes

Teadustöö

"Eksploratiivse käitumise regulatsioonis osalevate molekulaarsete mehhanismide käitumuslik ja neurogeneetiline uurimus närilistel"