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SILVA SÜTT

Role of endocannabinoid system and *Wfs1* in regulation of emotional behaviour: behavioural, pharmacological and genetic studies

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LIST OF ORIGINAL PUBLICATIONS

1. **Sütt S**, Raud S, Areda T, Reimets A, Kõks S, Vasar E. Cat odour-induced anxiety-a study of the involvement of the endocannabinoid system. Psychopharmacology (Berl). 2008 Jul; 198 (4):509–20. Epub 2007 Sep 20.
2. Raud S, **Sütt S**, Plaas M, Luuk H, Innos J, Philips MA, Kõks S, Vasar E. Cat odour exposure induces distinct changes in the exploratory behavior and Wfs1 gene expression in C57Bl/6 and 129Sv mice. Neurosci Lett. 2007 Oct 16; 426(2):87–90. Epub 2007 Aug 31.
3. **Sütt S**, Raud S, Abramov U, Innos J, Luuk H, Plaas M, Kõks S, Zilmer K, Mahlapuu R, Zilmer M, Vasar E. Relation of exploratory behavior to plasma corticosterone and Wfs1 gene expression in Wistar rats. J Psychopharmacol. 2009 Apr 3. [Epub ahead of print]
4. Raud S, **Sütt S**, Luuk H, Plaas M, Innos J, Kõks S, Vasar E. Relation between increased anxiety and reduced expression of alpha1 and alpha2 subunits of GABA(A) receptors in Wfs1-deficient mice. Neurosci Lett. 2009 Aug 28; 460(2):138–42. Epub 2009 May 27.

Contribution of the author:

1. The author performed all qRT-PCR gene expression analyses, statistical analysis and was responsible for writing the manuscript.
2. The author performed qRT-PCR gene expression analysis, statistical analysis and participated in writing the manuscript.
3. The author performed qRT-PCR gene expression analysis, statistical analysis, ELISA measurements of corticosterone and was responsible for writing the manuscript.
4. The author performed qRT-PCR gene expression analysis, calculated an algorithm for choosing the best housekeeper gene, performed statistical analysis and participated in writing the manuscript.

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AEA	*N*-arachidonoylethanolamine, anandamide
2-AG	2-arachidonoylglycerol
Ala	Alanine
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of variance
AOB	Accessory olfactory bulb
Arg	Arginine
ATF6	Activating transcription factor 6
B2M	β_2 microglobulin
BLA	Basolateral amygdala
BNST	Bed nucleus of stria terminalis
CaM	Calmodulin
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
CeA	Central nucleus of amygdala
CHOP	C/EBP homologus protein
CNS	Central nervous system
CREB	cAMP response element-binding
CRH	Corticotropin-releasing hormone
Ct	Cycle threshold
Dagla	Diacylglycerol lipase-alpha
DIDMOAD	Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness
DMSO	Dimethyl sulfoxide
dlPAG	Dorsolateral periaqueductal gray
dPAG	Dorsal periaquaductal gray
DRN	Dorsal raphe nucleus
EC	Endocannabinoid
ECS	Endocannabinoid system
EPM	Elevated plus-maze
ER	Endoplasmic reticulum
GABA	Gamma-aminobutyric acid
Gabra1	Gamma-aminobutyric acid receptor subunit alpha-1
Gabra2	Gamma-aminobutyric acid receptor subunit alpha-2
Gad	Glutamate decarboxylase
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Glu	Glutamic acid
GPR	Glucose-regulated protein
HEA	High exploratory activity
HPA	Hypothalamic-pituitary-adrenal axis

Hprt1	Hypoxanthine-guanine phosphoribosyltransferase
FAAH	Fatty acid amide hydrolase
IRE1	Inositol-requiring enzyme
LC	Locus coeruleus
LEA	Low exploratory activity
MeA	Medial nucleus of amygdala
MGLL	Monoacylglycerol lipase
MRN	Median raphe nucleus
mRNA	Messenger ribonucleic acid
Nape-pld	N-acyl phosphatidylethanolamine phospholipase D
NMDA	N-methyl-D-aspartate
PAG	Periaqueductal gray
PERK	Protein kinase-like ER kinase
Pro	Proline
PTSD	Posttraumatic stress disorder
PVN	Paraventricular nucleus
qRT-PCR	Quantitative real-time polymerase chain reaction
Δ^9-THC	Δ^9-tetrahydrocannabinol
TAR	Total antioxidant response of plasma
Thr	Threonine
TMT	2,4,5 Trimethylthiazoline
TPX	Total peroxide concentration of plasma
Trp	Tryptophan
UPR	Unfolded protein response
Wfs1	Wolfram syndrome 1 gene or protein in any species other than human
WFS1	Wolfram syndrome 1 gene or protein in human
Wolframin	Wfs1 protein
XBP1	X-box DNA-binding protein

INTRODUCTION

This is a human nature, but probably not only human, to feel anxiety in the stressful and threating situations. It is obviously a normal reaction to stress because it helps to cope with the difficult circumstances and it is important for adaptation in the novel environment. Long lasting anxiety, which is not associated with the actual risk, is pathological and alters homeostasis of the body in different ways (Engelmann et al., 2004; Korte, 2001). Symptoms of anxiety include alterations in autonomic nervous system (changes in blood pressure and heart rate) as well as in neuroendocrine responses (release of hormones from pituitary and adrenal gland) (LeDoux, 2003; Korte, 2001). Neurotransmitters like serotonin, γ-aminobutyric acid (GABA) and noradrenaline are the key molecules which imbalance may cause pathological anxiety (Lowry et al., 2008; Millan, 2003). For example, the imbalance between GABAergic inhibition and glutamatergic excitation seems to play a role in the development of different anxiety disorders (Herman et al., 2004; Takahashi et al., 2009). It is well-known that the benzodiazepines interacting with $GABA_A$ receptor subunits are employed in the clinical practice as anxiolytic medications (Blanchard et al., 2003; Haller, 2001). The modulation of activity of hypothalamic-pituitary-adrenal (HPA) axis plays an eminent role in the regulation of anxiety. The activation of HPA axis leads to the elevation of cortisol in humans and corticosterone in rodents, which is related to the increased level of anxiety (Engelmann et al., 2004). The development of anxiety disorders is complex depending on genetical, environmental and developmental factors. However, according to the contemporary understanding environmental factors play more apparent role in the development of anxiety disorders as compared to the genetic factors (Leonardo and Hen, 2006; Sullivan et al., 2000).

There is a number of different possibilities to induce anxiety-like state in rats and mice under experimental conditions. The predator odour is an innate stimulus in animals, inducing unconditioned fear, which is not controllable and not learned (Panksepp, 1998). In the laboratory, exposure of rats or mice to cloth impregnated with the cat odour elicits strong anxiety-like behaviour (freezing, avoidance of cloth with predator odour), even when they have never encountered such a stimulus in their life before (Dielenberg and McGregor, 1999, Blanchard et al. 2001, Calvo-Torrent et al., 1999, Panksepp, 1998). On the other hand, elevated plus-maze and zero-maze are classical ethological models for studying anxiety in rodents. Exploratory behaviour of rodents is characterized as a balance between novelty seeking and avoidance behaviour and it is correlated with the level of anxiety, which means that animals that explore open spaces actively are less anxious than animals avoiding the open parts of maze (Rodgers et al., 1997). The elevated plus-maze model is a feasible approach to screen anxiety under the influence of different pharmacological agents. The brain structures involved in the regulation of anxiety are the periaqueductal gray (PAG), amygdala, hippocampus and prefrontal cortex (Panksepp, 1998). The amygdala is a central structure of anxiety/fear circuits, it

extends influences from the temporal lobe (from central and lateral areas of amygdala) through the anterior and medial hypothalamus to the PAG and then down to the specific autonomic and behavioural areas of the lower brain stem and spinal cord, which controls the physiological symptoms of anxiety and fear (increase of heart rate, blood pressure, elevated startle response, freezing etc.) (Panksepp, 1998).

The herb *Cannabis sativa* (marijuana) has been used for a long time already due to its psychoactive properties. The main psychoactive component of marijuana, Δ^9-tetrahydrocannabidol (THC), was discovered by Gaoni and Mechoulam in 1964 and the first cannabinoid receptor (CB_1) was cloned in 1990 (Matsuda et al., 1990). CB_1 receptor is the most abundant G-protein coupled receptor in the brain being highly expressed in brain structures involved in the regulation of anxiety and mood disorders (amygdala, hippocampus, anterior cingulate, prefrontal cortex and PAG) (Herkenham et al., 1990, Tsou et al, 1998a). Two endogenous cannabinoids, anandamide (Devane et al., 1992, Felder et al., 1993) and 2-arachidonyl-glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) have been discovered in the brain and peripheral tissues. They both are able to activate the CB_1 receptors. Involvement of endocannabinoids in the regulation of anxiety is controversial, because low doses of THC have anxiolytic and high doses of THC cause anxiogenic effect, depending on individual and environmental context (Viveros et al., 2005). It has been shown that CB_1 receptor gene disruption and the pharmacological blockade of CB_1 receptor increases anxiety in rodents (Haller et al., 2002, Patel and Hillard, 2006).

Wolfram syndrome 1 gene (WFS1) is a new potential target in the regulation of anxiety. Mutations of WFS1 cause a progressive neurodegenerative disorder – Wolfram syndrome (DIDMOAD). Moreover, the heterozygous carriers of WFS1 mutation are more susceptibile to mood disorders (Swift et al., 1998). Kõks and colleagues demonstrated that the exposure of male Wistar rats to cat odour produces an up-regulation of *Wfs1* mRNA in the amygdala (Kõks et al., 2004). Wfs1 mRNA and protein is densely expressed in the brain structures related to behavioural adaptation – the amygdaloid complex, bed nucleus of stria terminalis, nucleus accumbens, prefrontal cortex and hippocampus (Takeda et al., 2001; Luuk et al., 2008). Moreover, *Wfs1*-deficient mice display more prominent anxiety-like behaviour and stronger elevation of corticosterone levels in response to stress compared to their wild-type littermates (Luuk et al., 2009).

The general aim of present study was to reveal a role of new potential targets, endocannabinoids and wolframin, in the adaptation to a novel environment and regulation of anxiety. For that purpose, two main behavioural approaches, a model of cat odour-induced anxiety and selection of rodents according to their exploratory behaviour, were employed. Rimonabant, an antagonist of CB_1 receptors, was used to reveal a role of the endocannabinoid system (ECS) in the development of cat odour-induced anxiety. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in order to establish changes in the ECS, GABA and *Wfs1* gene expression as a result of

anxiogenic manipulations in mice and rats. Corticosterone levels and oxidative stress index, as endogenous indicators of stressful influences, were measured in the blood samples of rats after the elevated plus-maze exposure.

REVIEW OF LITERATURE

1.1. The general background of anxiety

Anxiety is a physiological response in adaptation to stress in the novel and unknown situation. The appropriate strategies, corresponding to stressors, are used for survival in threatening conditions (Haller, 2001). Active coping strategies (e.g. confrontation, fight, escape) are usually evoked if the stressor is controllable or escapable (threat of a predator or conspecific). Passive coping strategies (e.g. quiescence, immobility, decreased responsiveness to the environment) are usually elicited if the stressor is inescapable (Koolhaas et al., 1999). Active coping is related to the release of noradrenaline and adrenaline into the blood (activation of sympathoadrenal system) (Engelmann et al., 2004). On the contrary, passive coping is associated with the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Engelmann et al., 2004) (Figure 1). Anxiety disorders differ from physiological anxiety by the quantitative means, the ongoing anxiety responses are inadequately strong or lasting too long. Accordingly, experience of anxiety is excessive and is elicited even in the absence of potential threats. This kind of anxiety is maladaptive and sometimes it appears for no apparent reasons (Millan, 2003). Long-lasting anxiety definitely affects the homeostasis of body. These changes include autonomic (increase in heart rate, blood pressure etc.) and/or behavioural parameters (inhibition of ongoing behaviours, scanning, avoidance of the source of danger) (Blanchard et al., 2001). HPA activation increases the level of adrenocorticotropin hormone (ACTH) and subsequent release of glycocorticoids (cortisol in humans and corticosterone in rodents) in the circulating blood (Engelmann et al., 2004). Long-lasting aversive events may lead to a sustained activation of HPA-axis, which is linked to development of anxiety and mood disorders (McEven, 2000).

Anxiety can be divided in various forms, including “state” and “trait” anxiety (Belzung and Griebel, 2001). “State” anxiety, which is also called “normal” anxiety, is a change of individual response to the dangerous situation at a particular moment of time and it is increased by the presence of anxiogenic stimuli. “Trait” or “pathological” anxiety is persistent and it is related to the characteristics of personality. These persons are much more prone to respond to a various kind of stimuli and will tend to worry also in situations which for most individuals would not represent a source of threat (Belzung and Griebel, 2001; Kalin et al., 2001).

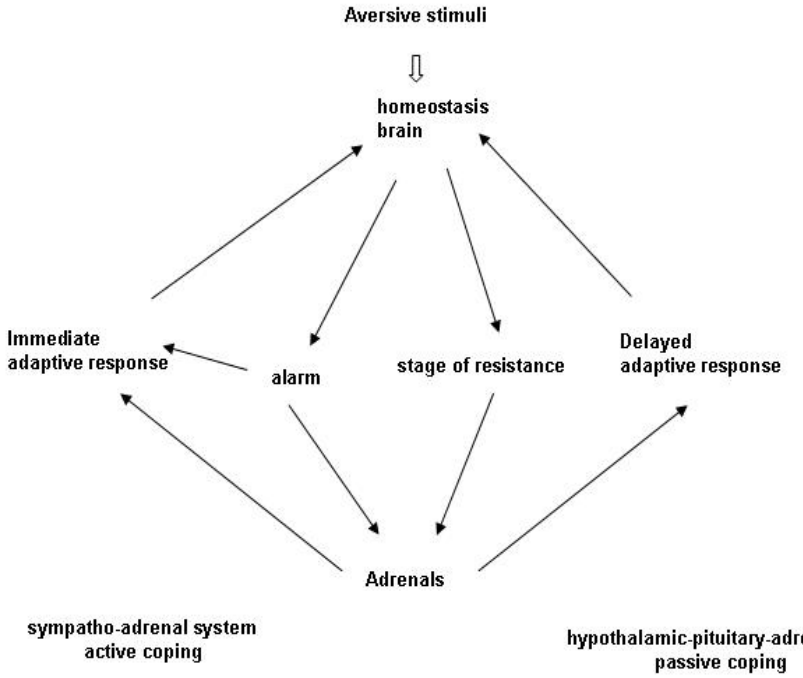

Figure 1. Neuroendocrine systems integrate adaptative responses to aversive stimuli. The passive coping strategies and active coping strategies are triggered by different pathways (Adapted from Engelmann et al., 2004).

1.2. The neural circuits of fear and anxiety

The experience of anxiety and fear is controlled by a modular neural system including regions in the brainstem, hypothalamus, and deeper parts of the temporal lobe (Davis et al., 1997; LeDoux, 2000). The key structure mediating anxiety and fear responses is amygdala in the brain (LeDoux, 2000, 2003). The amygdala possesses an extensive pattern of reciprocal connections with cortical, limbic, monoaminergic and other structures implicated in the emotional, cognitive, autonomic and endocrine response to stress (Millan, 2003; LeDoux, 2000; Kjelstrup, 2002). Amygdala complex is defined by several distinct groups of cells, including the lateral, basal, accessory basal nuclei, which is collectively termed basolateral amygdala. Basolateral amygdala has a crucial role in mediating anxiety and fear (Davis and Whalen, 2001; LeDoux, 2000). Several structures surrounding the basolateral amygdala, including the central, medial and cortical nuclei, are traditionally included into the 'amygdaloid complex' (Davis and Whalen, 2001). Immediately downstream of basolateral amygdala are the central nucleus of the amygdala (CeA) and bed nucleus of stria terminalis (BNST) (Walker et al., 2003; McDonald, 2003). The BNST and CeA are anatomically, neurochemically, cytoarchitectonically and embryologically

related and share similar functions (Alheid et al., 1995). The centromedial amygdala, BNST and neural column interconnecting these nuclei are called together as the "extended amygdala" (Alheid and Heimer, 1988; Heimer, 2003). The basolateral amygdala receives sensory information from the thalamus, hippocampus and cortex and projects to the CeA or BNST, which then activates or modulates synaptic transmission in hypothalamic and brainstem target areas that directly mediate specific signs of fear and anxiety (Figure 2.) (Davis and Whalen, 2001; Davis, 2006, Dielenberg et al., 2001; McGregor et al., 2004; Davis, 2000; LeDoux, 2000, 2003). CeA-mediated behaviours may represent stimulus-specific fear responses, whereas BNST-mediated behaviours are more concerned with anxiety (Davis, 2006; Davis and Shi, 1999; Davis et al., 1997). The basolateral amygdala (BLA) and medial nucleus (MeA) of amygdala are involved in modulating predator odour-induced unconditioned fear and contextual fear conditioning (Takahashi et al., 2007; Li et al., 2004), whereas lesions of the central amygdala (CeA) produce profound deficits in both the acquisition and expression of conditioned fear (Maren, 2001; Young and Leaton 1996; Goosens et al., 2000). Local infusion of the $GABA_A$ antagonist bicuculline or NMDA or AMPA into the basolateral nucleus of amygdala increases blood pressure and heart rate (Sanders and Shekhar, 1991; Soltis et al., 1997).

The locus coeruleus (LC) is a compact nucleus in the dorsal pons comprising the majority of noradrenergic neurons in brain (Aston-Jones and Bloom, 1981). Activation of locus coeruleus and increased release of noradrenaline in the hypothalamus, amygdala and locus coeruleus increases anxiety in animals (Tanaka et al., 2000). Serotonergic neurones originating in the raphe nuclei provide a massive input to the corticolimbic structures involved in the control of anxious states. The dorsal raphe nucleus (DRN) primarily innervates the frontal cortex, dorsal hippocampus and amygdala, while the median raphe nucleus (MRN) principally projects to the dorsal and ventral hippocampus, septum, nucleus accumbens and hypothalamus (Kirby et al., 2003; Millan, 2003).

It is apparent that the midbrain periaqueductal gray (PAG) is related to the stereotyped, reflexive, autonomic and behavioural fight/flight response to unconditioned fear (Bandler et al., 2000; Blanchard et al., 2001). The PAG is also an important effector nucleus for stress-induced freezing, because lesioning of either the PAG or the central nucleus of the amygdala results in complete abolition of this response (LeDoux et al., 1988; Amorapanth et al., 1999). The PAG contains distinct neural substrates mediating active or passive coping strategies. Active coping, related with defensive behaviours is evoked by activation of either the dorsolateral or lateral PAG (Bandler et al., 2000), whereas the ventrolateral PAG axis is involved in the occurence of conditioned freezing behaviour (Bandler et al., 2000; Keay and Bandler, 2001; Brandão et al., 2008). Unlike the amygdala and ventral PAG lesions, hippocampal lesions do not block the freezing behaviour that occurs immediately following the footshock (Kim et al., 1993).

The involvement of all cortical regions (insular, orbital, entorhinal, temporal, association, frontal, pre-frontal, cingulate and parietal) has been described in

response to fear and stress (Millan, 2003; Bechara et al., 2000; Grachev et al., 2002; Pralong et al., 2002). The connection between BLA and prefrontal cortex is critical for representation of unconditioned stimulus (Davis, 1992). The orbitofrontal cortex is important for desicion making (Bechara et al., 2000).

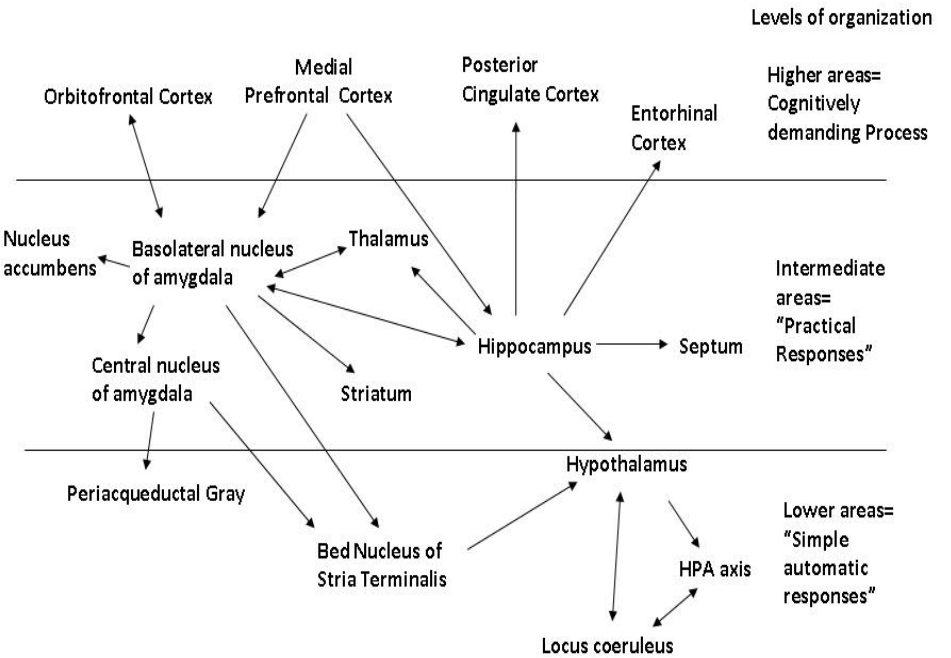

Figure 2. The simplified model of anxiety-related neural circuits (adapted from Finn et al., 2003; Millan 2003, Davis et al., 2003).

1.3. Animal models of anxiety

The animal models include both conditioned and unconditoned responses to aversive and threating stimuli. Also, the genetic models are available for studying the mechanisms of fear and anxiety (Finn et al., 2003). Most studies with animal models of anxiety have passed the validation with pharmacological compounds, where the $GABA_A$ receptor agonists benzodiazepine anxiolytics have been the „golden standards“ (Rodgers et al., 1997). Charles Darwin (1872) established that the defensive pattern of other species is essential evolutional precursor for human fear and anxiety reactions (Blanchard et al., 1991). Also, the neural structures (e.g. amygdala, PAG) involved in detecting danger and producing defence responses are similar among vertebrates (Davis et al., 1992). Being conserved during the evolution it is conceivable to study animal behaviour and taking lessons from that helps us to get a deeper understanding

about the human behaviour (LeDoux, 1995). There is a consensus that the animal models must follow some minimum requirements (Belzung and Griebel, 2001):

1. Face validity – the animal model must be identical to the behavioural and physiological responses observed in human. The isomorphism between human and animal behaviours has to be placed in the context of the theory of the evolution, suggesting that a given pattern may be selected according to its survival value.
2. Construct validity – the human and animal responses are homologous. The model is consistent with theoretical rationale underlying the human behaviour. The etiology of anxiety behaviour and biological factors underlying anxiety may be similar in animals and humans.
3. Predictive validity – the animal model should be sensitive to clinically effective pharmacological agents. Anxiogenic compounds should elicit opposite effects, while agents that have no effect in the clinical context should be ineffective in these models.

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

Table 1. Some commonly used animal models of anxiety

Unconditioned responses	**Conditioned responses**
Anxiety/defence test battery	Active/passive avoidance
Elevated plus-maze and zero-maze	Conditioned emotional response (CER)
Fear/defence test battery	Conditioned taste aversion
Free exploration	Conflict tests (pigeon and primates)
Holeboard	Defensive burying
Human treat (primates)	dPAG stimulation
Light/dark exploration	Fear potentiated startle
Open field	Four plate test
Social competition	Geller-Seifter conflict
Social interaction	Learned helplessness
Ultrasonic vocalization (pups)	Ultrasonic vocalization (adults)

1.3.1. Cat odour-induced anxiety in rodents

The predator-pray relationship is an innate mechanism and evolutionally important adaptation in natural selection. Adequate responses to predator's stimuli are ecologically relevant for animal's survival (Dielenberg and McGregor, 1999, Blanchard et al., 2001). Currently, it has been shown in many different studies that the predator odour, like exposure to predator itself, can increase anxiety-like behaviour (Adamec and Shallow, 1993; Blanchard et al., 2003; Dielenberg et al., 1999; Li et al., 2004; McGregor et al., 2002; McGregor and Dielenberg,

1999). Odours of predator trigger a range of behavioural, endocrine and neuro-chemical changes in rodents (Adamec et al., 2006; Blanchard et al., 1998; 2003). Two commonly used predator odours for rodents are cat odour (urine, fur) (Blanchard et al., 2003, Kõks et al., 2002, 2004) and TMT, a synthetic compound of fox faeces (Vernet-Maury et al., 1992; Wallace and Rosen, 2000). The chemical components of the cat odour stimulus that give rise to anxiety are currently unknown making standardisation of use of this stimulus across studies difficult (McGregor and Dieleberg, 1999). Compared to TMT, it has been found that only cat odour can elicit anxiety-like behaviour in the EPM, inhibit activity in the novel environment and adaptation (McGregor et al., 2002; Staples et al., 2008b). Therefore, the TMT is probably merely aversive signal but not anxiogenic (Blanchard et al., 2003; McGregor et al., 2002).

Cat odour is usually obtained from the domestic animals, and it is presented to rats as worn collar or cloth that has been rubbed against cat fur/skin (Blanchard et al., 2003). Cat odour increases freezing, immobility and risk assessment, heightens responses to the aversive stimuli, makes animals avoid the cloth impregnated with cat odour (Blanchard et al., 2003; Dielenberg et al., 1999, 2001; Li et al., 2004, Adamec et al., 1998, McGregor et al., 2002) and induces marked neuro-endocrine changes (Morrow et al., 2000). Laboratory rodents never seen cat before develop anxiety-like state after exposure to cat odour or living cat (Blanchard et al., 2003). By comparing different sources of cat odour, it has been shown that in a novel environment the cat fur odour elicited more behavioural inhibition (freezing, decrease of rearings) than the cat urine (Muñoz-Abellán et al., 2008; Blanchard et al., 2003). Also, rats exposed to the cat fur/skin odour showed anxiety-like behaviour in the elevated plus-maze even one week after initial exposure to the stressor indicating a long-lasting anxiety and fear conditioning (Blanchard et al., 2003; Muñoz-Abellán et al., 2008). Although, it has been shown that cat odour exposure affects fear conditioning one week after the initial exposure, it does not cause long-lasting anxiety-like behaviour in the EPM (Muñoz-Abellán et al., 2009). The exposure of rodents to cat odour elicits HPA axis activation immediately after the initial exposure to the cat fur odour, but long-term anxiety effect being present in behavioural studies is not accompanied by the elevated levels of ACTH and corticosterone in the circulating blood (File et al., 1994; Muñoz-Abellán et al., 2008). It is likely that the long-term behavioural inhibition after initial stressor may not always reflect increased anxiety in rodents. Rather, it may be an example of cautious behaviour in any novel situation, based on the previous experience that challenge to the cat odour stressor was associated with exposure to unknown environment (Muñoz-Abellán et al., 2008). Probably the activation of the HPA axis reflects rather fear conditioning than the long-lasting anxiety-like behaviour in the EPM (Muñoz-Abellán et al., 2008).

c-Fos-immunohistochemistry has been used extensively to identify the neural substrates when rats or mice are exposed to the cat odour (Dieleberg et al., 2001; McGregor et al., 2004). c-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors. Expression of c-fos

is an indirect marker of neuronal activity, because c-fos is often expressed when firing of neurons is increased (Herrere and Robertson, 1996). The exposure to cat odour induces c-fos activation in the different brain structures, which include the medial amygdala (MeA), BNST, various medial hypothalamic structures, PAG (Dieleberg et al., 2001; McGregor et al., 2004), prelimbic region of medial prefrontal cortex and ventral part of lateral septum (McGregor et al., 2004). Although the CeA plays a critical role in the production of conditioned fear response (Davis and Whalen, 2001; LeDoux, 2000), the cat odour exposure activated MeA, without changing the activity of CeA (Dielebelg et al., 2001; McGregor et al., 2004; Takahashi et al., 2007). Also, the ibotenic acid lesions of the BLA produced a significant reduction in freezing when rat was exposed to the cat odour (Takahashi et al., 2004). The exposure to cat odour increased the c-fos expression in the glomerular, mitral, and granule cell layers of the posterior accessory olfactory bulb (AOB) (McGregor et al., 2004; Staples et al., 2008a; 2008b), which receives the inputs from the vomeronasal organ. The activation of the accessory olfactory system supports the hypothesis that cat odour may be processed as a pheromone-like stimulus (Panksepp, 1998). The evidence shows that after lesioning of the vomeronasal nerve rats do not display anxiety-like state after the cat odour exposure, but the disruption of the main olfactory system did not change the cat odour induced anxiogenic effect (Panksepp, 1998).

1.3.2. Exploratory models of anxiety

Elevated plus-maze (EPM) is an ethologically based and pharmacologically validated test of anxiety-like behaviour. Exposure of rodents to the EPM itself is stressful and anxiogenic. EPM is used as a screening test for putative anxiolytic compounds and as a general research tool in neurobiological research of anxiety (Pellow et al., 1985). Exposure to the EPM usually induces suppression of exploratory behaviour in rodents. Exploratory behaviour is the tendency to explore or investigate a novel environment. It is considered as motivation not clearly distinguishable from curiosity. Handley and Mithani (1984) described the assessment of anxiety behaviour of rodents by using the ratio between time spent on open arms and total time of experiment. Unlike other behavioural assays used to assess anxiety responses that rely upon the presentation of noxious stimuli (i.e., electric shock, food/water deprivation, loud noises, exposure to predator odour, etc.) that typically produce a conditioned response, the EPM is based on rodents' proclivity toward dark, enclosed spaces (approach) and an unconditioned fear of heights and open spaces (avoidance). It has been shown that according to the exploratory behaviour in the EPM male Wistar rats can be divided into low exploratory activity (LEA) and high exploratory activity (HEA) (Rägo et al., 1988). HEA rats are more motivated of "novelty" seeking, despite this activity elicits in these animals higher stress responses. HEA rats showed an increased basal CRH mRNA in the PVN and

low levels of CRH in the CeA compared to LEA animals (Landgraf and Wigger, 2002). A decrease of glucocorticoid receptor mRNA in the hippocampus of HEA rats demonstrates a possible role of these receptors in individual differences of novelty-seeking behaviour (Kabbaj et al., 2000).

Another exploratory model of anxiety is the elevated zero-maze. The elevated zero-maze is a modification of EPM model of anxiety in rodents, which incorporates both traditional and novel ethological measures in the analysis of drug effects. The novel design comprises an elevated annular platform with two opposite enclosed quadrants and two open, removing any ambiguity in interpretation of time spent on the central square of traditional design and allowing uninterrupted exploration (Shepherd et al. 1994). Anxiety is measured by the amount of time spent by rodents on the open arms.

2. Novel targets in the study of anxiety

2.1. Endocannabinoid system

2.1.1 Overview of the endocannabinoid system

Endocannabinoid system comprises of cannabinoid receptors, endogenous ligands (endocannabinoids) and enzymes responsible for the synthesis and degradation of endocannabinoids. The endocannabioid signaling systems are related with many different functions – pain perception, emotions, cognition and motivations (Piomelli, 2003). Two subtypes of high affinity cannabinoid receptors have been cloned and characterized so far: CB_1 receptors (Devane et al., 1992; Matsuda et al., 1990) and CB_2 receptors (Munro et al., 1993; Mechoulam et al. 1995; Sugiura et al. 1995). It has been shown that anandamide has affinity to GPR55, an orphan G protein-coupled receptor, suggesting that this receptor might represent a novel target of cannabinoid action (Sawzdargo et al, 1999; Ryberg et al. 2007; Lauckner et al., 2008). CB_1 receptor is one of the most abundantly expressed G-protein coupled receptors in the brain, primarily located on the axons and synaptic terminals of neurons (Mackie, 2005). CB_1 receptors are present at high density within the olfactory bulb, neo- and paleocortical regions (neocortex, pyriform cortex, hippocampus, and amygdala), several parts of basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex, and brainstem nuclei (Herkenham et al., 1990, Tsou et al, 1998a). CB_2 receptors are mainly located in the immune and blood cells, regulating immune responses (Klein et al., 1998). The main lipid endogenous cannabioinoids, which activate cannabinoid receptors, are anandamide (Devane et al. 1992) and sn-2 arachidonyl-glycerol (2-AG) (Suguira et al, 1995). In addition, two other ligands for cannabinoid receptors have been established: 2-AG-ether (noladin ether) and O-arachidonoylethanolamine (virodhamine).

CB_1 and CB_2 receptors are G-protein-coupled receptors (Di Marzo et al., 1998, Dewey, 1986). The activation of CB_1 or CB_2 receptors inhibits adenylate cyclase (AC) (Piomelli, 2003; Felder et al, 1993; Vogel et al., 1993; Bayewitch et al., 1995), N-, Q-, L-type Ca^{2+} channels (Mackie et al, 1993; Felder et al., 1993; Bayewitch et al., 1995; McAllister and Glass, 2002) and TASK-1 K^+ channels (Maingret et al., 2001). CB_1 receptor agonists can inhibit glutamatergic transmission in the cerebellum (Levenes et al., 1998) and hippocampus (Shen et al., 1996; Misner and Sullivan, 1999; Sullivan, 1999), whereas GABAergic synaptic transmission is inhibited by endocannabinoids in the substantia nigra (Chan et al., 1998), striatum (Szabo et al., 1998), medulla (Vaughan et al., 1999) and hippocampus (Hoffman and Lupica, 2000).

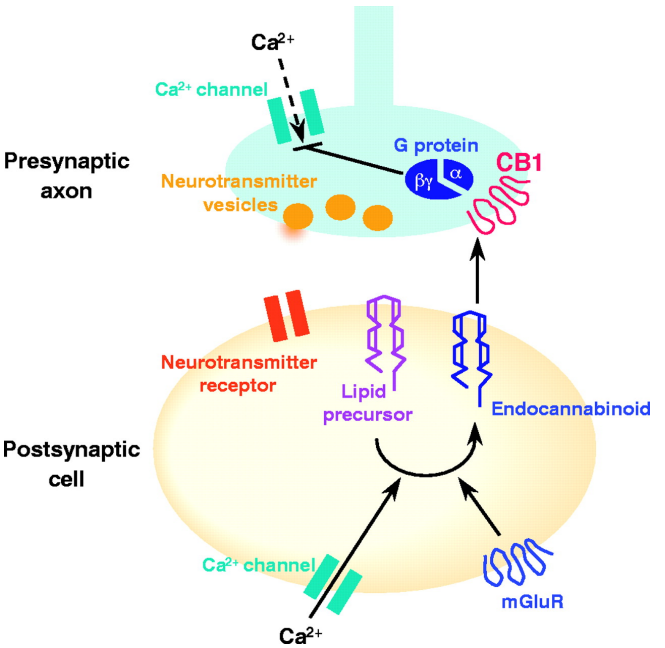

Figure 3. Retrograde signaling by endocannabinoids. Postsynaptic depolarization opens voltage-dependent Ca^{2+} channels; postsynaptic Ca^{2+} then activates enzymes that synthesize endocannabinoids from lipid precursors. Activation of postsynaptic metabotropic glutamate receptors (mGluRs) can also generate endocannabinoids, possibly by the activation of phospholipase C, generating diacylglycerol, which is then cleaved by diacylglycerol lipase to yield 2-arachidonylglycerol. Endocannabinoids then leave the postsynaptic cell and activate presynaptic CB_1 receptors. G-protein activation liberates $G^{\beta\gamma}$, which then directly inhibits presynaptic Ca^{2+} influx. This decreases the probability of release of a vesicle of neurotransmitter (adapted from Wilson and Nicoll, 2002).

2.1.2. Endocannabinoids synthesis, release and degradation

Unlike traditional neurotransmitters that are stored in synaptic vesicles, the endocannabinoids are synthesized "on demand" through cleavage of membrane precursors at post-synaptic sites of neurons after increase in neural activity and calcium ion influx, and they are then released into the synaptic cleft (Di Marzo et al., 1998). This "on-demand" model implicates the participating biosynthetic and degradative enzymes as key regulators of lipid signaling tone. Endocannabinoids act as retrograde synaptic messengers (Figure 3). They are released from the postsynaptic neurons activating CB_1 receptors on presynaptic axons, the activation of which inhibits pre-synaptic Ca^{2+} influx into the axonal terminals and, therefore, reduces the release of neurotransmitters (acetylcholine, GABA, glutamate, noradrenaline) (Piomelli, 2003; Wilson and Nicoll, 2002). The physiological outcome of this inhibition clearly depends upon which cell populations are being activated. For example in the hippocampus, the pre-synaptic inhibition of Ca^{2+}-channels on the axonal terminals, synapsing with the pyramidal neurons, has been correlated with the ability of endocannabinoids to inhibit the long-term potentiation (McAllister and Glass, 2002). This mechanism may be one way by which cannabinoids alter learning and memory.

Anandamine (N-arachidonylethanolamide), an arachidonic acid derivative was first discovered in porcine brain and was identified as a first endogenous ligand for the CB_1 receptor (Devane et al., 1992, Felder et al., 1993). Anandamide is derived from combination of arachidonic acid and ethanolamine coupled through an amide linkage (Felder et al., 1993). Anandamide can bind in addition to CB_1 and CB_2 receptors also to the third cannabinoid receptor GPR55 (Ryberg et al., 2007; Lauckner et al., 2008; Sawzdargo et al., 1999) and the vanilliod type I channel (TRPV) receptors (Cristino et al., 2006). Anandamide produces most of its pharmacological effects through the CB_1 receptor, but its influence on the locomotor activity seems to be mediated via the non-CB_1 receptor mechanisms (Wise et al., 2007). Anandamide transport across cellular membranes involves a protein carrier molecule, although the possible carrier molecule has not been isolated and characterized to date (Hillard and Jarrahian, 2000). The major biosynthetic pathway for the synthesis of anandamide and other N- acylethanolamines consists of two enzymatic reactions. The first step is generation of *N*-arachidonoyl phosphatidylethanolamines (NAPEs), which are produced through the transfer of arachidonic acid from the *sn*-1 position of phospholipids (e.g., phosphatidylcholine, PC) to the primary amine of phosphatidylethanolamine. This reaction is catalyzed by *N*-acyltransferase (NAT), which is a Ca^{2+}-dependent enzyme (Cadas et al., 1997, Sugiura et al., 1996; Liu et al., 2006; Astarita and Piomelli, 2009). The second step is the direct conversion of NAPE to anandamide by a NAPE-specific phospholipase D (PLD). *Nape-pld* has been cloned (Okamoto et al., 2004) and the purified protein has been characterized (Wang et al., 2006). NAPE-PLD belongs to the zinc metallohydrolase family of the β-lactamase fold (Okamoto et al., 2004). Unexpectedly, it has been shown that no significant reduction of the level of

anandamide was found in the brain in the *Nape-pld* knockout mice (Leung et al., 2006). Furthermore, tissues from *Nape-pld*−/− mice contained an enzymatic activity capable of converting NAPE to anandamide in a Ca^{2+}-independent manner (Leung et al., 2006), suggesting existence of parallel biosynthesis pathways. Other possible ways for the synthesis of anandamide contain the hydrolysis of NAPE by a NAPE specific phospholipase A1/A2 (e.g., hydrolase-4, ABHD-4) to form the intermediates lyso-NAPE and glycerophosphoanandamide, which are then cleaved by PLD and the hydrolysis of NAPE catalyzed by a NAPE-specific phospholipase C to yield the intermediate phosphoanandamide, which can then be cleaved by a lipid phosphatase such as protein tyrosine phosphatase (Liu et al., 2006; Astarita and Piomelli, 2009). Anandamide degradation requires its transport across the plasma membrane, which may occur by carrier-mediated facilitated diffusion (Fegley et al., 2004). Once inside the cell, anandamide is hydrolyzed to arachidonic acid and ethanolamine by intracellular, membrane-bound fatty-acid amide hydrolase (FAAH) (Cravatt et al., 1996), which is found predominantly in neurons postsynaptic to axon terminals expressing the CB_1 receptor (Tsou et al., 1998b).

2-arachidonoyl-glycerol (2-AG), the most abundant endocannabinoid in nervous and other tissues (Mechoulam et al., 1995; Sugiura et al., 1995), is synthesized in the presence of Ca^{2+} by the hydrolysis of diacylglycerol (DAG) by sn-1-specific DAG lipase (DAGL) (Bisogno et al., 2003). Differently from anandamide, the degradation of 2-AG to arachidonic acid and glycerol occurs via multiple enzymatic pathways. It is primarly degraded by a serine hydrolase – monoacylglycerol lipase (MGLL), which is originally purified and cloned from the adipose tissue (Karlsson et al., 1997; Dinh et al., 2002). MGLL is localized in the presynaptic axon terminals of the hippocampus, cerebellum, and amygdala, including the terminals of GABA-ergic interneurons (Gulyas et al., 2004). There are two other enzymes responsible for the hydrolysis of 2-AG – ABHD12 and ABHD6. Anandamide degrading enzyme FAAH is also involved in the hydrolysis of 2-AG activity (Blankmann et al., 2007). Probably the existence of multiple enzymatic pathways shows that in cells, where no MGLL is detected, other enzymes can take the responsibility for the hydrolysis of 2-AG, because all these different enzymes show distinct subcellular localization (Blankman et al., 2007).

2.1.3. Involvement of ECS in the regulation of anxiety

Role of endocannabinoids in the regulation of anxiety is rather controversial (Haller et al., 2002, Viveros et al., 2005) depending on the individual and environmental context and the dose of compounds administered. It has been shown that low doses of THC are anxiolytic, whereas high doses are ineffective in this respect or even anxiogenic (Patel and Hillard, 2006). CB_1 receptor mRNA is expressed densely and relatively specifically within the rat BLA, a region implicated in the extinction of conditioned fear, and only modest expres-

sion could be seen in the medial and central nuclei (Chhatwal et al., 2005). CB_1 receptors affect GABA- and glutamate-ergic systems, which play opposite role in the regulation of anxiety. Therefore, the anxiety-related effects of cannabinoids depend largely on the equilibrium between their effects on glutamate- and GABA-ergic neurotransmission (Haller et al., 2007). FAAH inhibitors can reduce anxiety-like behaviour in a variety of species and strains, and in a variety of anxiety tests (Cippitelli et al., 2008; Scherma et al., 2008; Moreira et al., 2008; Hill et al., 2007; Patel and Hillard, 2006; Rubino et al., 2008; Rutkowska et al., 2006). CB_1-knockout mice displayed increased anxiogenic responses in the light-dark box, plus-maze, and social interaction tests, an increased aggressive response in the resident-intruder test, and marked alterations in the HPA axis coupled with impaired action of known anxiolytic drugs such as buspirone and bromazepam (Haller et al., 2002, 2004; Martin et al., 2002; Urigüen et al., 2004). Stimulation of CB_1 receptors in rodents activates the HPA axis through the release of CRH (Weidenfeld et al., 1994; Wenger et al., 1997; Martín-Calderón et al., 1998; Manzanares et al., 1999; Marco et al., 2004), which could account for the anxiogenic-like action of high doses of cannabinoids (Rodriguez de Fonseca et al., 1996; Marín et al., 2003). In contrast, there are also examples of negative modulation of HPA function by endocannabinoids (Di et al., 2003; Patel et al., 2004).

2.2. WFS1 and emotional behaviour

2.2.1. WFS1 and Wolfram syndrome

Wolfram syndrome (DIDMOAD), first described by Wolfram and Wagner (1938), is a rare autosomal recessive disorder characterized by early-onset, non-autoimmune diabetes mellitus, optic atrophy, deafness and diabetes insipidus (Domenech et al., 2006). Wolfram syndrome is caused by mutations in the coding region of the *WFS1* gene, which is located in human on the short arm of chromosome 4 (4p16.3). *WFS1* encodes wolframin, an endoplamic reticulum (ER) transmembrane glycoprotein of 890 amino acid residues and molecular mass of ~100 kDa (Strom et al., 1998, Inoue et al., 1998, Takeda et al., 2001; Osman et al., 2003). Wolframin contains nine transmembrane segments, whereas the N-terminus is located in the cytoplasma and C-terminus in the ER lumen (Strom et al., 1998, Hofman et al., 2003). The mRNA and protein of Wfs1 are ubiquitously expressed, whereas the highest levels of expression are detected in the brain, heart, pancreatic β-cells and lung (Inoue et al., 1998). In Xenopus oocytes the level of cytosolic Ca^{2+} in the oocytes was increased by over-expression of wolframin (Osman et al., 2003). Also, wolframin regulates positively ER Ca^{2+} levels by increasing the rate of Ca^{2+} uptake (Takei et al., 2006; Takeda et al., 2001). In addition, it has been shown that N-terminal cytoplasmic domain of wolframin binds with Ca^{2+}/calmodulin (CaM) complex. The minimum region responsible for Ca^{2+}/CaM binding is located from Glu90

to Trp166 residues of WFS1. Moreover, three single mutations (Ala127Thr, Ala134Thr, and Arg178Pro) associated with Wolfram syndrome and being conserved in human and rat, completely abolished CaM binding of wolframin (Yurimoto et al., 2009). These results suggest that wolframin may be a novel ER Ca^{2+} channel or a regulator of ER Ca^{2+} channel activity (Yurimoto et al., 2009). Recently, the interaction between the C-terminus and transmembrane domain of wolframin and sodium-potassium ATPase β1 subunit in ER lumen has been shown (Zatyka et al., 2008).

The endoplasmic reticulum (ER) is responsible for the correct folding, assembly and post-translational modification of newly synthesized proteins, and a cellular calcium store. Unfolded or misfolded proteins are degradated by UPR induced response, which is mediated by IRE1, PERK and ATF6 signaling pathways (Lai et al., 2007). The disruption of *Wfs1* induces ER-stress and causes the apoptosis specifically in the pancreatic β-cells and neurons in the brain (Riggs et al., 2005; Yamada et al., 2006). The upregulation of WFS1 in response to ER sress in pancreatic β-cells is mediated via IRE1 and PERK signaling pathways (Fonseca et al., 2005). Pancreatic β-cells are responsible for insulin secretion and biosynthesis, playing important role in glucose metabolism. Chronic ER stress in beta cells induces CHOP expression and leads to apoptosis, which is responsible for the development of type 2 diabetes (Araki et al., 2003). As the symptoms of Wolfram syndrome include diabetes and psychiatric abnormalities, probably the role of WFS1 is to protect pancreatic β-cells and brain neurons from apoptosis.

Recently, it has been shown that valproate activates the *WFS1* promotor and induces *WFS1* mRNA expression in neuronal cells without activating other components of ER stress signaling (Kakiuchi et al., 2009). Valproate induces the dissociation of *WFS1* from GPR94 (the component of UPR response) increasing the free WFS1 level and therefore preventing cells from ER-stress (Kakiuchi et al., 2009).

Mutations of the *WFS1* gene, causing Wolfram syndrome, are mainly located in exon 8 and include stop, frameshift, deletions and missense mutations (Inoue et al., 1998). It has been shown that 60% of Wolfram syndrome patients have psychiatric symptoms with especially high prevalence of depression (Swift and Swift, 2005; Swift et al., 1990). Morever, the heterozygous carriers of *WFS1* mutations have a 26-fold higher likelihood for psychiatric hospitalization (Swift and Swift, 2000) and suicidal behaviour compared to non-carriers (Aluclu et al., 2006). Also, it has been shown that *Wfs1* is up-regulated in the CA1 and amygdala region in PTSD-like rats (Kesner et al., 2009) and after the exposure to the cat odour in the amygdaloid area (Kõks et al., 2002, 2004).

2.2.2. Localization of Wfs1 protein in the brain

Wolframin is expressed in specific brain areas mainly related to emotional behaviour and motivations (Takeda et al., 2001; Luuk et al., 2008). The regional distribution of Wfs1 protein and mRNA has been explored in the rat brain by immunohistochemistry and *in situ* hybridization histochemistry, respectively. Immunohistochemical analysis with anti-Wfs1-C antibody of the rat brain demonstrated the presence of Wfs1 immunoreactivity in the neuronal cells in the forebrain, midbrain and lower brainstem. The especially high density of immunostaining was observed in the hippocampus CA1 region, in the central amygdaloid nucleus, in the olfactory tubercle and neurons in the dorsal part of the lateral septal nucleus and the oval nucleus of the bed nuclear group of the stria terminalis (Takeda et al., 2001). X-Gal staining on *Wfs1*-β-glactosidase knock-in mice and immunohistochemical staining of Wfs1 established the highest expression in the forebrain structures (Luuk et al., 2008). Very high expression of wolframin was detected in the insular, perirhinal and postrhinal cortices, and also in the olfactory tubercle, nucleus accumbens, central amygdaloid nucleus and dorsal part of lateral bed nucleus of stria terminalis. In the hippocampus wolframin expression was detected only in the CA1 region and parasubiculum. In the diencephalon, midbrain and brainstem only moderate expression of wolframin was found (Luuk et al., 2008). The high expression of wolframin in neurons of the central extended amygdala, ventral striatum, hippocampus CA1, olfactory tubercles and superficial layer of allocortex (Takeda et al., 2001; Luuk et al., 2008) suggests a possible involvement of Wfs1 protein in emotional responses, especially in the regulation of anxiety and fear. The extended amygdala and lateral division of bed nucleus of stria terminalis share structural and functional similarities (Walker and Davis, 2008). Both these structures have very similar efferent connections to various hypothalamic and brain stem target areas known to be involved in specific signs and symptoms of fear and anxiety (Davis, 1998). Anxiety disorders are commonly treated by administration of bensodiazepine anxiolytics, which mediate their sedative, amnestic and anxiolytic actions via interaction with $GABA_A$ receptors (Wisden et al., 1992). The structures of the central extended amygdala are characterized by high expression of alpha2 subunit of $GABA_A$ receptors (Kaufmann et al., 2003). There is evidence that the anxiolytic as well as the stimulating actions of bensodiazepine anxiolytics are mediated via the alpha2 subunit of $GABA_A$ receptors, probably located in the extended amygdala (Kaufmann et al., 2003).

2.2.3. Phenotype of *Wfs1*-deficient mice

Currently two different *Wfs1*-deficient mice have been generated (Ishiara et al., 2004; Luuk et al., 2009) showing impaired behavioural (Kato et al., 2008; Luuk et al., 2009) and metabolic phenotype (Ishiara et al., 2004). Inactivation of the

Wfs1 gene by inserting a neomycin-resistence gene into the second exon of the *Wfs1* gene caused insufficient insulin secretion due to progressive β-cell loss in the pancreas and impaired stimulus-secretion coupling in the β-cells. However, the development of diabetes was dependent on the genetic background of mice. Back-crossing of mice from 129Sv to C57Bl/6 background significantly counteracted to the loss of β-cells and development of diabetes (Ishiara et al., 2004). Disturbances in glucose metabolism and insulin secretion were also shown in mice with the 8th exon invalidation (Luuk et al., 2009). Behavioural studies established similarities as well as differences between these two lines of *Wfs1*-deficient mice (Kato et al., 2008; Luuk et al., 2009). There are probably two main reasons for described differences. First, Luuk et al. (2009) performed their experiments with F2 hybrids with randomly mixed 129Sv and C57Bl/6 background, whereas Kato et al. (2008) did their studies after extensive back-crossings into C57Bl/6 background. It has been shown that backcrossing into C57Bl/6 significantly protects mice against the development of diabetes due to the lack of Wfs1 protein (Ishiara et al., 2004). On the other hand, the majority of mutations in the case of Wolfram syndrome occur in the 8th exon (Luuk et al., 2009). It is possible that the symptoms caused by the complete lack of Wfs1 protein are less severe compared to the presence of truncated Wfs1 protein (Kato et al., 2008). Invalidation of exon 2 of the *Wfs1* gene did not change the level of anxiety in the elevated plus-maze compared to wild-type animals. *Wfs1* knockout mice showed longer escape latency and lower number of avoidance during the active avoidance test. Also, longer latency to move in the passive avoidance test was established. No changes were detected in pain sensitivity and sensorimotor functions. All these behavioural changes mimic at least partly the symptoms of depression. The lack of immobility in the third day of the forced swimming test and decreased social interaction show retardation of emotionally triggered motion (Kato et al., 2008). Invalidation of exon 8 of the *Wfs1* gene impairs the adaptation of mice in a novel and stressful environment in terms of increased anxiety in ethological models (Luuk et al., 2009). Pre-treatment of mutant mice with diazepam (1 mg/kg), an anxiolytic agonist of $GABA_A$ receptors, antagonized increased anxiety and stress-induced vocalizations in *Wfs1*-deficient mice.

3. Concluding remarks

Recent studies have shown that endocannabinoid system is involved in the regulation of anxiety, pain, emotional and cognitive processes. CB_1 receptors are one of the most abundant G-protein coupled receptors in the CNS being highly expressed in the structures involved in the regulation of emotional behaviour (amygdala, hippocampus, anterior cingulate and prefrontal cortex, PAG). Endocannabinoids have emerged as potential targets for the medication of anxiety disorders (Tsou et al., 1998a; Kathuria et al., 2003; Katona et al.,

2006). Up till now the involvement of the endocannabinoid system in the regulation of anxiety remains to be controversial, because the low doses of THC cause anxiolytic, whereas high doses of a cannabinoid agonist elicit anxiogenic effects (Viveros et al., 2005).

The second emerging new target in the regulation of anxiety – *Wfs1* – plays a significant role in the adaptation to a novel environment. Mutations of the *WFS1* gene in humans cause a progressive neurodegenerative disorder – Wolfram syndrome (DIDMOAD) and the heterozygous carriers of *WFS1* mutation are more susceptibile to mood disorders (Swift et al., 1998). It has been shown that the exposure of male Wistar rats to cat odour produces an up-regulation of *Wfs1* mRNA in the amygdala (Kõks et al., 2004). Also, the Wfs1 mRNA and protein are densely expressed in the brain structures related to behavioural adaptation and emotions – the amygdaloid complex, bed nucleus of stria terminalis, nucleus accumbens, prefrontal cortex and hippocampus (Takeda et al., 2006; Luuk et al., 2008). In addition, *Wfs1*-deficient mice display higher anxiety-like behaviour and stronger elevation of corticosterone levels in a stressful environment compared to wild-type littermates (Luuk et al., 2009).

Based on the literature analysis the general aim of the present study was to reveal a role of new potential targets, endocannabinoids and wolframin, in the adaptation to a novel environment and regulation of anxiety. For that purpose, two behavioural approaches, a model of cat odour induced anxiety and selection of rodents according to their exploratory behaviour, were used. Rimonabant, an antagonist of CB_1 receptors, was used to reveal a role of ECS in the development of cat odour-induced anxiety. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in order to establish changes in the ECS, GABA and *Wfs1* gene expression due to anxiogenic manipulations in mice and rats. Corticosterone level and oxidative stress index as indicators of stressful influences were measured in the blood samples of rats after the elevated plus-maze exposure.

OBJECTIVES OF THE PRESENT STUDY

The general goal of the present study was to reveal a role of ECS and *Wfs1* in the adaptation to a stressful environment and regulation of anxiety in rodents. More specific questions were as follows:

1. To establish a possible role of the ECS genes in anxiety induced by predator odour and elevated zero-maze exposure. For that purpose gene expression analysis by qRT-PCR were performed in the mesolimbic area (*nucleus accumbens* and *tuberculum olfactorium*), amygdala and periaqueductal grey. In addition, a pharmacological manipulation with rimonabant, CB_1 receptor antagonist was performed (Paper I).
2. To establish differences in the cat odour response between two major mouse strains, 129Sv and C57Bl/6, displaying significant differences in the emotional behaviour as well as in the response to various pharmacological drugs. Also, to study a possible involvement of *Wfs1* in the regulation of behavioural differences (Paper II).
3. To establish the relation between the exploratory behaviour, and the markers of stress in the male Wistar rats divided into sub-groups (high and low exploratory activity animals) according to their exploratory behaviour. Simultaneously the expression of the ECS genes and *Wfs1* were measured in the brain structures of each group of rats (Paper III).
4. To establish a possible relation of GABA-related genes in the forebrain structures for increased anxiety and increased sensitivity to anxiolytic-like action of diazepam in female *Wfs1*-deficient mice (Paper IV).

MATERIALS AND METHODS

1. Animals (Papers I, II, III, IV)

In Papers I and III the experiments were performed on male Wistar rats (Scanbur BK, Sweden) weighing 300–350 g at the time of experiment. The experiments were performed 19 days after arrival of rats from the breeding company. In Paper III, 42 handling naive rats were subjected to the EPM experiment. Behavioural experiments were carried out between 10:00 and 15:00. Eight rats were taken randomly from the same cages and not subjected to the EPM exposure. These rats were not tested in the EPM, and they were used as the home-cage control group in the biochemical and gene expression studies.

In Paper II, the studies were performed in female C57Bl/6 (Bl6, Scanbur BK) and 129S6/SvEv/Tac (129Sv, Taconic) mice, 8–10 weeks old at the time of testing. These mice strains were chosen, because of their widespread use in the gene knockout experiments. For the experiment female mice were chosen as they tend to be more sensitive to predator odour stress than males (Adamec et al., 2006). The experiments in Paper IV were performed in wild-type female F2 hybrids [(129S6/SvEvTac x C57BL/6) x (129S6/SvEvTac x C57BL/6)] and in their *Wfs1*-deficient littermates. Mice were 8–12 weeks old at the time of testing. Breeding and genotype analysis of mice were carried out in the Department of Physiology, University of Tartu.

The animals were housed in groups of five to six (rats) or 10–12 (mice) in the animal house at the temperature of 20 ± 2°C in a silent room illuminated artificially from 7 a.m to 7 p.m. Tap water and food pellets were available *ad libitum*. The permission (No. 39, 7th of October, 2005) for the present study was given by the Estonian National Board of Animal Experiments in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

2. Generation of *Wfs1*-deficient mice harboring β-galactosidase transgene (Papers II, IV)

Wfs1 targeting construct was created by subcloning a 8.8 kb BamHI fragment from 129SvEv/TacfBr mouse genomic PAC clone 391-J24 (RPCI21 library, MRC U.K. HGMP Resource Centre, U.K.) including introns 6–7 and exons 7–8 of the *Wfs1* gene into pGem11 cloning plasmid (Promega, Madison, WI). A 3.7 kb NcoI fragment was replaced by an in-frame NLSLacZNeo cassette, deleting more than 90% of the 8th exon and 60% of the total coding sequence including 8 of the 9 predicted transmembrane domains. A pgk-TK negative selection cassette was cloned upstream of 5' genomic arm. The targeting construct was electroporated into W4/129S6 embryonic stem (ES) cells

(Taconic, Hudson, NY), which were selected for resistance to Neomycin and Gancyclovir. ES cell colonies were tested for homologous recombination by PCR using recombination-specific primer pair NeoR1 5' GACCGCTATCA GGACATAGCG and *Wfs1*_WTR1 5' AGGACTCAGGTTCTGCCTCA. PCR-product was sequenced to verify the integration site. ES cell clone 8A2 was injected into C57BL/6 blastocysts and heterozygous F1 mice were established by mating male chimeras with C57BL/6 female mice. F2 generation homozygous *Wfs1*-deficient animals were obtained by crossing heterozygous F1 mice. Mice were genotyped by multiplex PCR for both alleles using primers WfsKO_wtF2 5' TTGGCTTGTATTTGTCGGCC, NeoR1 5' GACCGCTAT CAGGACATAGCG and WfsKO_uniR2 5' CCCATCCTGCTCTCTGAACC.

3. Drugs (Paper I)

The action of rimonabant (0.3–3 mg/kg; Sanofi-Aventis), an antagonist of CB_1 receptors, was studied in the elevated zero-maze and motility boxes. Rimonabant was suspended in DMSO with two or three drops of Tween-85. Saline (0.9%) was added to this mixture to get a final dilution of 1:10 (DMSO/saline). All injections were performed intraperitoneally in a volume of 1 ml/kg body weight 15 min before beginning of the study.

4. Behavioural studies

4.1. The cat odour exposure (Papers I, II)

The procedure of exposure of rats or mice to the cloth impregnated with cat odour or clean cloth was conducted in two separate, but similar rooms with the same illumination intensity of 20 lx, humidity, ventilation, etc. All animals were subjected to gentle handling once daily for three consecutive days in these rooms before the experiment. All the studies were conducted between 0900 and 1700 hours. On the fourth day, after a 30-min habituation period in the exposure room, a cloth impregnated with cat odour or a clean cloth was placed on top of the cage for 30 min and each session was video recorded. The behaviour was analyzed from videotapes by an observer unaware of the manipulations performed with the rats or mice.

In Paper I, the effect of cat odour on the exploratory behaviour of rats was studied in two different experiments. In the first experiment, the exposure of rats to the cat odour or clean cloth was performed in groups in their home cages. Half of the rats (n=16) were exposed to a cloth impregnated with cat odour; whereas the other half (n=16) were exposed to a clean cloth. After the cat odour or clean cloth exposure, half of the rats (n=8) were placed into the elevated zero-maze located in the third room, different from the rooms where the

exposure to cat odour or a clean cloth was performed. These rats exposed and non-exposed to the elevated zero-maze were used for the gene expression studies. In the second experiment, the effect of rimonabant was measured in rats (n=64) exposed to cat odour in the individual motility boxes. All animals were subjected to gentle handling once daily for three consecutive days in the room (30 min each time) where the motility boxes were located. However, during the adaptation period, the rats were not exposed to the motility boxes. On the fourth day of the study, the animals were brought from the animal room to the laboratory where they were subjected to handling 60 min before the beginning of experiment. They were kept in their home cages. Injection of rimonabant or vehicle was performed 15 min before the beginning of the study. After that the rats were taken from their home cages and singly placed into a photoelectric motility box (448×448×450 mm). A control cloth or a cloth impregnated with cat odour was fixed by tape into one corner of the motility boxes exactly between the sensors detecting the vertical and horizontal locomotor activities of the rats. The illumination level of motility boxes was 10–15 lx. To avoid possible contamination of the motility boxes with cat odour, the experiment was performed in two consecutive days. In the first day, the effect of a clean cloth was studied using half of the rats (n=32), whereas in the second day, the other half (n=32) was exposed to a cloth impregnated with cat odour. Rats were used only once, and the separate groups of animals were confronted to the clean cloth and cloth impregnated with cat odour. The boxes were connected to a computer (TSE Technical & Scientific Equipment, Germany) and time in locomotion (s), total distance traveled (m), the number of rearings and corner entries to the cloth side were automatically recorded during a 30-min period. After that the rats were transported into the other room where the elevated zero-maze experiment was performed. The delay between the motility box test and the elevated zero-maze experiment was approximately 1 min.

In Paper II, the effect of cat odour exposure on the exploratory behaviour was investigated in two different mouse lines (129Sv and C57Bl/6). Both 129Sv and C57Bl/6 mice were divided into two groups. One group was exposed to a cloth impregnated with cat odour and the other to a clean cloth. Number of mice in each group was 16–17. Mice were individually exposed to either a clean or a cat odour impregnated cloth. The exposure was performed in a cage (25 cm × 40 cm × 15 cm) similar to animals' home cages. A mouse was placed in a cage and a clean or a cat odour impregnated cloth was placed in the corner of the cage on the bedding. The exposure to cat odour lasted for 30 min and the session was video-recorded. In mice the following behavioural parameters were analyzed: number of contacts with the cloth, duration of cloth contact, number of rearings, number of digging events, duration of digging, number of burying events and duration of burying.

4.2 The elevated zero-maze (Paper I)

Zero-maze exploration test is an unconditioned test of anxiety-like behaviour originally described by Sheperd et al. (1994). The apparatus comprised of a black Perspex annular platform (105 cm diameter, 10 cm width) elevated to 65 cm above ground level. Two closed quadrants were enclosed by black metal walls (27 cm high), the remaining two open quadrants were surrounded by a 1-cm high lip. Two open quadrants of the zero-maze were divided by lines into four equal parts. The behavioural parameters were recorded by an observer in the same room, 2 m away from the zero-maze. The behaviour of rats was recorded for 5 min and the following measures were taken: time spent in the open quadrant(s), number of open quadrant entries, line crossings, stretch-attend postures and head-dips. The experiments were carried out in a dimly illuminated room (10–15 lx).

4.3. The elevated plus-maze (Papers III, IV)

In Paper III, the EPM was used as described previously (Kõks et al., 1997) for the selection of rats according to their exploratory activity. The rats were selected into low and high exploratory activity groups after the completion of the EPM experiment based on the frequency of open and closed arm entries (Nelovkov et al., 2006). The illumination level was 110 lx, encouraging the exploration of open arms by rats and helping to get a better separation between the groups. As short-term isolation of rats increases their exploratory activity, the rats were kept in isolation for 15 min before the EPM exposure. The EPM study was carried out by an experienced person. Since the separation of animals into subgroups was performed after the completion of the experiment, this person was blind to whether the animals belonged to the high or low exploratory activity group.

In Paper IV, before testing in the elevated plus-maze, mice were kept in isolation for 15 minutes. EPM study was performed as described by Luuk et al. (2009). During a 5-min observation session the following measures were taken: (1) time spent in exploring the central square and open arms of the EPM, (2) the number of attempts to enter the central square from the closed arm, (3) the number of head-dipping and stretch-attend postures, (4) the number of line crossings, (5) the number of closed and open arm entries and (6) latency to enter into the central square. 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 EPM facing a closed arm. An arm entry was counted only when all four limbs of mouse 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 open and total arm entries are conventional measures of anxiety in the EPM (Pellow, et al., 1985; Lister, 1987; Rodgers et al., 1997). The head-dippings and stretch-attend

postures are ethological measures of anxiety (Rodgers, 1997). Head-dipping was counted as described previously (Espejo, 1997).

5. Measurement of corticosterone level (Paper III)

To control for naturally occurring fluctuations in corticosterone levels, naive rats not exposed to the EPM were used in parallel with animals exposed to the maze. The subsequent analysis of selected rats demonstrated that low, intermediate and high exploratory activity rats were distributed similarly over the time course of the EPM experiment. The comparison of corticosterone values in each time period established that the level of stress hormone was always significantly higher in animals exposed to the EPM compared to naive rats. Immediately after the EPM exposure or taking out from the home-cage the rats were decapitated and truncal blood (a mixture of arterial and venous blood) was collected into heparinized tubes. Blood samples were centrifuged after collection for 10 min at 1500 x g. Sera were stored at –20^{o}C until the corticosterone and systemic oxidative stress assays. For determination of stress hormone Corticosterone HS ELISA kit from Immunodiagnostic Systems (U.K.) was used according to manufacturer's instructions. The results of the experiment were analysed by means of software GraphPad Prism 4.

6. Oxidative stress index (Paper III)

Percent ratio of total peroxide concentration of plasma (TPX) to total antioxidant response of plasma (TAR) was accepted as oxidative stress index (OSI), an indicator of systemic oxidative stress (Horoz, et al., 2006). This has been widely used as a measure for characterization of oxidative stress. OSI = [(TPX, μmol/L)/ (TAR, μmol Trolox/L) x 100)]

6.1. Measurement of total antioxidant response of plasma

In this method hydroxyl radical (OH•), the most potent biological radical, was produced and the rate of the reactions was monitored by following the absorbance of coloured dianisidyl radicals (Erel, 2004). Antioxidants, present in the sample, suppressed the colour formation to a degree that is proportional to their concentrations. The suppression of the colour formation was calibrated with Trolox, which is widely used as a traditional standard for TAR measurement assays, so the results are expressed as in terms of millimolar Trolox equivalent per litre.

6.2. Measurement of total peroxide concentration of plasma

Total peroxide concentrations of plasma samples were determined using PeroXOquant TM Quantitative Peroxide Assay Kit, lipid-compatible formulation from PIERCE (Rockford, IL, USA). The kit detects plasma peroxide concentrations based on oxidation of ferrous to ferric ion in the presence of xylenol orange. In a sulfuric acid solution, the Fe^{3+} complexes with the xylenol orange dye were formed to yield a purple product with maximum absorbance at 560 nm. For TPX calibration dilution series from solution of H_2O_2 was made.

7. Gene expression studies (Papers I, II, III, IV)

7.1. Dissection of brain structures, RNA isolation and cDNA synthesis

The animals were decapitated immediately after behavioural studies. Brains were rapidly removed from the skull, and different brain structures were dissected and frozen in liquid nitrogen. Dissection of the rat brain was performed according to the rat brain atlas of Swanson (Swanson, 1998). Dissection of the amygdala was performed using a round-shape puncher. Dissection of the mice brain was performed according to coordinates obtained from the mouse brain atlas (Franklin and Paxinos, 1997). In Paper I the mesolimbic area (including the *nucleus accumbens* and *tuberculum olfactoriaum*), amygdala and periaqueductal gray (PAG) of rats were used. In Paper II, the mesolimbic area, temporal lobe (including the amygdala) and the prefrontal cortex of mice were used. In Paper III, the mesolimbic area, temporal lobe and hippocampus of rats were used. Since significant differences in corticosterone levels were established between high and low exploratory activity groups, the intermediate group was eliminated from gene expression studies (Paper III). In paper IV, the frontal cortex, mesolimbic area (including the *nucleus accumbens* and *tuberculum olfactorium*), hippocampus and temporal lobe (including the amygdala) of mice were used.

Total RNA was extracted individually from each brain structure of each mouse or rat using Trizol® Reagent (Invitrogen, USA) according to the manufacturer's protocol. First strand cDNA was synthesized by using poly $(T)_{18}$ oligonucleotides and SuperScript™ III Reverse Transcriptase (Invitrogen, USA).

7.2. Quantitative real-time-PCR

The gene expression studies in control odour exposed, cat odour exposed and experimentally naive animals were conducted in parallel (Papers I, II), Also, the experiments with high exploratory activity, low exploratory activity and home-

cage control group animals were conducted in parallel (Paper III). In Paper IV, experimentally naive animals and animals exposed to the elevated plus-maze, belonging to both genotypes, were used in parallel. For quantitative real-time PCR (qRT-PCR) analysis the ABI PRISM 7900HT Fast Real-Time PCR System equipment (PE Applied Biosystems, USA) and the ABI PRISM 7900 SDS 2.2.2 Software were used. Every reaction was made in four parallel samples to minimize possible errors. All reactions were performed in a final volume of 10 µl, using 50–100 ng of cDNA. The mRNA level of control group of rats and mice was always defined as one and the increase or decrease of mRNA amounts is shown as the fold change.

Choosing of housekeeper gene is the most important step for qRT-PCR analysis. The suitable housekeeper must be stable in different samples of tissue and not affected after the manipulations with animals. In the present study three candidates normalization genes, (*Hprt1*, *Gapdh* and *B2M*) were used as they are considered the most common housekeeper genes in the brain tissues. We estimated amplification efficiency (E) by formula

$$E = (10(-1/\text{slope}) - 1) \times 100\%$$

The amplification efficiency of reference genes was as follows: *Hprt1* – 96%, *Gapdh* – 67% and *B2M* – 92%. We determined the best suitable houskeeper gene using mathematical algoritm developed by Vandesompele and colleagues (2002). The method is based on the pair-wise comparison of multiple candidate reference genes. It means that the ratio of most stable housekeeper genes is the same. The stability of the gene is determined in standard deviation of logarithmic values of pairwise comparison. According to the internal gene-stability measure (M) of candidate gene the most stable genes were *Hprt1* (M=0.013) and *Gapdh* (0.014). The M value for *B2M* was 0.022. The intra-assay coefficient of variability for *B2M*, *Gapdh* and *Hprt1* genes was 0.62%; 0.11% and 0.06%, respectively. The inter-assay variability for *B2M*, *Gapdh* and *Hprt1* was 3.4%; 2.3% and 2%, respectively. However, since the amplification efficiency of *Hprt1* was higher compared to *Gapdh* we chose *Hprt1* as a housekeeper for the qRT-PCR analysis. In favour of *Hprt1* selection was also the fact that [Ct] for *Gapdh* resulted in a higher value between target and reference gene and, therefore, this made the following comparison between groups not so feasible as compared to *Hprt1*, because a higher value between the target and reference genes gives smaller differences in ratio calculation.

7.2.1. SYBR® Green assay (Papers I, III, IV)

Primers were designed with the Primer ExpressTM software (PE Applied Biosystems, Foster City, California, USA). Primers are always in exon-exon junction eliminating the possibility of amplifying the genome DNA. As described above, in all gene expression experiments *Hprt1* was used as the

housekeeper gene. All reactions were performed using the SYBR Green I qPCR™ Core Kit (Eurogentec, Belgium). Instructions of the equipment and reagent manufacturers were always followed. A melting curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. All samples to be compared were run in the same experiment. The amount of the target gene was compared to the housekeeper gene by means of the ΔΔCT method (Livak and Schmittgen, 2001).

Table 2. Sequences of primers used for qRT-PCR gene expression studies

Oligo name	Sequence of primers	Amplicon	Gene ID
Hprt1_for Hprt1_rev	5'-GCAGTACAGCCCCAAAATGG-3' 5'-AACAAAGTCTGGCCTGTATCCAA-3'	84 bp	NM_013556
CB_1 for CB_1_rev	5'-GGGTAGTCCCTTCCAAGAAAAG-3' 5'-GTCCATAAAGTTCTCCCCACAC-3'	154 bp	NM_012784
Faah_for Faah_rev	5'-CCTCAAGGAATGCTTCAGCTAC-3' 5'-ACTTCCATGGGTTCA TGGTC-3'	209 bp	NM_024132
Faah_for Faah_rev	5'–CCTGCTGAAGCCTCTGTTTCC–3' 5'– CGCATACTGTTGAGAAAGGCTG –3'	50 bp	NM_024132
Mgll_for Mgll_rev	5'-ATGACCATGTTGGCCATGG-3' 5'-CGTGCTGCAACAAATCTCTGAC-3'	87 bp	NM_138502
Nape-pld_for Nape-pld_rev	5'-CTTTTGACCTCGCGGCTATT-3' 5'-CATAAACCACCTTGGCTCATAAGC-3'	53 bp	NM_199381
Dagla_for Dagla_rev	5'-AAGAATGTCACTCTCGGGATGG-3' 5'-AGTGATGCAGACGCTGAGGAT-3'	63 bp	NM_001005886
Gabra1_for Gabra1_rev	5'-TGTACACCATGAGGTTGACCGT-3' 5'- GAAGTCTTCCAAGTGCATTGGG-3'	50 bp	NM_010250.3
Gabra2_for Gabra2_rev	5'- ATGGTCTCTGCTGCTTGTTCTTCT-3' 5'- AGCACCAACCTGACTGGGTC-3'	50 bp	NM_008066

In Paper I, we studied the gene expression levels of the ECS genes, including CB_1 receptors, the enzymes related to endocannabinoids biosynthesis (*Nape-pld*, *Dagla*) and degradation (*Faah*, *Mgll*). In Paper III, we selected genes the expression level of which changed most ion response to cat odour (Paper I) – CB_1 receptor, *Nape-pld* and *Faah*. In Paper IV, the *Gabra1* and *Gabra1* expression was studied in wild-type and *Wfs1*-deficient mice, in naive animals and mice exposed to the elevated plus-maze. The primer sequences are presented in Table 2.

7.2.2. Taqman assay (Papers II, III, IV)

For *Wfs1* (Papers II, III) as well as for *Gad1* and *Gad2* (Paper IV) mRNA expression we used Taqman assay (PE Applied Biosystems). The assays and the sequence of the *Hprt1* probe are given in Table 3.

Table 3. The Taqman assays and probes used in the study.

Assay name	Assay ID or sequence	Gene ID
Wfs1 mouse	Mm_01220326_m1	NM_011716
Wfs1 rat	Rn_01453284_m1	NM_031823
Gad1 mouse	Mm_00725661_s1	NM_008077
Gad2 mouse	Mm_01329282_m1	NM_008078
Hprt1 probe (VIC_TAMRA)	5'-VIC-AAGCTTGCTGGTGAAAAGGACCTCTCG TAMRA-3'	NM_012583 (rat) NM_013556 (mouse)

8. Statistical analysis (Papers I, II, III, IV)

The results of the behavioural, biochemical and gene expression studies are expressed as mean values ± SEM. For statistical analyses we used Statistica for Windows software (Statsoft, USA). The effect of cat odour exposure on the exploratory activity (Paper I) and EPM studies in Paper IV were analysed using Student's t-test. In case of two independent variables, such as cat odour exposure and treatment with rimonabant in Paper I, two-way analysis of variance (ANOVA) was used. Two-way ANOVA was also applied for measuring the effect of cat odour exposure on the behaviour of C57Bl/6 and 129Sv mice (Paper II). The behavioural, biochemical and gene expression studies were analysed by means of one-way analysis of variance (ANOVA). The correlation between EPM exploration and biochemical parameters was analysed by means of Pearson r correlation coefficient (simple linear correlation test) (Paper III). In Paper I, gene expression results were analyzed by means of two-way ANOVA (cat odour and zero-maze exposure as independent variables). In Paper IV, two-way analysis of variance was used where genotype and plus-maze exposure were the independent variables. In all papers, *post hoc* comparisons after significant one-way or two-way ANOVA were performed using either Tukey HSD or Newman-Keuls test.

RESULTS

EXPERIMENT I. CAT ODOUR EXPOSURE OF RATS CAUSED AN ANXIETY-LIKE STATE, INHIBITED EXPLORATORY ACTIVITY IN THE ELEVATED PLUS-MAZE AND INDUCED CHANGES IN ENDOCANNABINOID SYSTEM GENES (PAPER I)

1.1. Behavioural studies

1.1.1. Exposure of rats to cat odour

Rats were exposed to the cloth impregnated with the control odour or cat odour in their home cages. Cat odour exposed rats displayed significantly increased frequency of freezing and suppressed number of rearing compared to the clean cloth exposed rats (Table 4). The rats clearly avoided the cloth impregnated with cat odour. Differences in all behavioural measures were statistically significant ($p < 0.05$, Student's t-test).

Table 4. Effect of cat odour exposure (for 30 min) on the exploratory behaviour of male Wistar rats (Mean values ± S.E.M.).

Behavioural measure	Control odour	Cat odour
Number of freezing	0	20±1*
% of freezing	0	68±4*
Number of cloth touching	4±0.6	0*
Time in contact with cloth	38±9	0*
Number of rearing	19±2	0*

* $p < 0.01$, Student's t-test

1.1.2. Exploratory behaviour of rats in the elevated zero-maze

Exposure of rats to the elevated zero-maze after cat odour exposure demonstrated that cat odour induced significant suppression of exploratory behaviour in rats. The latency of first open part entry was significantly delayed after the cat odour exposure (Table 5). There was a dramatic reduction of open part entries, line crossings and time spent on open parts due to the cat odour exposure. Simultaneously with the spatio-temporal measures the ethological parameters of zero-maze exploration were affected after cat odour exposure. Predator odour exposure significantly reduced the number of head-dippings and increased the frequency of stretch-attend postures in rats. All these behavioural changes reflect increased anxiety.

Table 5. Effect of cat odour exposure on the exploratory activity in the elevated zero-maze.

Behavioural parameters	Control odour	Cat odour
Latency of the first open arm entry, sec	15±2	156±45**
Number of open arm entries	9.1±1.1	0.8±0.3**
Time in open arm, sec	137±11	26±11**
Number of line crossings	42±4	3±1**
Number of head-dips	16.4±1.3	2.0±0.4**
Number of stretch-attend postures	0.1±0.1	1.6±0.6*

* $p < 0.05$; ** $p < 0.01$, Student's t-test

1.2. Gene expression studies

1.2.1. Mesolimbic area

The zero-maze and the cat odour exposure increased the expression of *Faah*, the gene of enzyme responsible for the degradation of anandamide (Table 6). The application of two-way ANOVA and the following *post hoc* analysis of Tukey HSD (F1,20=3.18, p=0.09 [zero-maze]; F1,20=1.34, p=0.26 [cat odour]; F1,20=10.7, p<0.01 [cat odour × zero-maze]) confirmed statistically significant results. Exposure of rats to the zero-maze after challenge to cat odour significantly increased the expression level of *Nape-pld*, the enzyme responsible for the synthesis of anandamide (F1,20=2.65, p=0.12 [zero-maze]; F1,20=10.7, p<0.01 [cat odour]; F1,20=0.02, p=0.97 [cat odour × zero-maze]). The other changes in mesolimbic area did not reach the level of statistical significance.

1.2.2. Amygdala

Exposure to the elevated zero-maze did not change the expression of EC-genes (Table 7). The exposure of rats to cat odour decreased the expression level of *Nape-pld*, *Faah* and *Dagla* compared to control cloth exposure as revealed by means of two-way ANOVA and being statistically significant after *post hoc* analysis (p<0.05, Tukey HSD), (*Nape-pld*: F1,20= 2.58, p=0.12 [zero-maze]; F1,20=0.21, p=0.65 [cat odour]; F1,20=12.1, p<0.01 [zero-maze × cat odour]; *Faah*: F1,20=5.49, p<0.05 [zero-maze]; F1,20=0.45, p=0.51 [cat odour]; F1,20=9.65, p<0.01 [zero-maze × cat odour]; *Dagla*: F1,20=1.43, p=0.24 [zero-maze]; F1,20=4.89, p<0.05 [cat odour]; F1,20=4.89, p<0.05 [zero-maze × cat odour]). Interestingly, the subsequent exposure of rats to the elevated zero-maze abolished the changes of the same genes induced by the predator odour. The expression level of CB_1 receptor was significantly increased only after the application of both anxiogenic stimuli – cat odour and zero-maze exposure

(F1,20=3.98, p=0.06 [zero-maze]; F1,20=0.47, p=0.50 [cat odour]; F1,20= 6.89, p<0.05 [zero-maze × cat odour]) compared to the predator odour alone.

Table 6. The effect of cat odour and zero-maze exposure on the expression of the ECS genes in the mesolimbic structures.

Gene symbol	Control odour exposure	Control odour+zero-maze exposure	Cat odour exposure	Cat odour + zero-maze exposure
CB_1	1.04±0.05	1.22±0.12	1.23±0.12	1.09±0.12
Nape-pld	1.04±0.02	1.12±0.08	1.22±0.09	1.33±0.06*
Faah	1.02±0.01	1.36±0.07*	1.31±0.04*	1.25±0,10
Dagla	1.04±0.02	1.25±0.12	1.11±0.12	1.08±0.09
Mgll	1.02±0.02	0.90±0.10	1.15±0.15	0.85±0.05

* p<0.05 (compared to control group, Tukey HSD test after significant two-way ANOVA). Number of animals in each group was 6.

Table 7. The effect of cat odour and zero-maze exposure on the expression of ECS genes in the amygdala.

Gene symbol	Control odour exposure	Control odour+zero-maze exposure	Cat odour exposure	Cat odour + zero-maze exposure
CB_1	1.02±0.01	0.97±0.12	0.97±0.04	1.29±0.14$^+$
Nape-pld	1.02±0.08	0.82±0.11	0.69±0.07*	1.24±0.16$^+$
Faah	1.01±0.04	0.96±0.03	0.71±0.11*	1.15±0.05$^+$
Dagla	1.02±0.04	0.98±0.03	0.69±0.09*	0.98±0.07$^+$
Mgll	1.02±0.08	0.88±0.03	1.11±0.11	0.91±0.03

* p < 0.05 (compared to control group, Tukey HSD test after significant two-way ANOVA);
+ p < 0.05 (compared to the cat odour exposure). Number of animals in each group was 6.

1.2.3. Periaqueductal gray

The gene expression profile in the PAG was quite similar to that established in the amygdala. The exposure of rats to the elevated zero-maze did not modify the expression of ECS genes (Table 8), but the cat odour exposure reduced the expression of *Nape-pld*, *Faah* and *Mgll* (*Nape-pld*: F1,20=7.13, p<0.05 [zero-maze] F1,20=58.1, p<0.001 [cat odour]; F1,20=0.42, p=0.52 [cat odour × zero-maze]; *Faah*: F1,20=0.52, p=0.48 [zero-maze]; F1,20=9.36, p<0.01 [cat odour]; F1,20=1.56, p= 0.23 [cat odour × zero-maze]; *Mgll*: F1,20=0.24, p=0.63 [zero-maze]; F1,20=39.9, p<0.001 [cat odour]; F1,20=5.21, p<0.05 [cat odour × zero-maze]). All these findings were confirmed by the subsequent *post hoc* analysis (Table 8). Moroever, the predator odour exposure decreased the level of *Dagla*

in the PAG (F1,20=0.35, p=0.56 [zero-maze]; F1,20=7.10, p<0.05 [cat odour]; F1,20=2.89, p=0.10 [cat odour × zero-maze]), but it was not statistically significant. The subsequent analysis with Tukey HSD test revealed that the reduction of the level of *Dagla* was established only in rats exposed to the elevated zero-maze after the cat odour exposure (p<0.05). Similarly to the amygdala, the expression level of CB_1 receptor was significantly increased only after both anxiogenic stimuli (cat odour and zero-maze exposure) compared to the predator odour alone (F1,20=4.04, p=0.058 [zero-maze]; F1,20=2.26, p=0.15 [cat odour]; F1,20=16.4, p<0.001 [cat odour × zero-maze]). This finding was also confirmed by the subsequent *post hoc* analysis (Table 8).

Table 8. The effect of cat odour and zero-maze exposure on the expression of the ECS genes in the periaquaductal gray.

Gene symbol	Control odour exposure	Control odour-zero-maze exposure	Cat odour exposure	Cat odour + zero-maze exposure
CB_1	1.01±0.01	0.81±0.05	0.76±0.09	1.35±0.17^{+}
Nape-pld	1.03±0.02	1.13±0,09	0.60±0.09*	0.77±0.07*
Faah	1.02±0.01	0.99±0.13	0.66±0.04*	0.83±0.11
Dagla	1.02±0.01	1.12±0.09	0.93±0.10	0.72±0.02*
Mgll	1.03±0.01	1.12±0.03	0.75±0.10*	0.64±0.04*

* p < 0.05 (compared to control group, Tukey HSD test after significant two-way ANOVA);
\+ p < 0.05 (compared to cat odour exposure). Number of animals in each group was 6.

EXPERIMENT 2. ADMINISTRATION OF RIMONABANT INDUCED AN ANXIOGENIC EFFECT IN THE ELEVATED ZERO-MAZE, BUT DID NOT AFFECT CAT ODOUR-INDUCED CHANGES (PAPER I)

2.1. Cat odour exposure in the motility boxes

The cat odour exposure and the administration of rimonabant (0.3–3 mg/kg) reduced the frequency of rearing (F1,55=12.07, p<0.001 [cat odour]; F3,55=3.72, p<0.05 [treatment]; F3,55= 3.26, p<0.05 [cat odour × treatment]) and number of entries into the cloth's corner (F1,55=36.89, p<0.001 [cat odour]; F3,55=3.54, p<0.05 [treatment]; F3,55=2.87, p<0.05 [cat odour × treatment]) (Figure 4). The subsequent *post hoc* analysis with Newman-Keuls test revealed that the cat odour exposure significantly suppressed (p<0.05) the number of rearings and visits into the corner where the cloth was located. The lowest dose of rimonabant (0.3 mg/kg) reduced the number of rearing in the

control group, whereas the suppression of cloth's corner entries occurred after the administration of intermediate dose (1 mg/kg) of CB_1 receptor antagonist (Figure 4). However, pre-treatment of rats with rimonabant did not affect the suppression of the abovementioned behaviours induced by the predator odour. Two-way ANOVA demonstrated that the cat odour exposure also reduced the time in locomotion ($F1,55= 7.90$, $p<0.01$ [cat odour]; $F3,55=2.12$, $p=0.10$ [treatment]; $F3,55=0.26$, $p=0.85$ [cat odour × treatment]) and distance traveled ($F1,55=5.80$, $p<0.01$ [cat odour]); $F3,55=2.44$, $p= 0.07$ [treatment]; $F3,55=0.35$, $p=0.78$ [cat odour × treatment]) (Figure 4). Newman-Keuls *post hoc* test confirmed that these parameters were significantly affected by the predator odour. However, the mentioned parameters were markedly decreased if the rats were pre-treated with rimonabant (1–3 mg/kg) before the cat odour exposure. It should be noted that the distance traveled was also significantly reduced if the control rats were treated with the highest dose (3 mg/kg) of CB_1 antagonist (Figure 4).

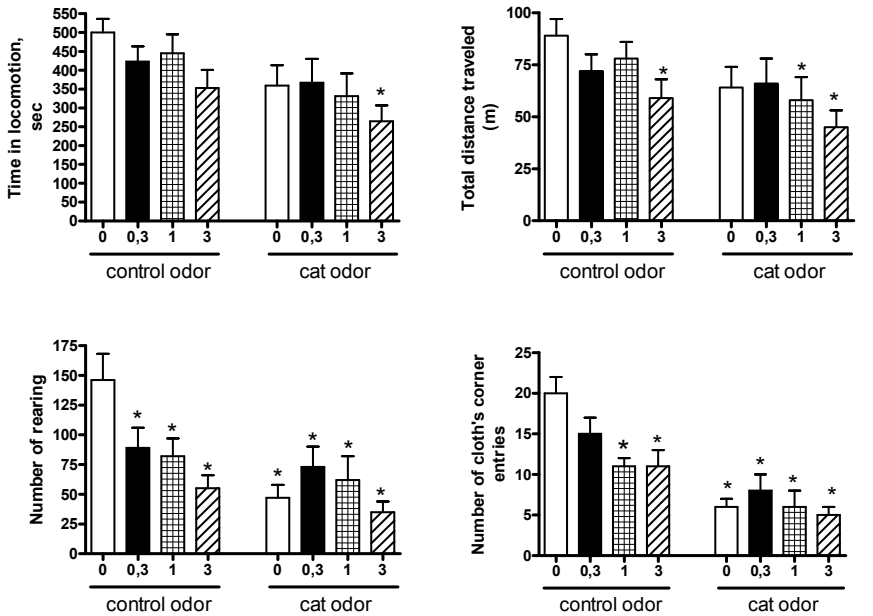

Figure 4. The effect of rimonabant (0.3–3 mg/kg) on the exploratory activity of male Wistar rats exposed to the control or cat odour in the motility boxes (mean values± SEM). White bars saline; black bars rimonabant 0.3 mg/kg; hatched bars rimonabant 1 mg/kg; striped bars rimonabant 3 mg/kg. * $p<0.05$ (compared to the control odour vehicle-treated group, Newman- Keuls test after significant two-way ANOVA)

2.2. Challenge to the elevated zero-maze after cat odour exposure

The exposure of rats to cat odour reduced the frequency of open quadrant entries ($F1,55=4.08$, $p<0.05$ [cat odour]; $F3,55=1.40$, $p=0.25$ [treatment]; $F3,55=2.41$, $p=0.07$ [cat odour × treatment]), time spent on open quadrant ($F1,55= 8.13$, $p<0.01$ [cat odour]; $F3,55=1.65$, $p=0.19$ [treatment]; $F3,55=2.10$, $p=0.11$ [cat odour × treatment]) and number of line crossings ($F1,55=3.12$, $p<0.05$ [cat odour]; $F3,55=1.49$, $p=0.22$ [treatment]; $F3,55=2.60$, $p=0.06$ [cat odour × treatment]) (Figure 4). By contrast, cat odour exposure and treatment with rimonabant had no effect on the number of head-dippings. The subsequent *post hoc* analysis confirmed that the established reduction in the exploratory behaviour caused by the predator odour was statistically significant. Pre-treatment of rats with rimonabant (0.3–3 mg/kg) also reduced the exploratory behaviour of the control group in the zero-maze. As opposed to the motility box test, the lowest dose of rimonabant (0.3 mg/kg) did not cause any significant changes in the exploratory behaviour of rats exposed to a clean cloth. The intermediate dose (1 mg/kg) of CB_1 receptor antagonist markedly reduced the number of open quadrant entries, whereas the highest dose (3 mg/kg) also decreased the time spent in open quadrants and number of line crossings (Figure 5). Pre-treatment of rats with rimonabant did not modify cat odour-induced changes of exploratory activity.

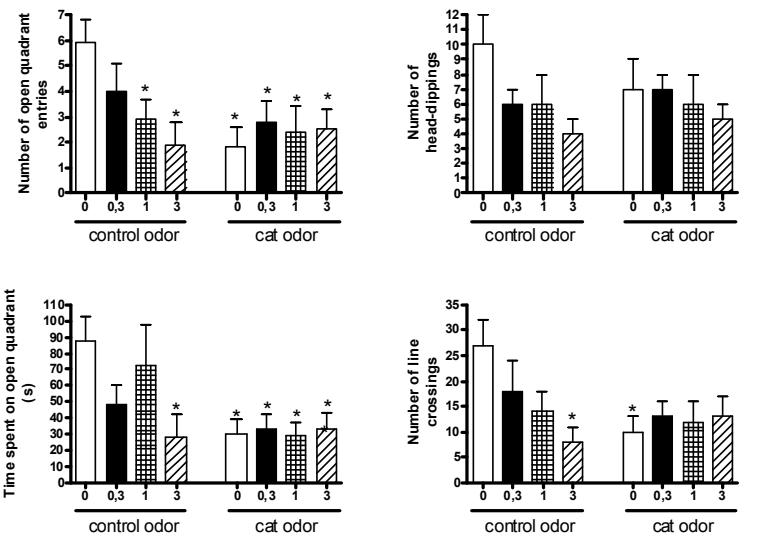

Figure 5. The effect of rimonabant (0.3–3 mg/kg) on the exploratory behaviour of male Wistar rats in the elevated zero-maze after exposure to control or cat odour (mean values±SEM). White bars saline; black bars rimonabant 0.3 mg/kg; hatched bars rimonabant 1 mg/kg; striped bars rimonabant 3 mg/kg.
* $p<0.05$ (compared to the control odour vehicle-treated group, Newman-Keuls test after significant two-way ANOVA)

EXPERIMENT 3. CAT ODOUR EXPOSURE INDUCED AN ANXIETY-LIKE STATE AND REDUCED *Wfs1* mRNA EXPRESSION IN THE MESOLIMBIC AREA AND TEMPORAL LOBE IN THE C57BL/6 STRAIN, BUT NOT IN 129Sv MICE (PAPER II)

3.1. Behavioural studies

3.1.1. Exposure to cat odour in C57Bl/6 and 129Sv mice

In the presence of a control cloth, C57Bl/6 mice exhibited significantly higher exploratory activity than 129Sv mice (Table 9). Nearly all measured parameters of exploratory behaviour were significantly higher in C57Bl/6 mice (Newman-Keuls test, $p < 0.05$). Only time spent in contact with the control cloth did not differ between C57Bl/6 and 129Sv mice. The exposure to cat odour suppressed exploratory activity in C57Bl/6 mice as evidenced by reduced horizontal and vertical exploratory activity and digging behaviour. However, cat odour exposure did not change the number and duration of cloth contacts in C57Bl/6 mice. By contrast, cat odour exposure had no effect on the exploratory activity of 129Sv strain mice and tended to increase the number and duration of cloth contacts. Two-way ANOVA revealed a significant strain effect for the number of contacts with cloth [$F1,63 = 65.7$, $p < 0.001$ (strain); $F1,63 = 1.48$, $p = 0.23$ (exposure); $F1,63 = 0.56$, $p = 0.46$ (strain × exposure)] and duration of cloth contact [$F1,63 = 6.96$, $p < 0.01$ (strain); $F1,63 = 1.98$,$p = 0.16$ (exposure); $F1,63 = 1.85$, $p = 0.18$ (strain × exposure)]. Contrary to our expectations, 129Sv mice spent significantly longer time in contact with the cat odour impregnated cloth than individuals from C57Bl/6 strain (Table 9). Two-way ANOVA established significant strain as well as strain and exposure interaction effects for the following parameters of exploratory behaviour: number of transitions [$F1,63 = 43.3$, $p < 0.001$ (strain); $F1,63 = 3.39$, $p = 0.07$ (exposure); $F1,63 = 16.5$, $p < 0.001$ (strain × exposure)], number of rearing [$F1,63 = 206.0$, $p < 0.001$ (strain); $F1,63 = 2.68$, $p = 0.11$ (exposure); $F1,63 = 4.29$, $p < 0.05$ (strain × exposure)], number of digging [$F1,63 = 56.9$, $p < 0.001$ (strain); $F1,63 = 11.9$, $p = 0.001$ (exposure); $F1,63 = 10.1$, $p < 0.01$ (strain × exposure)], duration of digging [$F1,63 = 37.3$, $p < 0.001$ (strain); $F1,63 = 6.41$, $p = 0.01$ (exposure); $F1,63 = 9.0$, $p < 0.01$ (strain × exposure)] and number of burying [$F1,63 = 55.0$, $p < 0.001$ (strain); $F1,63 = 0.48$, $p = 0.49$ (exposure); $F1,63 = 5.33$, $p < 0.05$ (strain × exposure)]. For time spent in burying, only strain effect was established [$F1,63 = 57.8$, $p < 0.001$ (strain); $F1,63 = 1.02$, $p = 0.32$ (exposure); $F1,63 = 1.64$, $p = 0.21$ (strain × exposure)]. *Post hoc* analysis demonstrated that cat odour exposure suppressed exploratory behaviour in C57Bl/6 mice, but not in 129Sv strain (Table 9)

Table 9. The effect of cat odour exposure on the behaviour of 129Sv and C57Bl/6 mice

Behavioural parameters	129Sv clean cloth	129Sv cat odour	C57/Bl6 clean cloth	C57/Bl6 cat odour
Number of contacts with the cloth	18 ± 2.8	25 ± 2.5	48 ± 3.7*	49 ± 4.1
Duration of cloth contact (s)	462 ± 122	683 ± 96‡	360 ± 33	364 ± 34
Number of transitions	20 ± 1.6	25 ± 1.4	43 ± 3.4*	30 ± 1.8†
Number of rearing	24 ± 5.2	26 ± 3.5	96 ± 5.2*	79 ± 3.3†
Number of digging	6.3 ± 1.1	5.8 ± 0.6	21 ± 2.1*	12 ± 1.1†
Duration of digging (s)	10 ± 2.3	11 ± 1.5	38 ± 4.5*	21 ± 3.1†
Number of burying	3.6 ± 1.0	2.2 ± 0.5	7.8 ± 0.9*	10.2 ± 0.9†
Duration of burying (s)	4 ± 1.2	3 ± 1.1	16 ± 2.6*	20 ± 2.2

Mean values ± S.E.M. are presented in the table. Number of mice in each group was 16–17. * $p < 0.05$ (compared with 129Sv clean cloth group, Newman-Keuls test after the significant two-way ANOVA).
† $p < 0.05$ (compared with the respective clean cloth group).
‡ $p < 0.05$ (compared with C57Bl/6 mice exposed to cat odour).

3.2. Gene expression studies

3.2.1 Mesolimbic area

Expression level of *Wfs1* gene did not differ significantly in the mesolimbic area of C57Bl/6 and 129Sv mice not exposed to cat odour (Table 10). After cat odour exposure, significant differences in *Wfs1* gene expression were established between C57Bl/6 and 129Sv strains (Table 10). Two-way ANOVA (strain and exposure as independent factors) revealed both strain and strain x exposure effects [$F1,42 = 8.97$, $p < 0.01$ (strain); $F2,42 = 0.55$, $p = 0.58$ (exposure); $F2,42 = 3.68$, $p < 0.05$ (strain × exposure)] One-way ANOVA demonstrated a significant inhibitory effect of cat odour on the expression of *Wfs1* gene in the mesolimbic area ($F2,21 = 3.53$, $p < 0.05$) of C57Bl/6 animals.

3.2.2. Temporal lobe

After cat odour exposure, differences in *Wfs1* expression were observed between C57Bl/6 and 129Sv mice [F1,42 = 2.61, p = 0.11 (strain); F2,42 = 0.13, p = 0.88 (exposure); $F2,42 = 4.97$, $p < 0.01$ (strain × exposure)]. Further analysis demonstrated that the established differences were mostly due to changes in the gene expression in C57Bl/6 mice, but not in 129Sv strain. One-way ANOVA demonstrated a significant inhibitory effect of cat odour on the expression of *Wfs1* gene in the temporal lobe ($F2,21 = 3.72$, $p < 0.05$) of C57Bl/6 animals. The subsequent use of Tukey HSD test demonstrated that the effect of cat odour exposure was significant ($p < 0.05$) when compared to naive and clean cloth exposed mice in the temporal lobe, and to naive animals (Table 10).

3.2.3. Prefrontal cortex

In the prefrontal cortex only strain effect was established [$F1,42 = 8.95$, $p < 0.01$ (strain); $F2,42 = 0.55$, $p = 0.58$ (exposure); $F2,42 = 0.53$, $p = 0.59$ (strain × exposure)]. However, the *post hoc* test did not establish any statistically significant differences between the groups (Table 10).

Table 10. The effect of cat odour exposure on the expression of *Wfs1* in the prefrontal cortex, temporal lobe and mesolimbic area of C57Bl/6 and 129Sv mice.

Brain structure	C57Bl/6 (naive)	C57Bl/6 (clean cloth)	C57Bl/6 (cat odour)	129Sv (naive)	129Sv (clean cloth)	129Sv (cat odour)
Prefrontal cortex	1.00 ± 0.10	0.83 ± 0.18	0.92 ± 0.15	1.20 ± 0.11	1.18 ± 0.17	1.41 ± 0.12
Mesolimbic area	1.00 ± 0.08†	0.81 ± 0.14	0.57 ± 0.09*	0.91 ± 0.15	1.22 ± 0.12	1.19 ± 0.18
Temporal lobe	1.00 ± 0.06†	1.02 ± 0.11†	0.72 ± 0.09*	0.91 ± 0.09	1.00 ± 0.11	1.24 ± 0.16

Mean values ± S.E.M. are presented in the table. Number of mice in each group was 8.
* $p < 0.05$ (compared to 129Sv mice exposed to cat odour, Tukey HSD test after significant two-way ANOVA).
† $p < 0.05$ (compared to C57Bl/6 mice exposed to cat odour, Tukey HSD test after significant one-way ANOVA).

EXPERIMENT 4. EFFECT OF EXPOSURE TO THE ELEVATED PLUS-MAZE ON THE LEVEL OF CORTICOSTERONE AND EXPRESSION OF *Wfs1* GENE DEPENDS ON THE EXPLORATORY ACTIVITY OF MALE WISTAR RATS (PAPER III)

4.1. Behavioural studies

4.1.1. Selection of rats according to their exploratory behaviour in the EPM

According to the exploratory activity the rats (42) were divided into three groups. Animals making two or three open arm visits (2.3±0.2) were selected into the high exploratory activity group (10 rats). Rats making two to three closed arm entries (2.5±0.5) were selected into the low exploratory group (10 rats). From the remaining 22 rats intermediate group consisting of ten animals was randomly selected. These animals frequently explored the closed arm (7.0±0.5) and six of them also performed one open arm visit. In comparison with the other groups, the rats with high exploratory activity displayed shorter

latency to enter the central square (F2,27=6.1, p<0.01) and lower open arm avoidance (number of open arm entries: F2,27=90.1, p<0.0001; time on open arms: F2,27=19.2, p<0.0001; number of closed arm entries: F2,27=12.2, p<0.001; ratio between open and total arm entries: F2,27=93.2, p<0.0001) (Table 11). Also, high exploratory animals performed more line crossings (F2,27=18.4, p<0.0001); number of protected head-dippings (F2,27=8.5, p<0.01); unprotected head dippings (F2,27=26.8, p<0.0001), total head-dippings (F2,27=19.3, p<0.0001) and number of stretch-attend postures (F2,27=2.21, p<0.13) compared to low exploratory animals (Table 11). The intermediate group had higher locomotor activity and spent more time on the central square compared to the low exploratory activity group. Rats with low exploratory activity performed more attempts to enter the central square from the closed arm compared to the high exploratory activity group (number of attempts to enter into the central square: F2,27=7.7, p<0.01; time on central square: F2,27=13.0, p<0.001). Significant correlations were found between the ratio of open and closed arm entries with the number of open arm entries (r=0.93, p<0.0001), time spent in exploring the open arms (r=0.92, p<0.0001) and the number of unprotected head-dippings (r=0.86, p<0.0001). Also, two measures of locomotor activity, the number of closed arm entries and line crossing were in significant correlation (r=0.88, p<0.0001). There was no significant correlation between the parameters of anxiety and locomotor activity in the EPM.

Table 11. Selection of rats according to their exploratory activity in the elevated plus-maze.

Behavioural parameters	**Low**	**Intermediate**	**High**
Latency to enter central square, sec	159±44	56±7*	25±5*
Number of open arm entries	0±0	0.6±0.2*	2.3±0.2*, **
Time on open arms, sec	0±0	6±2	31±6*,**
Number of closed arm entries	2.5±0.5	7.0±0.8*	5.9±0.7
Ratio between open and total arm entries, x100	0±0	8±3	29±2*,**
Number of line crossings	9±1	20±2*	23±2*
Number of unprotected head-dippings	0±0	1.4±0.5	4.5±0.7*, **
Number of total head-dippings	3.6±0.9	8.2±1.1*	12.3±1.6*
Number of stretch-attend postures	2.0±0.5	1.6±0.4	0.8±0.3
Number of attempts to enter into the central square	2.6±0.3	1.5±0.3	0.6 ±0.2*
Number of risk assessment behaviours	4.6±0.7	3.1±0.5	1.4 ±0.3*
Time on central square, sec	2±1	31±6*	33±4*

* – p<0.05 (compared to low exploratory group, Tukey HSD test after significant one-way ANOVA);
** – p<0.05 (compared to intermediate exploratory group). Number of animals in each group was 10.

4.2. Corticosterone levels

Short-term isolation and EPM exposure significantly increased the level of corticosterone in the serum of all groups compared to home-cage controls ($F3, 28=13.0$, $p<0.0001$) (Figure 6A). In the high exploratory activity group there was more pronounced elevation of the corticosterone level compared to low exploratory activity animals (Tukey HSD test after significant two-way ANOVA, $p<0.05$). Also, the level of corticosterone significantly correlated with the number of open arm entries ($r=0.56$, $p<0.001$), number of line crossings ($r=0.60$, $p<0.001$) and number of unprotected head-dippings ($r=0.59$, $p<0.001$), reflecting the relation between the increased exploratory activity and levels of corticosterone.

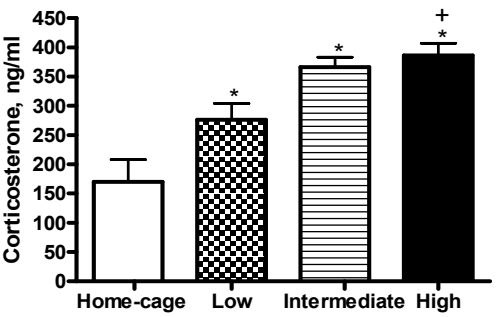

Figure 6. Effect of EPM exposure on the level of corticosterone.
∗ $p<0.05$ (compared to home-cage control animals, Tukey HSD after significant two-way ANOVA), + $p<0.05$ (compared to low activity group). The number of rats in each group was 8.

4.3. Oxidative stress index

There were no correlations between the exploratory behaviour and systemic oxidative stress in rats exposed to the EPM.

4.4. Gene expression studies

4.4.1. ECS genes

We did not find any differences of expression levels of ECS genes in the mesolimbic area, temporal lobe and hippocampus of the selected rats.

4.4.2. *Wfs1* gene

The expression level of the *Wfs1* gene was significantly reduced in high exploratory activity rats compared to home-cage control and low exploratory activity animals in the mesolimbic area ($F2,18=7.2$, $p<0.01$) and in the hippocampus ($F2,18=7.3$, $p<0.01$) (Figure 7). *Wfs1* gene expression was 1.4-fold higher in low exploratory rats compared to high exploratory activity group in both brain areas.

On the contrary, in the temporal lobe the expression level of the *Wfs1* gene was significantly reduced in low exploratory activity rats ($F2,18=11.4$, $p<0.001$) (Figure 7). *Wfs1* gene expression was 1.6-fold lower in low exploratory activity rats compared to high exploratory activity group.

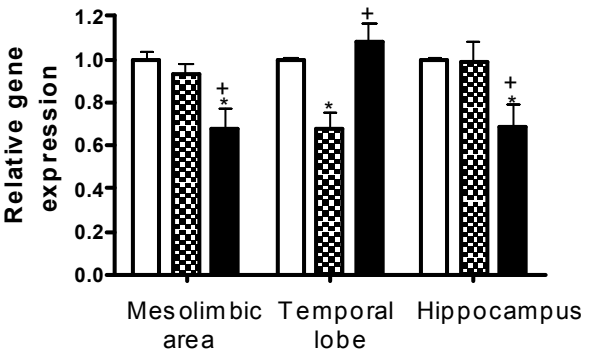

Figure 7. *Wfs1* gene expression in the mesolimbic area, temporal lobe and hippocampus of rats selected according to their exploratory activity. White bars – home-cage controls; hatched bars – low exploratory activity group; black bars – high exploratory activity group.
*$p < 0.05$ (compared to home-cage control animals, Tukey HSD test after significant one-way ANOVA); +$p < 0.05$ (compared to low activity group). The number of rats in each group was 7.

EXPERIMENT 5. *Wfs1*-DEFICIENT MICE SHOW INCREASED ANXIETY IN THE ELEVATED PLUS-MAZE AND DECREASED LEVEL OF EXPRESSION OF *GABRA1* AND *GABRA2* GENES COMPARED TO THEIR WILD-TYPE LITTERMATES (PAPER IV)

5.1. Behavioural studies

In the plus-maze test, *Wfs1*-deficient mice displayed increased anxiety-like behaviour in terms of increased open arm avoidance and affected risk assessment behaviours. Homozygous animals made significantly less open arm entries as compared to wild-type mice (Student's t-test, $p<0.05$) (Figure 8). Also, a similar tendency was observed for other conventional measures of anxiety: time spent on open arms ($p=0.07$) and ratio between the open and total arm entries ($p=0.07$). Additionally, the genotype differences were noticeable for the ethological measures of anxiety. Namely, *Wfs1*-deficient mice performed significantly more stretch-attend postures compared to their wild-type littermates ($p<0.05$) (Figure 8). The number of head-dippings was also reduced, but remained statistically not significant in genetically modified animals ($p=0.09$).

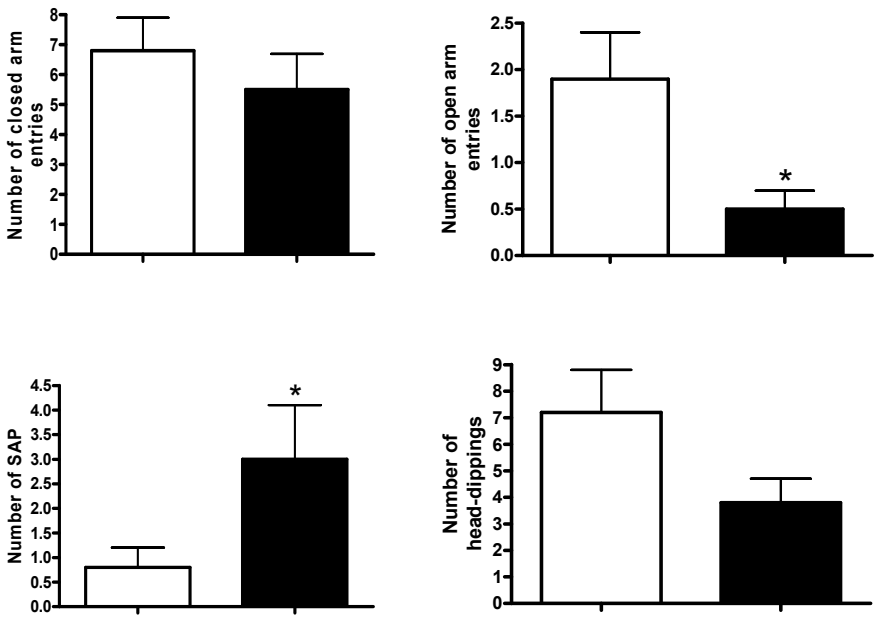

Figure 8. Exploratory behaviour of *Wfs1*-deficient mice in the elevated plus-maze. White bars: wild-type mice; black bars: homozygous mice.*$p < 0.05$ (compared to wild-type mice, Student's *t*-test for independent samples).

5.2. Gene expression analysis

We established significant genotype as well as genotype and elevated-plus-maze exposure interactions for the *Gabra1* and *Gabra2* genes in the frontal cortex and temporal lobe (Figure 9). Genetic invalidation of the *Wfs1* gene induced a remarkable reduction of the *Gabra1* and *Gabra2* genes in the temporal lobe (*Gabra1*: $F1,28 = 8.97$, $p = 0.0058$; *Gabra2*: $F1,28 = 1.80$, $p = 0.19$) and frontal cortex (*Gabra1*: $F1,28 = 7.09$, $p = 0.02$;. *Gabra2*: $F1,28 = 3.28$, $p = 0.03$) (Figure 9). The exposure of wild-type mice to the elevated plus-maze also reduced the expression level of these genes in the frontal cortex (*Gabra1 :* $F1,28 = 2.03$, $p = 0.17$; *Gabra2:* $F1,28 = 0.03$, $p = 0.85$) and significantly in the temporal lobe (*Gabra1:* $F1,28 = 23.7$, $p = 0.00004$; *Gabra2:* $F1,28 = 5.57$, $p = 0.03$). The exposure of *Wfs1*-deficient mice to the elevated plus-maze did not cause a further reduction in the expression of the *Gabra1* and *Gabra2* genes compared to the experimentally naive mutant mice in the frontal cortex (*Gabra1*: $F1,28 = 3.59$, $p = 0.07$; *Gabra2:* $F1,28 = 6.02$, $p = 0.02$) and temporal lobe (*Gabra1:* $F1,28 = 9.42$, $p = 0.0058$; *Gabra2:* $F1,28 = 5.43$, $p = 0.03$).

Genetic invalidation of the *Wfs1* gene or exposure of wild-type mice to the elevated plus-maze did not affect the expression of the *Gad1* and *Gad2* genes in the frontal cortex [*Gad1*: $F1,28 = 2.06$, $p = 0.16$ (genotype); $F1,28 = 0.82$, $p = 0.37$ (plus-maze exposure); $F1,28 = 0.05$, $p = 0.82$ (genotype × plus-maze exposure). *Gad2*: $F1,28 = 0.94$, $p = 0.34$ (genotype); $F1,28 = 2.53$, $p = 0.13$ (plus-maze exposure); $F1,28 = 0.66$, $p = 0.42$ (genotype × plus-maze exposure)] and temporal lobe [*Gad1*: $F1,28 = 3.73$, $p = 0.07$ (genotype); $F1,28 = 0.92$, $p = 0.35$ (plus-maze exposure); $F1,28 = 0.003$, $p = 0.95$ (genotype × plus-maze exposure). *Gad2*: $F1,28 = 2.63$, $p = 0.12$ (genotype); $F1,28 = 0.32$, $p = 0.57$ (plus-maze exposure); $F1,28 = 0.21$, $p = 0.65$ (genotype × plus-maze exposure)] (Figure 10).

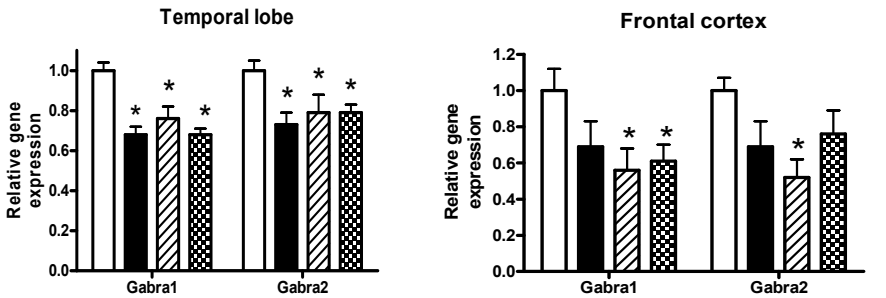

Figure 9. Effect of the *Wfs1* gene invalidation and plus-maze exposure on the expression of the *Gabra1* and *Gabra2* genes in the frontal cortex and temporal lobe. Gray bars: wild-type mice; hatched bars: wild-type mice exposed to the plus-maze; stripped bars: homozygous mice; white bars: homozygous mice exposed to the plus-maze.

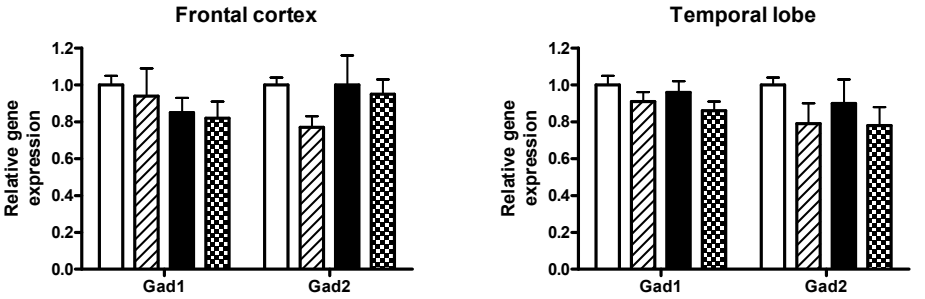

Figure 10. Effect of the *Wfs1* gene invalidation and plus-maze exposure on the expression of the *Gad1* and *Gad2* genes in the frontal cortex and temporal lobe. White bars: wild-type mice; stripped bars: wild-type mice exposed to the plus-maze; black bars: homozygous mice; hatched bars: homozygous mice exposed to the plus-maze.

DISCUSSION

6.1. Cat odour induced anxiety (Papers I, II)

6.1.1. Cat odour induced anxiety, reduced exploratory activity and altered the expression of the ECS genes in male Wistar rats (Paper I)

We obtained the same results as in numerous previous studies (Blanchard et al., 2003; Dielenberg et al., 2001; Li et al., 2004; Adamec et al., 1998; McGregor et al., 2002) in that the exposure of male Wistar rats to a natural unpleasant stimulus – cat odour – induced a robust anxiogenic-like response. After cat odour exposure the rats displayed significantly increased freezing and none of animals touched the cat odour-impregnated cloth. Subsequent exposure of rats to the elevated zero-maze, an exploratory model of anxiety, demonstrated a significant inhibition of exploratory behaviour in rats after the predator odour exposure. This finding is in good accordance with the previous studies demonstrating reduced exploratory activity of rodents after the predator odour stress in the elevated plus-maze and light/dark box exploration test (Adamec and Shallow 1993; Hebb et al., 2002, 2003).

Cat odour, which is a strong stressor, induced distinct changes in the ECS genes in the different brain structures of rats. In the mesolimbic area (involving *tuberculum olfactorium* and *nucleus accumbens*), the predator odour tended to increase the expression of the ECS genes. However, only the increase of the *Faah* gene, an enzyme responsible for the degradation of anandamide, was statistically significant. By contrast, in the amygdala and PAG, the levels of the ECS genes were decreased after the predator odour exposure. In both brain structures, the gene expression of *Nape-pld* and *Faah*, the enzymes responsible for the metabolism of anandamide, was significantly reduced. The cat odour exposure also decreased the expression of *Dagla* in the amygdala and *Mgll* in the PAG. These enzymes are responsible for the synthesis and breakdown of 2-AG, respectively. The decreased level of 2-AG synthesis in the amygdala and breakdown in the PAG reflect the differences in the endocannabinoid signaling pathway in these brain structures. It could be speculated, that the lower activation of CB_1 receptors may be one reason for the cat odour-induced freezing behaviour. The cat odour tended to decrease the expression of the ECS genes in the amygdala and PAG, but increased their expression in the mesolimbic area. In the mesolimbic area only the level of anandamide was changed after the exposure to predator odour. On the contrary, in the amygdala and PAG, the level of 2-AG and anadamide were changed. Therefore, it looks like the amygdala and PAG play the pivotal role in the regulation of anxiety mediated by the ECS. Assumingly, these distinct changes in the expression of the ECS genes in the mesolimbic area, amygdala and PAG after the cat odour exposure could be attributed to a different role of these brain regions in response to predator odour. The amygdala and PAG are involved in the

regulation of innate mechanisms of anxiety (Adamec et al., 2003; Wallace et al., 2004; Moreira et al., 2007), whereas the mesolimbic area plays a role in the mechanisms of motivation (Robbins and Everitt 1996; Kelley, 2004).

The injection of cannabinoids may induce either anxiolytic or anxiogenic-like effect depending on the environmental context and the dose administred (Viveros et al., 2005). The administration of THC at low doses into the pre-frontal cortex or ventral hippocampus induced in rats an anxiolytic-like response in the elevated plus-maze, whereas higher doses caused anxiogenic-like effect. On the contrary, low doses of THC in the basolateral amygdala produced an anxiogenic-like response, whereas high doses were ineffective. All these effects are probably triggered by a CB_1-dependent mechanism and are closely linked to the modulation of CREB activation (Rubino et al., 2007). Based on the different expression patterns of endocannabinoid genes in the various brain structures, it is likely that the established bidirectional effects can be mediated by distinct activation of CB_1 receptors in the brain structures after stressful stimuli.

The combination of two stressful events (cat odour and subsequent exposure to the elevated zero maze) did not potentiate the effects of each other on the expression of the ECS genes. The only exception was *Nape-pld* in the meso-limbic area. This leads probably to the elevation of the anandamide level and enhancing the endogenous cannabinoid signaling. It has been shown previously that elevated level of anandamide is anxiolytic for rodents (Kathuria et al., 2003) and the exploratory activity after cat odour may be rewarding for animals. In the amygdala and PAG, the following exposure to the zero-maze tended to antagonise changes caused by the cat odour exposure. In the amygdala downregulation of *Nape-pld*, *Faah* and *Dagla* after exposure to the cat odour was antagonised by the subsequent exposure to the elevated zero-maze, and in the PAG the expression of *CB_1* receptor showed the same effect. The PAG is responsible for a stereotyped, reflexive, autonomic and behavioural "fight or flight" response to unconditioned fear (Lang et al., 1998; Millan, 2003). It has been shown that direct administration of anandamide into the dorsolateral PAG has an anxiolytic-like effect in the elevated plus-maze (Moreira et al., 2007) and pre-treatment of anandamide or anandamide transport inhibitor (AM404) into the dorsolateral PAG attenuates freezing in the contextual fear conditioning (Resstel et al., 2008). 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 a global response to threat (Millan, 2003). Therefore, one may conclude that the established differences in gene expression reflect a greater plasticity of the EC mechanisms in the amygdala than in the other brain areas in response to subsequent aversive stimuli.

6.1.2. Rimonabant, a CB_1 receptor antagonist, did not affect cat odour-induced anxiogenic-like effect (Paper I)

In various previous studies it has been shown that the pharmacological blockade of CB_1 receptor by rimonabant or invalidation of *CB_1* gene (Maccarrone et al., 2002; Martin et al., 2002; Haller et al., 2004) inhibited the exploratory behaviour of rodents in the ethological models of anxiety (Navarro et al., 1997; Arévalo et al., 2001). It is known that the exploratory activity of rats is regulated by two opposing factors: neophobia and exploratory drive (Montgomery, 1955; Rodgers and Dalvi, 1997). Therefore, we assumed that the anti-exploratory effect of rimonabant can be more easily established in highly motivated rats and the exposure of rats to cat odour was not performed in their home cages, but in a novel, unknown environment to facilitate the exploratory drive in animals. As we expected, the administration of rimonabant induced a dose-dependent reduction of exploratory activity of control rats in the motility boxes. Already 0.3 mg/kg of rimonabant was effective to reduce the frequency of rearing in the motility boxes. The anxiogenic-like profile of rimonabant was also confirmed in the elevated zero-maze where the CB_1 receptor antagonist significantly decreased the exploratory behaviour of rats. The cat odour exposure also decreased the exploratory activity of rats in the motility boxes and in the elevated zero-maze. Pre-treatment with rimonabant before the cat odour exposure did not reverse the anxiogenic- like behaviour in the motility boxes and zero-maze. Although in a previous study (Griebel et al., 2005) it has been shown that rimonabant has anxiolytic- and antidepressant-like effect after mild chronic stress. Probably, the lack of effect of the CB_1 antagonist can also be explained in the light of the above-described gene expression data. Namely, the exposure of rats to the cat odour decreased the expression of several ECS genes in the amygdala and PAG. Therefore, it is difficult to believe that the further inhibition of the EC system by rimonabant would antagonise the behavioural changes caused by the cat odour.

6.1.3. Cat odour induced distinct changes in exploratory activity and *Wfs1* expression in C57Bl/6 and 129Sv mice (Paper II)

This study definitely confirms previously reported differences in the exploratory behaviour of C57Bl/6 and 129Sv strains (Võikar et al., 2001; Rodgers et al., 2002). The significant genetic differences between these two mice strains have been underlined by transgenic studies where the various genetic invalidations induce different phenotypic effects in C57Bl/6 and 129Sv mice. For example, Holmes and colleagues (2003) have demostrated that the genetic invalidation of serotonin transporter gene induced anxiety-like effect in C57Bl/6 mice, but not in 129Sv strain. Also, the glucose metabolism is different in C57Bl/6 and 129Sv mice (Berglund et al., 2008). In the present study C57Bl/6 mice were significantly more active in the open-field test compared to 129Sv strain. Cat

odour exposure induced anxiety-like state in C57Bl/6 mice by reducing horizontal and vertical exploratory activity and digging behaviour. In addition, C57Bl/6 mice made more attempts to cover the cat odour impregnated cloth with bedding than the control cloth. These results are in agreement with previous study (Belzung et al., 2001) showing that C57Bl/6 mice belong to the inbreed mice strains responding with increased anxiety to the exposure of cat odour. In contrast, cat odour exposure had no effect on the exploratory activity of 129Sv mice, but tended to increase time spent in contact with cloth impregnated with cat odour. Therefore, 129Sv mice belong to the inbreed mice strains not responding to the cat odour. It is possible that these non-responding mice strains have lost during the extensive breeding the genes responsible for responding to cat odour. On the other hand, the mice strains, lacking response to cat odour, are characterized by increased anxiety and reduced exploratory behaviour in a novel environment. One could speculate that the genes involved in the response to cat odour have a wider impact on the regulation of behaviour. It is likely that they belong to the molecular mechanisms responsible for adaptation in a novel environment and for coping strategies in stressful circumstances.

Kõks and coworkers (2002; 2004) demonstrated that the exposure of male Wistar rats to cat odour induces an up-regulation of the *Wfs1* gene in the amygdaloid area. Wfs1 protein is probably involved in adaptation to a novel and aversive environment (Luuk et al., 2009). In the present work, distinct mRNA expression changes of *Wfs1* were found in C57Bl/6 and 129Sv strains after cat odour exposure. Predator odour reduced *Wfs1* mRNA expression level in the mesolimbic structure and temporal lobe (including amygdala) of C57Bl/6 mice compared to the control group animals and the 129Sv strain. The Wolfram syndrome patients display psychiatric symptoms with especially high prevalence of depression (Swift and Swift, 2005; Swift et al., 1990) and the heterozygous carriers of *WFS1* mutations have a 26-fold higher likelihood of psychiatric hospitalization (Swift and Swift, 2000). Taking into account the previous studies with rats (Kõks et al., 2002; 2004) the current work confirms the role of *Wfs1* in the regulation of anxiety. The different behavioural response of C57Bl/6 and 129Sv to the cat odour exposure may be related to the established differences in the expression of the *Wfs1* gene. Dynamics of the *Wfs1* gene expression and exploratory behaviour observed in the present experiment suggest that the down-regulation of *Wfs1* in C57Bl/6 mice might be related to increased anxiety in this particular strain.

6.2. Relation of exploratory behaviour to plasma corticosterone level and gene expression changes in rats and mice (Papers III, IV)

6.2.1. High exploratory activity rats displayed higher plasma corticosterone level compared to low exploratory activity rats (Paper III)

The results of the present study are in agreement with previous studies showing that male Wistar rats display significant differences in the exploratory activity in the EPM (Harro et al., 1990; Rägo et al., 1991; Kõks et al., 1997; Schwarting et al., 1998). Exploratory activity is an important component of behaviour of rodents, which shows a tendency to explore or investigate a novel environment. It is related to the level of anxiety, because the animals with low anxiety level show higher exploratory activity compared to animals displaying higher anxiety level (Rodgers et al., 1997). Moreover, high anxiety rats selected by means of the EPM display rather similar behavioural characteristics in other tests of anxiety. For example, they have higher levels of object burying in marble burying task and slower acquisition in avoidance learning (Ho et al., 2002). The reason for these behavioural differences is not clear, but could be attributed to some extent to the existence of social hierarchy among the rats (Raab et al., 1986; Kozorovitskiy and Gould, 2004). Rodents with a lower ranking in the hierarchy are submissive and display passive coping strategies, whereas animals with a higher position display active coping strategies in stressful conditions (Landgraf and Wigger, 2002). Therefore, variations in exploratory behaviour probably reflect stress coping differences in the population of Wistar rats. It has been proposed by various studies that the exploratory activity as an indirect measure of coping style might explain individual vulnerability to anxiety (Steimer et al., 1997; Koolhaas et al., 1999). The differences in coping strategies are related to certain neurochemical and neuroendocrinological changes. It has been shown that animals with higher basal anxiety had a reduced level of 5-hydroxy-tryptamine in the ventral striatum, whereas no differences were established in the respective levels of dopamine and noradrenaline (Schwarting et al., 1998).

In the present work we used a lower illumination level compared to Nelovkov et al. (2006) resulting in remarkably higher exploratory activity of rats. In spite of this we established similar correlations between the spatial and temporal parameters of open arm exploration as in the mentioned study (Nelovkov et al., 2006). However, there were also differences, because the frequency of closed arm entries, a measure of locomotor activity, did not significantly correlate with the parameters of open arm exploration as it was demonstrated by Nelovkov et al. (2006). Therefore, probably a higher illumination level induces stronger anxiety and, therefore, suppresses the locomotor activity of rats.

In the present study we found that the level of corticosterone was significantly higher in high exploratory activity rats (actively exploring the open arms) than in low exploratory activity rats. It has been shown that time of stress

onset in relation to the phase of endogenous corticosterone basal level may determine the physiological response (Windle et al., 1998). Namely, rats exposed to noise stress in rising endogenous corticosterone phase responded with an additional release of corticosterone. Those in which basal corticosterone levels were falling at stressor onset showed little or no corticosterone response. In our study, blood for corticosterone measurements was collected after the behavioural testing due to the sensitivity of the EPM test to preceding manipulations. Nonetheless, the variations in blood corticosterone levels were similar between the different groups at selected time points. High exploratory activity rats always had higher stress hormone levels as compared to naive and low exploratory activity animals. Therefore, it is apparent that the established differences in corticosterone levels are caused by the EPM exposure rather than from the existing biological rhythms. Our findings are in good agreement with the results of Pellow et al. (1985) demonstrating a stronger elevation of corticosterone after exposure of rodents to open arms as compared to closed arms. As a matter of fact, in the current study a strong positive correlation was established between corticosterone levels and exploratory behaviour. Interestingly, Rodgers et al. (1999) established significant correlations between stress hormone level and risk assessment behaviours. Probably variations in experimental conditions, mostly due to different illumination conditions and short-term isolation before the EPM exposure, are responsible for different outcomes in these studies. Thus, one may conclude that apparently more aversive conditions are necessary to find correlations between corticosterone levels and the exploration of open arms.

Although recent evidence suggests that stressful events also increase the markers of oxidative stress in the brain of rodents (Chakraborti et al., 2007; Horoz et al., 2006), we found neither statistically significant differences in the OSI between groups of rats nor correlations between the parameters of the EPM exploration and OSI. Probably stronger or chronic stress is necessary to induce a substantial elevation of the oxidative stress marker.

6.2.2. EPM exposure significantly changed the mRNA level of the *Wfs1* gene, but not ECS genes in different brain structures (Paper III)

The exposure of rats to the elevated plus-maze did not change the expression of the ECS genes in the temporal lobe, hippocampus and mesolimbic structure (Paper III). This is a significant difference from the study (Paper I), where the rats were exposed to cat odour. Correspondingly, the exposure of rats to the plus-maze is not strong enough stress to reduce the expression of the ECS genes. This statement is in good accordance with the previous studies, where the elevated plus-maze alone has been not a stressor strong enough to change the expression of pre-proCCK mRNA levels in the amygdala and hippocampus (Pratt and Brett, 1995). By contrast, pre-treatment of rats with FG7142, an anxiogenic inverse agonist of benzodiazepine receptors, significantly elevated

the expression of pre-pro-CCK in these areas (Pratt and Brett, 1995). Also, the level of pCREB immunoreactivity in the amygdala, BNST and PAG was not changed after the elevated plus-maze exposure. However, the exposure of rats to the EPM after predator odour markedly increased the level of pCREB immunoreactivity in the right lateral PAG (Blundell and Adamec, 2006).

Involvement of the *Wfs1* gene in the adaptation to a novel environment (Luuk et al., 2009) and in psychiatric disorders (Swift et al., 1990) has been shown. Exposure to the EPM changes the mRNA expression level of the *Wfs1* gene in different brain structures in a distinct manner. In low exploratory activity rats, displaying increased anxiety, the *Wfs1* gene expression was decreased in the temporal lobe (including amygdala), however, in high exploratory rats, exhibiting high exploratory drive, the *Wfs1* mRNA expression was decreased in the mesolimbic area and hippocampus compared to the control group. The temporal lobe contains the amygdala and limbic cortical region that play an important role in the regulation of fear and anxiety (LeDoux, 2000). The *nucleus accumbens* in the mesolimbic area participates in motivations (Groenewegen, et al., 1996) and hippocampus has a role in learning and memory (Rudy and O'Reilly, 1999; Burgess et al., 2002). The amygdala, *nucleus accumbens* and CA1 region of the hippocampus are rich in Wfs1 protein (Takeda et al., 2001; Luuk et al., 2008; Becker et al., 2008; Kato et al., 2008). Molecular studies demonstrate that Wfs1 protein is responsible for Ca^{2+} homeostasis (Takei et al., 2006) and maturation of Na^{+}/K^{+}-ATPase β1 subunit (Zatyka et al., 2008). Therefore, assumingly the Wfs1 protein plays a role in the excitation-secretion coupling necessary for the release of increased amounts of hormones and neurotransmitters needed in stressful circumstances. Probably the altered expression of the *Wfs1* gene reflects distinct changes in the activity of particular brain structures in response to stress.

6.2.3. Reduced exploratory activity in *Wfs1*-deficient mice is related to a decrease of mRNA expression of *Gabra1* and *Gabra2* genes (Paper IV)

Wfs1-deficient mice display remarkably increased frequency of anxiety-like behaviours in the elevated plus-maze compared to wild-type littermates. It has been shown previously that pre-treatment of mutant mice with diazepam (1 mg/kg), an anxiolytic drug acting via $GABA_A$ receptors, antagonized increased anxiety and stress induced vocalization in *Wfs1*-deficient mice (Luuk et al., 2009). Therefore we studied gene expression changes of $GABA_A$ receptor subtypes (*Gabra1*, *Gabra1*) and enzymes responsible for the synthesis of GABA (*Gad1*, *Gad2*). *Wfs1*-deficient mice had decreased mRNA expression of *Gabra1* and *Gabra1* genes in the temporal lobe and frontal cortex compared to their wild-type littermates. The mRNA expression of *Gabra1* and *Gabra1* genes in the hippocampus and mesolimbic area were not changed in different groups. Also, there were no differences in the mRNA expression of *Gad1* and *Gad2* genes in temporal lobe and frontal cortex. These results show that increased

anxiety in *Wfs1*-deficient mice is probably related to decreased levels of *Gabra1* and *Gabra1* expression and reduced GABA-ergic neurotransmission in these particular brain regions. The lack of changes in the expression of *Gad1* and *Gad2* demonstrates that the invalidation of the *Wfs1* gene does not have a major impact on the synthesis of GABA in the brain. It has been shown that chronic GABA-treatment decreases the level of $GABA_A$ receptors in the CNS and uncoupling of GABA and bensodiazepine binding sites (Montpied et al., 1991; Roca et al., 1990) and it is regulated by the level of transcription (Lyons et al., 2000). Later, it has been revealed that the down-regulation of $GABA_A$ subunit receptors mRNA occurs due to GABA-induced elevation of intracellular Ca^{2+} levels (Lyons et al. 2001). Wfs1 protein modulates Ca^{2+} levels in the intracellular space (Takei et al., 2006), probably the elevated release of GABA due to heightened sensitivity to stress of *Wfs1*-deficient mice is responsible for the down-regulation of $GABA_A$ receptor subunits. A similar down-regulation of the *Gabra1* and *Gabra1* genes in the temporal lobe and frontal cortex was established in response to exposure to the elevated plus-maze in wild-type animals. This finding further underlines the possible role of stress in the down-regulation of $GABA_A$ receptor subunits established in *Wfs1*-deficient mice. These results are in favor of a relation between increased anxiety and reduced expression of subunits of $GABA_A$ receptors in the frontal cortex and temporal lobe in *Wfs1*-deficient mice. This could also be a reason for higher sensitivity of *Wfs1*-deficient mice toward the anxiolytic-like action of diazepam and alcohol.

6.3. Concluding remarks and future perspectives

The present study underlines the role of two distinct targets – ECS and *Wfs1* – in the adaptation to a novel environment and in the regulation of emotional behaviour. The changes in activity of the ECS are apparently related to the intensity of stress. Exposure to cat odour, but not to the exploratory models of anxiety (elevated plus-maze and zero-maze), caused a down-regulation of the ECS genes in the amygdala and periaqueductal gray, in the brain structures, playing a direct role in the regulation of fear and anxiety (Table 12). Therefore, the exposure of rats to cat odour is apparently a stronger stressor than exposure to the exploratory models of anxiety. The exploratory activity after strong stress is probably rewarding for rodents, which probably explains the antagonizing effect of the elevated zero-maze exposure after cat odour-induced inhibition of the ECS gene expression in the amygdala (Table 12). The prominent role of the ECS in the regulation of anxiety was further elaborated by experiments with rimonabant, an antagonist of CB_1 receptors. In the present study, the blockade of CB_1 receptors with rimonabant did not induce anxiolytic-like actions in response to cat odour-induced anxiety, as proposed by Griebel and colleagues (2005). By contrast, rimonabant tended to increase anxiety in rodents if given alone. Taking into account the reduced expression of the ECS genes in various brain structures due to cat odour exposure it is very unlikely that the blockade

of CB_1 receptors is not a right mechanism to ease anxiety. This is in line with recent developments with rimonabant in the drug market. Rimonabant, prescribed for control of body weight, caused in the certain patients severe emotional side-effects and, therefore, a drug has been withdrawn from the market. Nevertheless, according to the present study, the ECS remains a promising and attractive target for the development of drugs, reducing unwanted consequences of severe stress and easing symptoms of emotional disturbances.

Wolframin is another molecular target for better understanding of brain mechanisms of stress and psychiatric disorders. First of all, the *Wfs1* gene looks like a good and reliable marker to follow the ongoing molecular changes in the brain regions in response to emotional stress. This was established both in the case of cat odour as well as in the case of plus-maze exposure (Table 12). Moreover, the relation of the *Wfs1* gene to the regulation of emotional behaviour was further demonstrated by experiments where mice lacking the *Wfs1* gene were employed. Genetic invalidation of the *Wfs1* gene in mice induces severe alterations in the ability to cope with stress in threating and aversive conditions. Alterations in the emotional behaviour of *Wfs1*-deficient mice can be connected to reduced expression of *Gabra1* and *Gabra1* genes, subunits of $GABA_A$ receptors mediating the effects of anxiolytic drugs, in the frontal cortex and limbic structures. Therefore, homozygous and heterozygous *Wfs1*-deficient mice are definitely good models for studying the mechanisms of pathological anxiety. Our studies strongly support the importance of further experiments in order to establish the neuroanatomical and -chemical mechanisms linking the central amygdala with the paraventricular nucleus (involved in the control of hypothalamic-pituitary-adrenal axis) and with the periaqueductal gray (mediating various stress evoked behavioural responses, including stress-induced analgesia).

The results of present work indicate that strong stress – cat odour exposure – induces changes in the expression of the ECS and *Wfs1* genes. A possible interaction between the ECS and *Wfs1* genes in the regulation of anxiety should be investigated in the future, whereas the *Wfs1*-deficient mice are a good model for that purpose.

Table 12. Overview of gene expression studies of the ECS genes and *Wfs1* in different brain structures after elevated zero-maze/plus-maze exposure or cat odor exposure. **ML** – mesolimbic structure, **TL** – temporal lobe (including amygdala), **HC** – hippocampus, **AMY** – amygdala, **PAG** – periaqueductal gray

Gene symbol	**Elevated zero-maze or plus-maze exposure**			**Cat-odor exposure**			**Cat-odor and elevated zero-maze exposure**		
	ML	**TL**	**HC**	**ML**	**AMY**	**PAG**	**ML**	**AMY**	**PAG**
CB_1								↑	↑
Nape-pld					↓	↓	↑	↑	↓
Faah	↑			↑	↓	↓		↑	
Dagla					↓			↑	↓
Mgll						↓			↓
Wfs1	↑	↓	↑	↓	↓				

→ Compared to control group animals
→ Compared to cat odour group animals
→ C57Bl/6 exposed to cat odour compared to 129Sv animals
→ Low exploratory activity rats compared to high exploratory activity animals

Third noticeable outcome of the present study is linked to two major inbred mouse lines, C57Bl/6 and 129Sv, employed in the generation of transgenic animals. Indeed, differently from C57Bl/6 mice, 129Sv strain did not respond to cat odour with increased anxiety and failed to display alterations in *Wfs1* gene expression established in the limbic structures of C57Bl/6 mice. Therefore, one could speculate that 129Sv strain has lost (or their function is suppressed) during the extensive breeding the mechanism responsible for the adequate response to cat odour. However, this mechanism seems to play a much wider role in the adaptation of mice to a novel environment, because 129Sv strain usually responds with passive coping strategies to aversive conditions. Therefore, further studies targeting the molecular mechanisms of adaptation in 129Sv and C57Bl/6 may substantially extend our knowledge about the active and passive strategies in a novel stressful environment.

CONCLUSIONS

1. Alterations in the expression of the ECS genes are dependent on the strength of stressor. Only the strong stressor – cat odour – reduced the expression of the ECS genes in the amygdala and periaqueductal gray, the brain structures playing a key role in the mechanisms of fear and anxiety. Pre-treatment of rats with rimonabant induced anxiogenic-like action on the exploratory behaviour of rats both in the elevated zero-maze and open-field tests.
2. Differently from C57Bl/6 mice, 129Sv animals did not respond to the cat odour exposure with increased anxiety. Increased anxiety in C57Bl/6 mice was accompanied by suppressed expression of the *Wfs1* gene in the temporal lobe and mesolimbic area, reflecting a role of this protein in the mechanisms of anxiety.
3. An increase in the corticosterone levels positively correlated with the exploratory behaviour of male Wistar rats in the elevated plus-maze. High exploratory activity rats had reduced expression of *Wfs1* mRNA in the mesolimbic area (related to motivation and motor activity) and hippocampus (related to learning and memory), whereas low exploratory activity rats displayed inhibited expression in the temporal lobe (related to anxiety and emotional learning). These results reflect a wider role of the Wfs1 protein in the context of adaptation to a novel and challenging environment.
4. Female *Wfs1*-deficient mice display increased anxiety and sensitivity to the anxiolytic-like action of diazepam and alcohol. These behavioural and pharmacological alterations are related with reduced expression of *Gabra1* and *Gabra1* genes in the frontal cortex and temporal lobe. Therefore, it is likely that GABA-ergic neurotransmission of these mice is corrupted in the brain areas responsible for the regulation of anxiety.

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SUMMARY IN ESTONIAN

Endokannabinoidide süsteemi ja *Wfs1* roll emotsionaalse käitumise regulatsioonis: käitumuslikud, farmakoloogilised ja geneetilised uuringud

Sissejuhatus

Ärevus on normaalne reaktsioon vastuseks stressile uues keskkonnas või uute tingimustega kohanemisel. Kauakestev või liiga intensiivne ärevusseisund on patoloogiline, põhjustades inimestel probleeme igapäevaeluga toimetulekul. Ärevushäired on maailmas üha sagedamini esinevad psühhiaatrilised probleemid, ületades sageduselt isegi depressiivseid seisundeid. Ärevus vallandab organismis erinevaid psühholoogilisi ja füsioloogilisi mehhanisme. Tavaliseks muutuseks organismis on kortisooli taseme tõus, mis on seotud hüpotaalamuse-ajuripatsi-neerupealise (HPA) telje aktiveerumisega. Olulisemad ärevuse regulatsioonis osalevad ajustruktuurid on mandelkeha, ajukoor, ajuveejuha ümbritsev hallaine (PAG), hipokampus. Erinevate virgatsainete (γ-aminovõihape, serotoniin, noradrenaliin, dopamiin) tasakaalu muutused närviimpulsside edastamisel emotsionaalses ajus on seotud patoloogilise ärevuse tekkega. Ärevushäirete põhjused on komplekssed, kuid geneetilistest teguritest enam mängivad rolli keskkondlikud tegurid. Ärevushäirete uurimine on maailmas olulise tähelepanu all, prekliinilised ja kliinilised uuringud püüavad leida uusi sihtmärke ajus ja paremaid ravimeid haiguse leevendamiseks.

Endokannabinoidide süsteem on üks võimalik märklaud, mida on seostatud ärevuse, hirmu, valu ja teiste emotsioonide regulatsiooniga. Kannabinoidi esimest tüüpi retseptor (CB_1) on enim levinud G-valguga seotud retseptor ajus, mille ekspressioon on kõrge just emotsionaalse käitumisega seotud ajupiirkondades nagu mandelkeha, eesmine vöökäär, prefrontaalkoor, hipokampus, PAG. Erinevate katsetega on näidatud, et endogeenset endokannabinoidi anandamiidi lagundava ensüümi (FAAH) inhibeerimine ajus avaldab närilistele anksiogeenset toimet (Kathuria jt., 2003).

WFS1 on transmembraanne endoplasmaatilise retiikulumi valk, mille täpne funktsioon on senini veel teadmata. On näidatud, et Wfs1 mängib rolli valkude liikumises, töötlemises ja Ca^{2+} rakusisese taseme regulatsioonis. Samuti on leitud *Wfs1* mRNA ekspressiooni taseme tõus rottide mandelkehas vastuseks kassilõhnale (Kõks jt., 2004). Wolfram'i sündroom, mis on põhjustatud mutatsioonidest *WFS1* geenis, on harvaesinev retsessiivne pärilik neurodegeneratiivne haigus. Haigusega kaasnevad psühhiaatrilised häired ja diabeet. Samuti on leitud, et *WFS1* mutatsiooni heterosügootsetel kandjatel on 26 korda suurem risk haigestuda psühhiaatrilistesse haigustesse (Swift ja Swift, 2000, 2005).

Uurimistöö põhieesmärgid

Käesoleva uurimistöö eesmärkideks oli välja selgitada ECS-i geenide ja *Wfs1* roll ärevuse regulatsioonis ja stressitingimustega kohanemisel. Kitsamad eesmärgid olid järgmised:

1. Välja selgitada ECS geenide roll kassilõhnale eksponeeritud loomadel. ECS-i geenide ekspressiooni uuringuid teostasime ajustruktuurides, mis on seotud motivatsiooniga (mesolimbiline piirkond [naalduv tuum ja haisteköbruke]) ja ärevuse kaasasündinud mehhanismidega (mandelkeha ja PAG). Teiseks uuriti CB_1 retseptori antagonisti toimet isaste Wistar liini rottide uudistamiskäitumise aktiivsusele ja kassilõhnast tingitud ärevusele.
2. Välja selgitada 129Sv ja C57Bl/6 liini hiirte võimalikke käitumislikke erinevusi kassilõhnale eksponeerimisel. Lisaks käitumiskatsetele teostasime *Wfs1* mRNA ekspressiooni qRT-PCR-ga mesolimbilises piirkonnas, oimusagaras (sisaldab mandelkeha) ja prefrontaalkoores.
3. Leida seoseid kortikosterooni taseme, oksüdatiivse stressi intensiivsuse ja uudistava käitumise aktiivsuse vahel isastel Wistar liini rottidel. Välja selgitada ECS-i geenide ja *Wfs1* ekspressiooni võimalikke muutusi rottide erineva uudistava käitumise korral mesolimbilises struktuuris, oimusagaras ja hipokampuses.
4. Leida seoseid *Wfs1*-puudulikkusega hiirte suurenenud tundlikkusele diasepaami anksiolüütilise toime suhtes ja GABA-ergilise süsteemi geenide ekspressiooni vahel eesajus paiknevates emotsionaalset käitumist reguleerivates ajustruktuurides (otsmikukoor, mesolimbiline piirkond, hipokampus ja oimusagar).

Katseloomad ja meetodid

Katseloomadeks olid isased Wistar liini rotid, C57Bl/6 ja 129Sv taustaga hiired ja F2-põlvkonna *Wfs1*-puudulikkusega hiired (Luuk jt., 2009). Ärevuse ja hirmu esilekutsumiseks kasutati kassilõhnaga immutatud riidetükki. Uudistava käitumise aktiivsust testiti tõstetud pluss-puuri või null-puuriga. CB_1 retseptori antagonist- rimonabant (0,3–3 mg/kg) manustati rottidele intraperitonaalselt 15 minutit enne eksperimendi algust. Kortikosterooni määramiseks kasutati *Corticosterone HS ELISA* komplekti (Immunodiagnostic Systems, UK).

Peale kassilõhnale eksponeerimist või uudistava käitumise testimist loomad dekapiteeriti. Ajud eemaldati ja külmutati vedelas lämmastikus. Eraldati erinevad ajustruktuurid, mis on seotud ärevuse regulatsiooniga (prefrontaalkoor, oimusagar koos mandelkehaga, hipokampus, mesolimbiline struktuur – naalduv tuum ja haisteköbruke, ajuveejuha ümbritsev hallaine). Totaalse RNA eraldamiseks kasutati Trizol® reagenti (Invitrogen, USA) ja cDNA sünteesimiseks polü $(T)_{18}$ oligonukleotiide ja SuperScript™ III pöördtranskriptaasi (Invitrogen, USA) vastavalt etteantud protokollidele.

Esimeses eksperimendis uuriti ECS-i geenide ekspressiooni (CB_1 retseptor ning EC metabolismis osalevad ensüümid *Faah*, *Nape-pld*, *Mgll* ja *Dagla*). Rotid jaotati nelja gruppi: kodupuurist võetud kontrollgrupp, tõstetud null-puuris käinud loomad, kassilõhnale eksponeeritud loomad ning nii kassilõhnale kui ka seejärel tõstetud null-puurile eksponeeritud rotid. Teises eksperimendis teostati CB_1, *Nape-pld*, *Faah* ja *Wfs1* mRNA ekspressioon pluss-puuris erineva uudistava käitumisega rottidel. Kolmandas eksperimendis uuriti *Wfs1* ekspressiooni C57Bl/6 ja 129Sv liini hiirtel. Hiired jaotati kontrollrühmaks ja kassilõhnale eksponeeritud loomadeks. Neljandas eksperimendis teostati GABA-ergilise süsteemi geenide ($GABA_A$ retseptori alaühikud alfa1 ja alfa2, *Gad1* ja *Gad2*) ekspressioon *Wfs1*-puudulikkusega hiirtel ja metsiktüüpi hiirtel.

Peamised tulemused

Kassilõhn põhjustab närilistel tugevat ärevust ja on hea mudel uurimaks ärevuskäitumise molekulaarseid mehhanisme. Käesolevas töös leidsime ECS-i geenide ja *Wfs1* mRNA eskpressiooni muutusi kassilõhnale eksponeeritud loomadel. Mesolimbilises struktuuris oli anandamiidi lammutava ensüümi (*Faah*) mRNA ekspressioon suurenenud kassilõhnale eksponeeritud loomadel võrreldes kontrollgrupiga. Seevastu mandelkehas ja PAG-is olid anandamiidi metabolismis osalevate ensüümide (*Nape-pld* ja *Faah*) ekspressioon vähenenud kassilõhnale eksponeeritud loomadel võrreldes kontrollgrupiga. Amügdalas oli lisaks *Dagla* ja PAG-is *Mgll* mRNA ekspressioon vähenenud kassilõhnale eksponeeritud loomadel võrreldes kontrollgrupiga. Need tulemused viitavad, et ärevusega seotud ajustruktuurides toimub erinevate ECS-i geenide aktivatsioon.

Rottide uudistava käitumise järgi jaotati rotid madala, keskmise ja kõrge uudistamisaktiivsusega loomadeks. Stresshormooni kortikosterooni tase oli kõrge uudistamisaktiivsusega loomadel võrreldes vähem aktiivsete loomadega oluliselt suurem. Oksüdatiivse stressi indeksis erinevusi gruppide vahel polnud, samuti polnud tõstetud null-puur piisavalt tugev stress endokannabinoidide süsteemi geenide ekspressiooni muutuste esilekutsumiseks ajus. *Wfs1* mRNA ekspressioon oli oluliselt madalam mesolimbilises struktuuris ja hipokampuses kõrge uudistamisaktiivsusega loomadel võrreldes kontrollgrupiga, seevastu oimusagaras oli *Wfs1* mRNA ekspressiooni tase vähenenud madala uudistamisaktiivsusega loomadel võrreldes kontrollgrupiga. Need tulemused osutavad ilmselt erinevate ajustruktuuride aktivatsioonile erineva uudistava käitumisega rottidel. Madala aktiivsusega (ärevatel) rottidel domineerivad passiivsed stressiga toimetuleku mehhanismid. Nendel on eelkõige aktiveeritud oimusagaras paikneva mandelkeha närviringid. Kõrge aktiivsusega (mitteärevatel) loomadel on esiplaanil aktiivsed stressiga toimetuleku mehhanismid ja neil on aktiveeritud motivatsioonide (mesolimbiline piirkond) ja mäluga (hipokampus) seotud närviringid.

C57Bl/6 hiirtel põhjustab kassilõhnale ekponeerimine ärevuselaadse seisundi, mida ei esine aga 129Sv hiirtel. Samuti on C57Bl/6 hiirtel mesolimbilises

struktuuris ja oimusagaras oluliselt madalam *Wfs1* mRNA ekspressiooni tase võrreldes 129Sv liini hiirtega. Läbiviidud uuringud viitavad sellele, et 129Sv hiireliin kuulub, erinevalt C57Bl/6 hiirtest, kassilõhnale mittereageerivate liinide hulka. Võib oletada, et ristamiste käigus on 129Sv hiirtel kaduma läinud mehhanismid, mis on seotud reaktsiooniga kassilõhnale. Lisaks võib oletada, et need mehhanismid omavad olulist rolli ka stressiga aktiivses toimetulekus. 129Sv hiirtel domineerivad mehhanismid, mis on seotud passiivse kohanemisega stressile.

Wfs1-puudulikkusega hiired on ärevamad tõstetud pluss-puuri katses ja nad reageerivad tugevamini diasepaami anksiolüütilisele toimele võrreldes metsiktüüpi liigikaaslastega. Bensodiasepiinid avaldavad anksiolüütilist toimet $GABA_A$-retseptorite kaudu. Käesolevas töös leiti, et *Wfs1*-puudulikkusega hiirtel esineb $GABA_A$-retseptori alaühikute alfa1 ja alfa2 ekspressiooni langus oimusagaras ja frontaalkoores võrreldes metsiktüüpi liigikaaslastega, kusjuures hipokampuses ja mesolimbilises struktuuris *Wfs1* mRNA ekspressiooni muutusi ei esinenud. Samuti ei esinenud mRNA ekspressiooni erinevusi GABA-süntees sivate ensüümide geenide (*Gad1* ja *Gad2*) tasemetes *Wfs1*-puudulikkusega hiirtel võrreldes metsiktüüpi pesakonnakaaslastega. Käesolev uurimistöö ja eelnevad meie poolt avaldatud tulemused kinnitavad limbilistes struktuurides oleva Wfs1 valgu tähtsust kohanemisel uudses keskkonnas.

Järeldused

1. ECS-i geenide ekspressiooni muutus sõltub stressi tugevusest. Eksposits ioon tõstetud pluss-puurile ei ole piisav stress, et mõjutada isastel Wistar liini rottidel ECS-i geenide ekspressiooni. Rottide eksponeerimine kassilõhnale vallandab olulised muutused geenide ekspressioonis aju erinevates osades. Märkimisväärne on, et mesolimbilises struktuuris (naalduv tuum ja haistek öbruke) toimub paljude ECS-i geenide mRNA oluline tõus, sealjuures mandelkehas ja PAG-is on samade geenide ekspressioon vähenenud. See näitab, et erinevad ajuosad aktiveeruvad stressi mõjul erinevalt. CB_1 retseptori antagonisti rimonabandi manustamine avaldas anksiogeenset toimet rottide eksploratiivsele käitumisele nii tõstetud null-puuri kui ka avarvälja testis. Rimonabant ei mõjutanud kassilõhnale eksponeeritud loomade käitumuslikku mustrit.
2. C57Bl/6 ja 129Sv hiireliinid erinevad uudistava käitumise poolest. Kassilõhnale eksponeerimine vähendab oluliselt C57Bl/6 hiirte uudistavat käitumist, kusjuures 129Sv hiirtel käitumuslikke muutusi ei täheldatud. *Wfs1* mRNA ekspressioon oli vähenenud C57Bl/6 hiirtel peale kassilõhnale eksponeerimist, võrreldes kontrollgrupiga ja 129Sv liini hiirtega. Muutused oimusagara ja mesolimbilise piirkonna *Wfs1* mRNA ekspressioonis peegeldavad Wfs1 olulist tähendust ärevuse mehhanismides.
3. Eksploratiivse aktiivsuse järgi jaotatud Wistari liini rotid jaotati kahte alagruppi – kõrge ja madala uudistamisaktiivsusega loomad. Gruppide vahel

oksüdatiivse stressi indeksi ja ECS-i geenide ekspressiooni erinevusi ei leitud. Seevastu kortikosterooni tase oli oluliselt kõrgem pluss-puuri avatud osi aktiivselt uudistavatel rottidel võrreldes madala uudistamisaktiivsusega loomadega. Kõrge uudistamisaktiivsusega rottidel oli *Wfs1* geeni ekspressioon vähenenud mesolimbilises piirkonnas (seotud motivatsioonide ja liikumisaktiivsusega) ja hipokampuses (seotud mälu ja õppimisega). Madala uudistamisaktiivsusega loomadel oli *Wfs1* mRNA ekspressioon vähenenud oimusagaras (seotud ärevuse ja emotsionaalse õppimisega). Tulemused osutavad Wfs1 valgu laiemale tähendusele katseloomade kohanemises uudses ja ebameeldivas keskkonnas.

4. Emastel *Wfs1*-puudulikkusega hiirtel on suurem ärevus ning kõrgenenud tundlikkus diasepaami ja alkoholi anksiolüütilise toime suhtes. Need käitumuslikud ja farmakoloogilised nihked on seotud *Gabra1* ja *Gabra1* ekspressiooni vähenemisega otsmiku- ja oimusagaras. Sellepärast võib oletada, et *Wfs1*-puudulikkusega hiirtel on vähenenud GABA-ergiline närviülekanne ärevuse regulatsioonis osalevates ajustruktuurides.

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