DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
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Involvement of cholecystokinin in chronic pain mechanisms and endogenous antinociception

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II Gene expression profiling reveals upregulation of Tlr4 receptors in Cckb receptor deficient mice. Kõks S, Fernandes C, **Kurrikoff K**, Vasar E, Schalkwyk LC. Behav Brain Res. 2008 Mar 17;188(1):62–70.

III Stress-induced analgesia in mice: evidence for interaction between endocannabinoids and cholecystokinin. **Kurrikoff K**, Inno J, Matsui T, Vasar E. Eur J Neurosci. 2008 Apr;27(8):2147–55.

IV Interpretation of knockout experiments: the congenic footprint Schalkwyk LC, Fernandes C, Nash MW, **Kurrikoff K**, Vasar E, Kõks S. Genes Brain Behav. 2007 Apr;6(3):299–303.

Contribution of the author

1. The author designed the study, performed all the behavioural experiments, made the surgeries, harvested tissues, performed gene expression analysis, carried out the statistical analysis and wrote the manuscript.
2. The author made the surgeries, harvested tissues, performed the Affymetrix microarray gene expression experiment and performed the real-time PCR.
3. The author designed the study, performed all the behavioural experiments, harvested tissues, performed gene expression analysis, carried out the statistical analysis and wrote the manuscript.
4. The author made the surgeries, harvested tissues, performed the Affymetrix microarray gene expression experiment.

ABBREVIATIONS

2-AG	–	2-arachidonoylglycerol
BL	–	baseline
CB_1	–	cannabinoid type 1 receptor
CCI	–	chronic constriction injury
CCK	–	cholecystokinin
CCK_2	–	cholecystokinin type 2 receptor
$CCK_2^{-/-}$	–	CCK_2 receptor deficient homozygous mice
$CCK_2^{+/-}$	–	CCK_2 receptor deficient heterozygous mice
CNS	–	central nervous system
Ct	–	cycle threshold
DAG	–	diacylglycerol
DAGLa	–	sn-1-DAG lipase alpha
DAGLb	–	sn-1-DAG lipase beta
DRG	–	dorsal root ganglion
ES cell	–	embryonic stem cell
FAAH	–	fatty-acid amide hydrolase
i.p.	–	intraperitoneal
IP3	–	inositol-3-phosphate
MAGL	–	monoacylglycerol lipase
NAPE-PLD	–	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D
Oprd	–	opioid δ receptor
Oprk	–	opioid κ receptor
Oprm	–	opioid μ receptor
PDYN	–	pro-dynorphin
PENK	–	pro-enkephalin
PLC	–	phospholipase C
POMC	–	pro-opio-melanocortin
qRT-PCR	–	quantitative real-time PCR
s.c.	–	subcutaneous
SEM	–	standard error of mean
SHAM	–	sham-operated animals
SIA	–	stress-induced analgesia

INTRODUCTION

Cholecystokinin (CCK) is one of most abundant neuropeptides present in the central nervous system (CNS). Another interesting thing about CCK is its presence in so many important neuronal pathways and its co-localization with several classic neurotransmitters.

Of many roles that CCK has been associated with, regulation of pain sensitivity and endogenous analgesic mechanisms remains one of most interesting and important clinical aspects. Although involvement of CCK in chronic pain as a potential target system for the treatment of pain (and for counteracting morphine tolerance) has been recognised long ago (Panerai *et al.*, 1987), unfortunately, the majority of useful data to date has come from preclinical animal models and very few from clinical studies. Thus, there are numerous studies showing pain relieving, antiallodynic or opioid enhancing effects of CCK_2 antagonists (see Noble *et al.*, 1999 for a review) in rodents. There is also evidence that the nonselective CCK antagonist proglumide enhances the analgesic effect of morphine in patients with chronic pain of mixed aetiology (McCleane, 1998). However, the selective CCK_2 receptor antagonist L-365260 failed to increase the analgesic effect of morphine in human subjects with chronic neuropathic pain (McCleane, 2003). This suggests that some aspects of the CCK and/or opioid systems differ between rodents and humans. In the hope of finding new drug targets, new methodological aspects have recently been combined with pharmacological tools and attempts have been made to synthesize peptide analogs that would have agonist activity at opioid receptors and antagonist activity at the CCK receptors (Hruby *et al.*, 2006). Although progress has been made in developing such compounds, these have not been shown to be therapeutically useful in human studies. Clearly, there is a need to know more about the interactions between CCK and endogenous opioids. The first part of the thesis focuses on the role of CCK in neuropathic pain mechanisms and tries to add new information to the topic.

Endogenous antinociceptive mechanisms play an important role in the regulation of behaviour under stressful circumstances. Involvement of such mechanisms affects mobilisation of resources and decision making in threat situations, thus modifying coping with stress. Of course, the notion of "endogenous antinociceptive mechanisms" (described in the second part of the current thesis) does not imply something very different from "pain mechanisms" (the first part of the thesis). The distinction is rather methodological and involves different angle or approach to the same question. While the "pain mechanisms" type of study generally involves some model of clinical pain (i.e. nerve injury induced neuropathic pain), the "endogenous mechanisms" are studied as organism's ability to modify sensory (pain) information under specific environmental conditions (like stress). Obviously, the underlying "endogenous" mechanism is involved in both cases. One of the first explicit notations of stress-induced analgesia (SIA) came from observations of soldiers'

behaviour in the World War II (Beecher, 1959), where it was noted that even very badly wounded men in the battlefield behaved as if under the influence of morphine. SIA is especially useful in experimental conditions as it allows to measure subjective amount of stress in animals. The common assumption is that stronger stress induces more prominent SIA. The second part of the thesis concerns the role of CCK in endogenous analgesic mechanisms and applies experimental model of SIA.

Single gene knockout mice are extensively used in the behavioural studies. The common way of creating the transgenic animal is creating the knockout in 129sv strain (due to methodological reasons, obtaining the knockout in the 129sv strain is easier) and then backcrossing the 129sv line knockout to desired background (usually the C57BL/6 line). Until very recently the scientific community saw no problem with such methodology. Unfortunately, the backcrossing is not the best way of obtaining a knockout mouse line in C57BL/6 background, because even after extensive backcrossing, many genes still remain in the original (129sv) background and may influence the resulting "phenotype". The last part of the thesis addresses that question, taking the $CCK_2^{-/-}$ mouse line as an example.

REVIEW OF LITERATURE

The neuroanatomy of CCK, CCK_1 and CCK_2 receptors

CCK was first discovered in gastrointestinal tract (Ivy & Oldberg, 1928) as a digestion enhancing hormone. CCK is a linear peptide; its biologically active forms are synthesized from a pre-prohormone (pre-proCCK). The pre-proCCK is posttranslationally cleaved and resulting peptide variants of different length have all identical carboxy-terminus. The lengths of the CCK peptides vary from 4 to 58 amino acid residues. In addition, the tyrosine near the C-terminal end may or may not be sulphated. Sulphated CCK-8 is the predominant form in the CNS although small amounts of CCK-4 may also be present (Rehfeld, 1978a; b).

The regional distribution of CCK is heterogeneous with particularly high concentrations in the cerebral cortex, hippocampus, basal ganglia, hypothalamus, periaqueductal grey and spinal cord (Larsson & Rehfeld, 1979; Dockray, 1980; Arvidsson *et al.*, 1995). The main source of CCK in the dorsal horn of the spinal cord appears to be from superficial layers (Conrath-Verrier *et al.*, 1984). There are also projecting CCK-positive fibres in the superficial dorsal horn from supraspinal sites, such as the Edinger–Westphal nucleus (Maciewicz *et al.*, 1984), the nucleus raphe magnus (Mantyh & Hunt, 1984) and the periaqueductal grey (Skirboll *et al.*, 1983).

Two subtypes of CCK receptors (CCK_1 and CCK_2) exist. The vast majority of CCK receptors in the CNS are of the CCK_2 type, with CCK_1 receptors restricted to rather discrete regions (see Noble *et al.*, 1999 for a review). Radioligand studies, initially conducted in the rat, showed CCK_1 receptors to be mainly located in the interpeduncular nucleus, area postrema, and medial nucleus tractus solitarius (Moran *et al.*, 1986; Hill *et al.*, 1987). Studies in primates have revealed dramatic species differences, demonstrating a much higher prevalence and broader distribution of CCK_1 receptors in the monkey and humans than that in rodents (Hill *et al.*, 1988; Hill *et al.*, 1990). Thus, the prevalent receptor subtype in a primate spinal cord is CCK_1 (Hill *et al.*, 1988), a striking contrast with the rodents. Many more CCK_1 receptor-binding sites in the monkey have been located in addition to above described structures. These differences between species may have great clinical importance, considering above mentioned lack of success of applying results from animal behavioural studies into clinical practice.

Endogenous antinociceptive system

Endogenous opioid peptides and their receptors comprise the classically referred "endogenous antinociceptive system", because activation of this system inhibits transmission of pain signals (Reynolds, 1969; Basbaum *et al.*, 1976).

Three main types of opioid receptors have been found: μ, δ and κ. The genes encoding the receptors all belong to the G-protein coupled class of receptors. The μ receptor was originally defined on the basis of its high affinity for morphine (Martin *et al.*, 1976; Lord *et al.*, 1977). β-endorphin and enkephalins are thought to be preferred endogenous ligands for this class of receptors. The highest μ receptor densities are found in the thalamus, caudate putamen, neocortex, nucleus accumbens, amygdala, interpeduncular complex, and inferior and superior colliculi (Mansour *et al.*, 1987). The μ receptors, as well as δ and κ receptors, are also present in the superficial layers of the dorsal horn of spinal cord (Besse *et al.*, 1990). The δ opioid receptor was named according to the preparation (*vas deferens*) in which it was first characterized; enkephalins are considered the preferred endogenous ligands (Lord *et al.*, 1977). The highest densities are found in olfactory bulb, neocortex, caudate putamen, nucleus accumbens, and amygdala (Mansour *et al.*, 1987). Dynorphins are thought to be the main endogenous ligands for opioid κ receptors (Goldstein *et al.*, 1979). The κ receptors are located predominantly in the cerebral cortex, nucleus accumbens, claustrum and hypothalamus (Mansour *et al.*, 1987; Kitchen *et al.*, 1997).

The three major classes of endogenous ligands for opioid receptors are enkephalins (high affinity for μ and δ receptors), β-endorphin (high affinity for μ receptors), and dynorphins (high affinity for κ receptors). These peptides are generated from larger precursor molecules, which, in turn are synthesized from three separate genes: pro-enkephalin, pro-opio-melanocortin and pro-dynorphin, respectively. There are two additional potent and selective μ opioid activating peptides – endomorphines – which are also thought to be of endogenous origin, although the source of their synthesis is not known.

The endogenous antinociceptive system functions through descending inhibition from the brain to nociceptive relay neurons of the spinal cord. The midbrain periaqueductal grey matter (PAG) and brainstem rostroventral medulla (RVM) are important parts of the descending pathway. This can be concluded from studies showing that electrical stimulation (Reynolds, 1969) of these areas as well as injection of opiates into these areas (Basbaum *et al.*, 1976) produce profound analgesia, in naloxone reversible manner (Akil *et al.*, 1976). The midbrain PAG projects to the serotonergic and noradrenergic nuclei in the brainstem. These nuclei, in turn send descending projections to the dorsal horn of the spinal cord, where they inhibit nociceptive projection neurons both through direct synapses and through spinal interneurons (see Basbaum & Fields, 1984; Mason, 2005 for a review). In addition, a large number of brainstem, diencephalic (thalamic and hypothalamic) and telencephalic (cortical and

subcortical) structures modulate nociceptive processing through descending projections to the spinal dorsal horn, and in most cases their descending pain suppressive effect is relayed through PAG and RVM (see Pertovaara & Almeida, 2006 for a review). Both endogenous opioid peptides and their receptors are localised at key points in the pain modulating system. Thus, neurons containing enkephalin and dynorphin, as well as opioid receptors can be found in PAG, RVM and dorsal horn of the spinal cord (see Pertovaara & Almeida, 2006 for a review). β-endorphin is localized in neurons of hypothalamus; these neurons send projections to the PAG and RVM (Sim & Joseph, 1991).

CCK and analgesic mechanisms

Considering the abundance and wide-spread occurrence of CCK both in CNS and periphery, it is not surprising that CCK is involved in many physiological processes. These include modulation of anxiety and stress-related behaviour, regulation of feeding, nociception, memory, body temperature and reward-related behaviour (Crawley & Corwin, 1994; Dauge & Lena, 1998; Szelenyi, 2001; Rotzinger & Vaccarino, 2003; Moran, 2004).

What drew attention to the involvement of CCK in pain and analgesia mechanisms was discovery that the distribution of CCK-8 and CCK receptors paralleled that of endogenous opioids and opioid receptors in the CNS regions associated with nociceptive processing (Gall *et al.*, 1987; Pohl *et al.*, 1990; Wiesenfeld-Hallin *et al.*, 1999).

It is now well known that CCK-ergic control influences the antinociceptive efficacy of opioid peptides (Faris, 1985), exerting antagonistic effect to the antinociceptive action of opioid peptides (Noble & Roques, 1999). Administration of CCK attenuates, whereas selective CCK_2 receptor antagonists enhance morphine-induced antinociception in rodents (Faris *et al.*, 1983; Lavigne *et al.*, 1992; Noble *et al.*, 1995). In addition, pretreatment with CCK_2 receptor oligonucleotide antisense increases morphine-induced antinociception in mice (Vanderah *et al.*, 1994). Finally, opioid and CCK receptors, located on the terminals of a primary afferent exert opposing actions on neuronal activity (Ghilardi *et al.*, 1992; Stanfa *et al.*, 1994) and the opioid system appears also be able to regulate the release of CCK (see Noble & Roques, 1999 for a review). These findings confirm the existence of a functional antagonism by endogenous CCK and opioid systems in the regulation of pain sensitivity.

Overview of neuropathic pain

More important is that CCK is involved in chronic pain (patho)mechanisms. Damage to the nervous system leads to a chronic pain referred to as neuropathic pain. Ample evidence suggests that following tissue injury there are multiple changes in CNS function that contribute to the development and maintenance of chronic pain. Nerve fibres develop abnormal ectopic excitability at or near the site of nerve injury (Ochoa *et al.*, 1982). Several pain mediators (bradykinin, prostaglandins and cytokines) are involved in these ectopic discharges. Profound changes in expression of immediate-early genes are observed in DRG after the nerve injury (Zimmermann, 2001), probably followed by changes in the expression of other genes. Accordingly, sensitization of spinal dorsal horn cells and enhanced synaptic transmission has been described as wind-up (Davies & Lodge, 1987) or long-term potentiation (Liu & Sandkuhler, 1995). The resulting gene expression effects may also include programmed cell death such as apoptosis (Gillardon *et al.*, 1996a; Gillardon *et al.*, 1996b; Gillardon *et al.*, 1996c; Azkue *et al.*, 1998). One of the many studied immediate-early gene is *c-jun*. According to the results, hypothesis have been raised that the activation of *c-jun* in spinal dorsal horn neurons after nerve injury may trigger two different programs of transcriptional machinery, one of which supports regeneration of a primary afferent neuron and the other initiates apoptosis and kills the neuron (see Zimmermann, 2001 for a review). There is evidence that part of neuropathic pain and hyperalgesia is due to inefficiency of endogenous descending inhibitory systems (Zimmermann, 2001) and decreased efficacy of the spinal opioid system (von Knorring *et al.*, 1979; Panerai *et al.*, 1987; Lombard & Besson, 1989; Zajac *et al.*, 1989).

There are also peripheral mechanisms that have been described as a source of symptoms of chronic pain. Persistent abnormal excitability of sensory nerve endings in a neuroma is considered a mechanism of stump pain after amputation. In addition, spontaneous impulse activity originating predominantly from C-fibres has been described (Han *et al.*, 2000).

Although these processes of increasing nervous system excitability may be considered as a strategy to compensate functional deficits following nerve injury, its by-product is widespread nervous system sensitization resulting in pain and hyperalgesia.

Modelling neuropathic pain

Four main animal models for nerve injury-associated pain are widely used:

- Total nerve transection and ligation. This lesion results in immediate and irreversible interruption of electrical nerve conduction, followed by Wallerian degeneration of the axons distal to the lesion and sprouting of the

proximal axonal stumps in an attempt to regenerate the nerve fibre. This simulates clinical conditions of amputation.

- Partial nerve lesion with a tight ligation around part of the nerve fascicles (Seltzer *et al.*, 1990; Shields *et al.*, 2003), simulating the clinical condition of an accidental nerve bruise or accident-related nerve injuries.
- Chronic constriction injury (CCI) by placing several loose ligatures around the nerve leaving a lumen of less than the diameter of the original nerve (Bennett & Xie, 1988), simulating the clinical condition of chronic nerve compression such as the one that occurs in nerve entrapment neuropathy or spinal root irritation by a lumbar disk hernia.
- Tight ligation of a spinal nerve (Kim & Chung, 1992; Carlton *et al.*, 1994) or transection of one or several dorsal roots (rhizotomy) (Lombard *et al.*, 1979; Brinkhus & Zimmermann, 1983) resulting in complete deafferentation of one or several spinal segments, simulating nerve plexus and dorsal root injury.

There are three behavioural signs of neuropathic pain which are assessed:

- Autotomy – self-attack of the denervated leg. This results in self-mutilation and is assessed (as an autotomy score) by counting the number of wounds on the denervated extremity and by accounting for the size of wounds.
- Hyperalgesia – stronger withdrawal response to a noxious stimulus, compared to the healthy animals.
- Allodynia – withdrawal response to the non-noxious touching of the so-called "von Frey hair". These are a series of different calibrated filaments, each exerting certain pressure (expressed in grams) when applying to the animal's hindpaw (touching the paw with the filament, slightly bending it).

CCK and chronic pain

Neuropathic pain is relatively insensitive to morphine or other opioid treatment; this has been shown in both clinical studies (Portenoy *et al.*, 1990) and in laboratory settings (Bian *et al.*, 1995; Ossipov *et al.*, 1995). The basis of the reduced effectiveness is not known (see Przewlocki & Przewlocka, 2001 for a review), although it has been shown that CCK is able to modify morphine efficacy (Nichols *et al.*, 1995). The involvement of CCK is also supported by results showing that CCK antagonists proglumide and benzotript were able to inhibit the development of tolerance to morphine-induced analgesia (Panerai *et al.*, 1987) without affecting the occurrence of physical dependence of morphine. In another study, both CCK_2 receptor antagonist L-365260 and CCK_1 antagonist L-365031 prevented tolerance to morphine analgesia in the rat (Dourish *et al.*, 1990), again, without modifying the physical dependence.

Data from the animal models show that peripheral nerve injury is associated with an elevated level of CCK mRNA in the dorsal root ganglions (Xu *et al.*,

1993). CCK_2 receptor antagonist L-365260, given spinally, restores the effectiveness of morphine in the nerve-ligated rats (Nichols *et al.*, 1996) in naltrindole (opioid δ receptor antagonist) reversible way, suggesting that δ opioids are involved in regulation of some aspects of nerve-injury induced pain. In this study, administration of CCK_2 receptor antagonist alone did not alter allodynia. However, in another study, pretreatment with CCK_2 receptor antagonist, but not CCK_1 antagonist, attenuated the symptoms of mechanical allodynia in rat formalin test (a murine model of inflammatory pain) (Yamamoto & Nozaki-Taguchi, 1996). In another study, using rat spinal nerve ligation model, bilateral microinjection of the CCK_2 receptor antagonist L-365260 directly into the RVM reversed tactile allodynia and microinjection into the PAG restored the potency and efficacy of morphine in spinal nerve ligated rats (Kovelowski *et al.*, 2000).

With the advance of modern biotechnological methods, the function of single genes can be studied in novel ways. Thus, the CCK_2 receptor knockout ($CCK_2^{-/-}$) mouse line has been created (Nagata *et al.*, 1996). Upregulation of opioid system (Pommier *et al.*, 2002) and elevated thresholds for nociceptive stimuli (Veraksits *et al.*, 2003) have been established in the CCK_2 receptor deficient mice.

$CCK_2^{-/-}$ mice as a tool to study a function of neuronal CCK

$CCK_2^{-/-}$ mice were first generated in 1996 (Nagata *et al.*). The mutant vector replaced a part of exon 2 and exons 3–5 of the CCK_2 gene. This replacement deleted most of the seven membrane-spanning CCK_2 receptor except for the first 108 amino acids, containing the first membrane-spanning region. The expression of CCK_2 is absent in several CNS regions, as confirmed by binding analysis (Nagata *et al.*, 1996; Koks *et al.*, 2001).

The phenotype of the $CCK_2^{-/-}$ animals has been extensively studied since 1996 in several independent laboratories. Generally, the knockout animals are fertile and show no apparent signs of abnormalities. However, differences in feeding behaviour and body weight (Weiland *et al.*, 2004), metabolism (Miyasaka *et al.*, 2002a), immune system deficiencies (Weiland *et al.*, 2007), anxiety (Dauge *et al.*, 2001b; Miyasaka *et al.*, 2002b; Raud *et al.*, 2003; Horinouchi *et al.*, 2004; Raud *et al.*, 2005; Areda *et al.*, 2006), locomotor activity (Dauge *et al.*, 2001a; Dauge *et al.*, 2001b; Koks *et al.*, 2001; Pommier *et al.*, 2002; Runkorg *et al.*, 2003; Weiland *et al.*, 2004; Runkorg *et al.*, 2006), cognitive functions (Sebret *et al.*, 1999; Dauge *et al.*, 2001b; Koks *et al.*, 2001; Raud *et al.*, 2003) and reward-related behaviour (Dauge *et al.*, 2001a; Pommier *et al.*, 2002; Runkorg *et al.*, 2003; Runkorg *et al.*, 2006) have been described.

From current point of view, differences in pain behaviour and endogenous analgesia mechanisms are most interesting. The first reported result claimed

increased pain sensitivity in the $CCK_2^{-/-}$ mice (Pommier *et al.*, 2002). However, subsequent works have contradicted this result. Closer inspection shows that methodological aspects, rather than CCKergic mechanisms underlie the contradiction. Pain sensitivity was measured using a common tool (hot plate), but in unconventional way. The study incorporated only a secondary parameter – the jump latency, and did not present the traditional, primary parameter – shake/lick latency at all. Later, decreased pain sensitivity in hot plate was established (Veraksits *et al.*, 2003).

Neuropathic pain in humans

Chronic neuropathic pain is common in clinical practice. Patients with conditions as diverse as diabetic polyneuropathy, human immunodeficiency virus (HIV) sensory neuropathy, poststroke syndromes, and multiple sclerosis frequently experience daily pain that greatly impairs their quality of life (see Dworkin *et al.*, 2003 for a review). Chronic neuropathic pain syndromes are divided into 2 groups based on whether the CNS damage has occurred at central or peripheral location of the nervous system (Table 1).

The efficacy of gabapentin, lidocaine patch, opioid analgesics, tramadol hydrochloride and tricyclic antidepressants have been consistently demonstrated in multiple randomized controlled trials as the first-line medications for neuropathic pain. There are also several second-line medications that are used when patients do not have a satisfactory response to treatment with the mentioned first-line medications alone or in combination.

Despite much effort, medications providing effective and tolerable treatment of neuropathic pain have not appeared in the past 2 decades. Thus, more information is needed about the pathophysiology of the neuropathic pain in order to discover new drug candidates. Of course, new methods may appear which allow totally new approach to older results. One example of a recent pharmacological tool is presented by Hruby (2006), where single ligand that could act as both agonist at opioid receptors and antagonist at CCK receptors was designed.

Table 1. Common Types of Neuropathic Pain (according to Dworkin et al., 2003)

Peripheral neuropathic pain
Acute and chronic inflammatory demyelinating polyradiculoneuropathy
Alcoholic polyneuropathy
Chemotherapy-induced polyneuropathy
Complex regional pain syndrome
Entrapment neuropathies (eg, carpal tunnel syndrome)
HIV (human immunodeficiency virus) sensory neuropathy
Iatrogenic neuralgias (eg, postmastectomy pain or postthoracotomy pain)
Idiopathic sensory neuropathy
Nerve compression or infiltration by tumor
Nutritional deficiency–related neuropathies
Painful diabetic neuropathy
Phantom limb pain
Postherpetic neuralgia
Postradiation plexopathy
Radiculopathy (cervical, thoracic, or lumbosacral)
Toxic exposure–related neuropathies
Tic douloureux (trigeminal neuralgia)
Posttraumatic neuralgias
Central neuropathic pain
Compressive myelopathy from spinal stenosis
HIV (human immunodeficiency virus) myelopathy
Multiple sclerosis–related pain
Parkinson disease–related pain
Postischemic myelopathy
Postradiation myelopathy
Poststroke pain
Posttraumatic spinal cord injury pain
Syringomyelia

Endogenous analgesic mechanisms and stress

Brain pathways that project from the amygdala to the midbrain PAG and brainstem RVM and descend to dorsal horn of the spinal cord are involved in production of SIA (Walker & Hohmann, 2005). Endogenous opioid peptides have been associated with SIA as its chemical mediators (Lewis *et al.*, 1980; Akil *et al.*, 1986), but other, non-opioid mediators of SIA are known to exist (Terman *et al.*, 1986b). Later, genetic invalidation of CB_1 receptors has been shown to alter SIA mechanisms (Valverde *et al.*, 2000). Thus, Hohmann (2005) showed that development of the non-opioid SIA could be prevented by blocking the cannabinoid CB_1 receptors and enhanced by increasing the brain contents of endocannabinoid ligands, 2-arachidonoylglycerol (2-AG) and anandamide. The resulting hypothesis was that endocannabinoids may be one of the non-opioid mediators of SIA.

As explained in the previous section, CCK plays a role in the regulation of opioid tone in the CNS and consequently in pain sensitivity (Stanfa *et al.*, 1994) and there exists a functional antagonism between opioid peptides and CCK. It remains yet to be revealed whether similar interactions exist also between endocannabinoids and CCK. Several works have shown cellular colocalisation of CCK and CB_1 receptors in several CNS regions (Marsicano & Lutz, 1999; Hohmann, 2002). The physiological relevance of the colocalisation is largely unknown, although some very recent results show that CCK and endocannabinoids interact in extinction learning (Chhatwal *et al.*, 2009).

Overview of endocannabinoid system

Two cannabinoid receptors, the CB_1 and CB_2 have been cloned (Devane *et al.*, 1988; Munro *et al.*, 1993), the CB_1 being mostly distributed within CNS. To date, there are thought to be two main endogenous endocannabinoid ligands, 2-arachidonoylglycerol (2-AG) and N-arachidonoyl ethanolamine (anandamide) (for a review, see Di Marzo, 1999). These ligands are thought to be synthesized and released on-demand and activate cannabinoid CB_1 receptors with high affinity (Marsicano *et al.*, 2003). For the biosynthetic and degradation biochemical pathways there are many unknown issues to be revealed in the future. However, an enzyme N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) is implicated in the biosynthesis of anandamide (Okamoto *et al.*, 2004) and two sn-1-DAG lipases (DAGLα and DAGLβ) are responsible for the generation of 2-AG (Bisogno *et al.*, 2003). The ligands are metabolized by distinct hydrolytic pathways, 2-AG being hydrolyzed by monoacylglycerol lipase (MGLL) (Dinh *et al.*, 2002), whereas anandamide is preferentially hydrolyzed by fatty-acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996).

The endocannabinoid pain modulating sites are similar to the ones as described for the endopioids. Similarly to opioid receptors, cannabinoid receptor CB_1 is present in the same pain modulation areas: dorsal horn of the spinal cord, PAG and RVM. Cannabinoids suppress noxious stimulus evoked neuronal activity in nociceptive neurons in the spinal cord and thalamus (Hohmann *et al.*, 1995; Martin *et al.*, 1996; Hohmann *et al.*, 1998). *In vivo* electrophysiological studies of brainstem descending control of pain have also suggested the role of cannabinoids in pain modulation (Meng *et al.*, 1998; Maione *et al.*, 2006). Interestingly, certain stress paradigms induce analgesia which can be prevented by administering cannabinoid CB_1 antagonist rimonabant but can not be blocked by opiate antagonists or by CB_2 antagonist (Terman *et al.*, 1986a; Hohmann *et al.*, 2005) and SIA can be enhanced by inhibition of endocannabinoid hydrolysis (Hohmann *et al.*, 2005). In the latter study, it was established that electric foot-shock -induced stress elevated the levels of the two main endocannabinoid lipids, 2-AG and anandamide, in the midbrain PAG in rats. Thus, there is evidence for the involvement of cannabinoids in stress analgesia mechanisms.

AIMS OF THE STUDY

The general aim of current study was to reveal more information about the mechanisms of nociceptive processing in the mouse central nervous system. As there is increasing evidence about the involvement of CCK in regulation of pain, our aim was to study the effect of invalidation of CCK signal in the CNS on pain processing, investigating neuropathic pain and stress-induced analgesia in detail. More specific tasks were as follows:

1) To describe the neuropathic pain phenotype in $CCK_2^{-/-}$ mice. More specifically, to study the interaction of CCK with the opioid system in the development of neuropathic pain, and therefore, to map the opioid-related gene expression in $CCK_2^{-/-}$ and wild-type mice in brain regions associated with pain processing.
2) To conduct an exploratory gene expression study in order to find new target genes (in addition to opioid-related genes) that might be important in neuropathic pain mechanisms at the level of medulla oblongata and midbrain.
3) To describe the stress-induced analgesia phenotype of the $CCK_2^{-/-}$ mice. More specifically, to study the interaction of CCK with the endocannabinoids in the development of stress-induced analgesia, using the pharmacological blockade of cannabinoid receptors.
4) Finally, to study the effect of congenic footprint after backcrossing the CCK_2 receptor deficient mice to the C57BL/6 genetic background.

6

MATERIALS AND METHODS

Animals

CCK_2 receptor deficient mice ($CCK_2^{-/-}$) were used throughout the thesis. CCK_2 receptor was originally knocked out in J1 ES cells, derived from 129S4/Jae mice (Nagata *et al.*, 1996). Homologous recombination was targeted to replace a part of exon 2 and exons 3, 4 and 5. 129sv/C57BL6 background animals were subsequently backcrossed 10 times to C57BL/6Bkl (Scanbur-BK) in Tartu. Homozygous knockout and wild-type animals were obtained by intercrossing heterozygous stock. Housing was at 20 ± 2° C under a 12-h, lights on at 0700 cycle, water and food *ad libitum*. The University of Tartu Animal Care Committee approved all animal procedures (EC Directive 86⁄609⁄EEC).

Behavioural experiments

Assay of mechanical sensitivity

We used TouchTest® (North Coast Medical, Inc) monofilaments (bending forces 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 and 2.0 g) for the measurement of mechanical sensitivity. Number of animals in each group was 16 (Paper 1). Mice were placed into individual transparent (16 x 23 x 14 cm) chambers positioned on a metal mesh floor. Mechanical sensitivity was assessed, using up-down method (Chaplan *et al.*, 1994). Each filament was applied to the left hindpaw (i.e. the hindpaw was touched by the filament so that the filament slightly bended) 4 times for 0.5…1 s with an inter-stimulus interval of about 5 s. The response was considered as a "positive" when the mouse withdrew its hindpaw at least 2 times out of 4 applications. In this case a next weaker filament was similarly used. Otherwise, a next stronger filament was used, until "positive" response was acquired. Next, the direction of stimulus presentation was reversed (i.e, next weaker filament was used), until the first "negative" response was seen. This second crossing of threshold was stated as the threshold of mechanical sensitivity. Thresholds were measured on 7 consecutive days and the average value of the last 5 days was used for analysis (Mogil *et al.*, 1999).

Statistical differences were analysed with one-way ANOVA and differences between factor levels with Tukey *post-hoc* test.

The effect of L-365260 and naloxone on mechanical sensitivity

The doses of 0.01, 0.1 and 1 mg/kg of L-365260 (Merck Sharp & Dohme) and 0.1, 1 and 10 mg/kg of naloxone (Sigma) were used. The naloxone dose of 1 mg/kg was chosen as a dose that has been shown to exert a biological activity, blocking all opioid receptors (Marchand *et al.*, 2003); the other doses were chosen to cover as broad dose spectrum as possible. All drugs were injected 15 min before the beginning of an experiment via i.p. route.

To reduce the number of animals in the experiment, all animals received all doses of naloxone and L-365260 and the vehicles (see "Drugs" section for detailed information about the vehicles). Only one injection was delivered per day and the interval between the injections was 3 days (to reduce any carryover effects as much as possible). The number of animals in each group was 16. The animals were assigned to drug groups for both naloxone and L-365260 injection experiments according to the principle of Balanced Latin Square (Table 2).

The dose effects were analysed using 2-way ANOVA (genotype x drug dose), followed by Tukey *post-hoc*.

Table 2. The experiment plan for testing the effects of naloxone and L-365260 to mechanical sensitivity. All animals received all possible injections. The combinations were sequenced according to the Balanced Latin Square for both naloxone and L-365260 injection experiments.

	Day1	**Day4**	**Day7**	**Day10**
Animal1	Vehicle	Dose1	Dose2	Dose3
Animal2	Dose3	Vehicle	Dose1	Dose2
Animal3	Dose2	Dose3	Vehicle	Dose1
Animal4	Dose1	Dose2	Dose3	Vehicle
…	…	…	…	…

Neuropathic pain model

Paper I

Only $CCK_2^{-/-}$ and wild-type mice were used in the neuropathic pain model (number of animals was 6...12 in each group). Mice were anaesthetized with 200 µl of mixture containing 50 µl of Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen Animal Health Ltd), 50 µl of Dormicum® (midazolam 1.25 mg/ml, Roche) and 100 µl of water (dilution 1:1:2, respectively). The chronic constriction injury (Bennett & Xie, 1988) was chosen because of its wide acceptance as a reliable and reproducible model for neuropathic pain. Briefly, the left common sciatic nerve was exposed laterally at the mid-thigh level and 3 loose ligatures with an interval of about 1 mm were placed with 7–0 non-absorbable monofilament thread. The nerve was not

constricted. The wound was closed in layers. For half of the animals, sham operation was performed. In this case, the sciatic nerve was exposed, but not ligated. The preoperative and postoperative mechanical sensitivity scores were obtained as described in the "Assay of mechanical sensitivity" section.

The postoperative behaviour was first analysed quantitatively, comparing preoperative and postoperative mechanical sensitivity values. For this repeated measures ANOVA (day x genotype – surgery) and Tukey *post-hoc* was used.

Second, the postoperative behaviour was analyzed qualitatively, to specifically assess the rate of development of hypersensitivity. Thus, we transformed our data into a binary form (1="hypersensitive" and 0="not-hypersensitive"). The criteria for being included to the hypersensitive group were derived from the normal distributions of the baseline mechanical sensitivity values; the inclusion levels were calculated separately for the different genotypes. The criterion for being included as "hypersensitive" was calculated as equal or less than the minimum value of normal (preoperative) mechanical sensitivity (≤0.4 g for the wild-type and ≤0.6 g for the $CCK_2^{-/-}$ animals). Therefore, the individual animal's score for a given postoperative day was transformed either to "0=not-hypersensitive" or "1=hypersensitive". From these data, each animal acquired a cumulative hypersensitivity score (sum of all postoperative days). Last, genotype and surgery effects were tested for the cumulative hypersensitivity scores using 2-way ANOVA (genotype x surgery) and Tukey *post-hoc*.

Paper 2

Chronic constriction injury, a neuropathic pain model (anaesthetization, CCI operation and sham operation), was performed exactly as described in the previous section. The number of animals in each group (wild-type, knockout; CCI and sham) was 4.

The only difference, compared to the previous section was that the animals' mechanical sensitivity was not measured postoperatively, to avoid any confounding effect of algesia testing on gene expression. As the CCI surgery induces hypersensitivity in the majority of mice (Figure 5) and we have observed and documented exactly the same postoperative dynamics of hypersensitivity several times, it is probably not a problem. We confirmed that all of the wild-type CCI-treated animals exhibited general signs of hindpaw hypersensitivity such as avoiding using the ipsilateral paw.

Assay of SIA

In order to induce SIA, inescapable electric foot-shocks (0, 0.2, 0.4, 0.6, 0.9 mA, alternative current, for 3 min) were administered to the animals by means of an active avoidance testing system (TSE Systems, Germany). Withdrawal latencies in the radiant-heat tail flick test (Plantar Test 7371, TSE Systems, Germany) were measured before (baseline) and after the delivery of foot-shocks and an increase in the post-stress latency was a measure of SIA. The post-stress latencies were measured immediately after termination of the stress (termination of foot-shocks). Removal of the tail from the heat source terminated the application of thermal stimulation. Ceiling tail flick latencies were 30 s. Restraint tubes (opaque plastic cylinders, inner diameter 28 mm, length 90 mm, with a small hole in the closed end to allow breathing) were used for tail flick measuring. Mice were habituated with the tubes for 5 days prior to the actual experiment.

Wild-type and $CCK_2^{-/-}$ animals were used. The number of animals was 6–12; in 0.9 mA stress group only 5 animals of $CCK_2^{-/-}$ genotype were used. Application of the 0.9 mA stress was necessary only for the $CCK_2^{-/-}$ animals as wild-type animals displayed near-ceiling analgesia already at the stress level of 0.6 mA. The reason for using only 5 animals was because we verified that all of these displayed ceiling analgesia and applied the strong stress to as few animals as possible.

Each animal was used only once (received only one application of stress). 2-way ANOVA (genotype x stress intensity) and Tukey *post-hoc* was used to analyse the data.

The effect of rimonabant and naloxone on the development of SIA

The doses of 0.1, 1 and 3 mg/kg of CB_1 receptor antagonist rimonabant (Sanofi-Aventis) and 0.01, 0.1, 1 and 10 mg/kg of opioid receptor antagonist naloxone (Sigma) were used. The naloxone dose of 1 mg/kg was chosen as a dose that has been shown to exert a biological activity, blocking all opioid receptors (Marchand *et al.*, 2003); the other doses were chosen to cover as broad dose spectrum as possible. Drugs were injected i.p. 30 min before the beginning of stress application (3 min electric foot-shock, 0.6 mA). The number of animals in rimonabant experiment was 8–12 and in naloxone experiment 8–10.

2-way ANOVA (genotype x drug dose) and Tukey *post-hoc* was used to analyse the data.

Tissue dissection, RNA preparation and gene expression analyses

Paper I

We used a different set of CCI mice for the gene expression analyses. The surgery of chronic constriction injury was performed exactly as described in the previous section. Again, we used only $CCK_2^{-/-}$ and wild-type mice (N=6). Baseline mechanical sensitivity, as well as the development of hypersensitivity was monitored as described in previous section. Mice were sacrificed on the 9th day after surgery and nervous tissue from the lumbar segments of the spinal cord was dissected and rapidly frozen in liquid nitrogen. Tissues were pooled according to the animal group ($CCK_2^{-/-}$ and wild-type, CCI and sham-operated) and mRNA extracted, using RNeasy midi kit (Qiagen) according to the manufacturer's protocol. First strand cDNA was synthesized, using First Strand cDNA Synthesis Kit (Fermentas). Due to the pooling, gene expression analysis reflects the mean levels of the 6 animals' gene expression.

Expression levels of the following gene transcripts were measured: pro-opiomelanocortin (POMC), opioid receptors μ, δ, κ (Oprm, Oprd and Oprk), CCK_1 receptor (CCK_1) and pre-proCCK (CCK). Quantitative real-time PCR (ABI Prism 7000 SDS, Applied Biosystems) was used for the detection of gene expression level. The PCR reaction was performed in a final volume of 20 μl, using 0.5–50 ng of cDNA. For the POMC, CCK_1 and CCK, we used TaqMan® Universal PCR master mix (Applied Biosystems), Applied Biosystems pre-designed POMC, CCK_1 and CCK primers and probes (see Table 3 for the Applied Biosystems assay numbers). PCR was set up using the following steps: 95°C for 10 min; 95°C for 15 sec and 60°C for 1 min, repeated for 40 cycles. For the opioid receptors, we used SYBR green (qPCR™ Core Kit, Eurogentec) based real-time PCR. The primer sequences are listed in Table 3. Prior to the quantification, primers were tested with cDNA dilution series. Melting curve analyses were performed throughout the quantification to check for the possible presence of primer dimers. PCR was set up using the following steps: 95°C for 10 min; 95°C for 20 sec, 55°C for 30 sec and 60°C for 1 min, repeated for 40 cycles. We used GAPDH (TaqMan® Rodent GAPDH Control Reagents, VIC™ Probe, Applied Biosystems) as an internal reference for the quantification analysis.

The samples were run as 4 technical replicates in each plate and every sample was further repeated 3 times. From these an average threshold cycle (Ct) was calculated. Gene expression levels were analysed by comparing the values of ΔCt (ΔCt = threshold cycle of target gene – threshold cycle of housekeeper), using 2-way ANOVA (genotype x surgery) and Tukey *post-hoc*. To obtain better eye-comparison for the graphical view, the ΔCt values were then

transformed to a linear scale (in the form of $2^{-\Delta\Delta Ct}$), where the wild-type sham-operated animal group is a reference (has its expression level set to 1.0).

Paper 2

Gene expression experiment using microarrays

Mice were killed by cervical dislocation on the 9th day after surgery and medulla, midbrain and lumbar region of the spinal cord were dissected and rapidly frozen in liquid nitrogen. Medulla and midbrain samples were used for the microarray experiments and 32 arrays were run (N=4 per group, 8 different groups): CCI wild-type midbrain, CCI knockout midbrain, sham wild-type midbrain, sham knockout midbrain, CCI wild-type medulla, CCI knockout medulla, sham wild-type medulla, sham knockout medulla. Lumbar region was used for the quantitative real-time PCR analysis. Total RNA was extracted using the guanidium thiocyanate method (Chomczynski & Sacchi, 1987) with TRIzol reagent (Invitrogen Life Technologies, UK).

Double-stranded cDNA was synthesized from 4 μg of total RNA by reverse transcription using T7-Oligo(dT) promotor primer and then biotin-labelled cRNA was made from the cDNA template by *in vitro* transcription (One-Cycle Target Labeling kit, Affymetrix, Santa Clara, USA). cRNA was fragmented and hybridized to the Mouse 430A 2.0 Gene Expression Array (Affymetrix, Santa Clara, USA). The arrays were subsequently washed, stained with phycoerythrin streptavidin and scanned according to standard Affymetrix protocols. The raw intensity data (cel files) were analyzed with dChip2005 using invariant-set normalization and the PM-MM model (Li & Wong, 2001).

Analysis of microarray data

Analysis of gene expression data was divided into two parts. In the first part, ANOVA for each probeset combined with Benjamini and Hochberg's false-discovery-rate (Benjamini & Hochberg, 1995) (FDR, controlled at q=0.05) was used to find genes with the strongest evidence of either an effect of genotype, surgery, or genotype x surgery interaction across the two brain regions (different tissue samples from the same animals were treated as replicates in the analysis). In the second part of the analysis we applied functional annotation to discover which biochemical pathways or biological processes were influenced by experimental manipulations (genotype effect, surgery effect and genotype x surgery interaction), using a longer list of genes with an uncorrected ANOVA p-value ≤ 0.01.

Gene expression experiment using real-time PCR

To obtain additional information on the activation of genes related to inflammation identified in the microarray study, we performed quantitative real-

time PCR (qRT-PCR) using the ABI PRISM 7900 HT Sequence Detection System equipment (PE Applied Biosystems, USA) and ABI PRISM SDS Software.

Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, UK). cDNA synthesis was performed using reverse transcriptase enzyme SuperScript III (Invitrogen). For all qRT-PCR experiments, hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as the endogenous reference (housekeeper) gene. HPRT was chosen as the endogenous reference gene because it is a constitutively and stably expressed gene in the mammalian brain and is frequently used as a reference gene (de Kok *et al.*, 2005). The Tlr4 and IL1b primers were designed with the Primer Express software (PE Applied Biosystems, USA). The primer sequences are presented in (Table 3). Primers were designed to cover exon–exon junctions, to exclude amplification of any possible contaminating genomic DNA. All reactions were performed using qPCR Core Kit for SYBR® Green I Master Mix (Eurogentec, Belgium). Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. RNA extractions, cDNA synthesis and PCR reactions were performed separately for each individual. All samples were run as 4 technical replicates in the same experiment. From these an average threshold cycle (Ct) was calculated. Gene expression levels were analysed by comparing the values of ΔCt (ΔCt = threshold cycle of target gene – threshold cycle of housekeeper), using 2-way ANOVA (genotype x surgery) and Tukey *post-hoc*. To obtain better eye-comparison for the graphical view, the ΔCt values were then transformed to a linear scale (in the form of $2^{-\Delta\Delta Ct}$), where the wild-type sham-operated animal group is a reference (has its expression level set to 1.0).

Functional annotation of differentially expressed genes

Functional annotation was applied to further describe the genetic networks activated after the induction of CCI and to find differences in this activation related to genotypes of animals. For functional analysis of the expression, PathwayExplorer (https://pathwayexplorer.genome.tugraz.at/) was used. This is a recently developed integrated web-based data mining system to explore large datasets for the functional analysis of gene expression profiles (Mlecnik *et al.*, 2005). After statistical analysis of the main factors (genotype and surgery) and interaction between the main factors, we uploaded appropriate lists of genes (uncorrected p-values <0.01) with their log2 ratio values to the PathwayExplorer website to identify pathways influenced by these factors. Pathways were ranked according to the number of mapped hits from our microarray candidate gene list. During analysis, lists of gene identifiers are annotated and summarized according to the shared categorical Gene Ontology data. Functional categories represented in a gene list relative to the representation within the transcriptome/proteome of a given species are then identified by Fisher's exact test ($p \leq 0.05$ indicates significant enrichment in the annotation category).

Subsequently, for illustrative purposes, the log2 ratio data from the entire microarray was mapped to the pathway with the most significant enrichment (MAPK pathway).

Paper 3

For the gene expression analysis, we measured the expression levels of following genes: pre-proCCK (CCK), CCK_2 receptor (CCK_2), cannabinoid CB_1 receptor, sn-1-DAG lipase alpha (DAGLa), sn-1-DAG lipase beta (DAGLb), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), monoacylglycerol lipase (MAGL), fatty-acid amide hydrolase (FAAH), opioid μ receptor (Oprm), opioid δ receptor (Oprd), opioid κ receptor (Oprk), pro-opio-melanocortin (POMC), pro-enkephalin (PENK) and pro-dynorphin (PDYN). The genes were chosen to cover CCK, endocannabinoid and end-opioid systems, as these are known to be related to stress and SIA mechanisms (Lewis *et al.*, 1980; Stanfa *et al.*, 1994; Hohmann *et al.*, 2005).

The expression of all the listed genes was measured in lumbar spinal cord, brainstem (medulla, pons), midbrain, striatum and mesolimbic area (nucleus accumbens and olfactory tubercle). The lumbar spinal cord, brainstem and midbrain were selected because these CNS regions are related to the regulation of nociceptive sensitivity (Walker *et al.*, 1999; Walker & Hohmann, 2005). The striatum and mesolimbic area were selected because these brain structures are involved in the integration of nociceptive and motivational processes (Ozaki *et al.*, 2002; Ansah *et al.*, 2007).

For the stress group, tissues were collected 20 min after termination of stress (stress assay described above). The control group was exposed to the foot-shock chamber, but foot-shocks were not delivered. The number of animals was 6 in all groups.

RNA extraction, cDNA synthesis was performed using reverse transcriptase enzyme SuperScript III (Invitrogen). qPCR Core Kit for SYBR Green I Master Mix (Eurogentec, Belgium) and TaqMan universal PCR mastermix (Applied Biosystems) was used for quantitative real-time PCR (ABI PRISM 7900 HT Sequence Detection System equipment, PE Applied Biosystems, USA).

Primers were designed (Primer Express software, PE Applied Biosystems, USA) to hybridise exon-exon junctions, to exclude amplification of any possible remains of genomic DNA. All the primer sequences (or, for the pre-designed Applied Biosystems primers, their Applied Biosystems assay numbers) are listed in (Table 3). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an endogenous reference gene. All the primers were tested for their quantitative properties (cDNA 2X dilution series resulted in 1 Ct shifts of the raw curves) and for their selectivity (PCR yielded only one product).

Table 3. Primer sequences or Applied Biosystems Assay numbers used for the Q-RT gene expression studies

Gene	Forward	Reverse	AB Assay No
CCK			Mm_0446170_m1
CCK_1			Mm_00438060_m1
CCK_2			Mm_00432329_m1
CB_1	CGTGTTCCACCGCAAAGATA	CCCACCCAGTTTGAACAGAAA	
DAGLa			Mm_01701557_m1
DAGLb	CGCTTATCCTAGGGATGGATGT	CCATGTTGGTCACACTTAACCTG	
NAPE-PLD	GCCATTCCCATCGGAGC	ATGCTGGTATTTCATAAACCACCTT	
MAGL	TGTCCTGCCAAATATGACCTTG	GGTCAACCTCCGACTTGTTCC	
FAAH			Mm_01191808_m1
Oprm	AACACCCCTCCACGGCTAAT	GGGTTGGCTGGTGGTTAGTTC	
Oprd	TTGGCATCGTCCGGTACAC	AGATGTAGATGTTGGTGGCGG	
Oprk	GCAGCCTGAATCCTGTTCTC	TCATCCCTCCCACATCTCTC	
POMC	CTTTGTCCCCAGAGAGCTGC	AACAAGATTGGAGGGACCCC	
POMC			Mm00435874_m1
PENK	ATGCAGCTACCGCCTGGTT	GTGTGCACGCCAGGAAATT	
PDYN	TTGGCAACGGAAAAGAATCTG	TGTGCGGCTTCATCATTCAT	
Tlr4	AAACTTGCCTTCAAAACCTGGC	ACCTGAACTCATCAATGGTCACATC	
IL1b	GTAATGAAAGACGGCACACCC	CTTGGGATCCACACTCTCCAG	
HPRT	GCAGTACAGCCCCAAAATGG	AACAAAGTCTGGCCTGTATCCAA	
GAPDH			TaqMan® Rodent GAPDH Control Reagents

The RNA extractions, cDNA synthesis, ΔCt calculation was performed exactly as described in previous section (for the Paper 2). PCR reactions were performed separately for each individual. Gene expression levels were analysed by comparing the values of ΔCt, using 2-way ANOVA (genotype x stress) and Tukey HSD *post-hoc* test. To better visualise gene expression changes in the graphs, the ΔCt values were then transformed into a linear scale ($2^{-\Delta\Delta Ct}$), where the wild-type non-stressed mice are the reference group (expression level set to 1.0). Thus, the gene expression graphs show relative expression levels compared to wild-type non-stressed mice. Gene expression graphs are presented only for genes and CNS regions where statistically significant stress-induced effects were observed. The expression level mean values as well as their SEM values for all the listed genes in all the five CNS regions are presented in the Appendix 1.

Drugs

L-365260 (Merck Sharp & Dohme), the antagonist of CCK_2 receptors was dissolved in saline with the help of few drops of Tween-85 (Sigma) (Paper 1). Naloxone (Sigma), the antagonist of opioid receptors was dissolved in saline (Papers 1 and 3). Rimonabant (Sanofi-Aventis) was dissolved in 5% DMSO with a help of a few drops of Tween-80 (Paper 3).

Statistical analysis

The variances in all the figures are expressed as mean ± SEM.

RESULTS

Paper I

Mechanical sensitivity phenotype of the $CCK_2^{-/-}$ mice

We first measured mechanical sensitivity in wild-type, $CCK_2^{+/-}$ and $CCK_2^{-/-}$ mice. The number of animals in each group was 16. One-way ANOVA for the mechanical sensitivity values showed a significant genotype effect ($F(2, 45)=47.2$, $p<0.0001$). $CCK_2^{-/-}$ and $CCK_2^{+/-}$ mice displayed mechanical hyposensitivity (Figure 1), i.e. their response threshold was higher than that of wild-type animals ($p<0.001$, Tukey *post-hoc*).

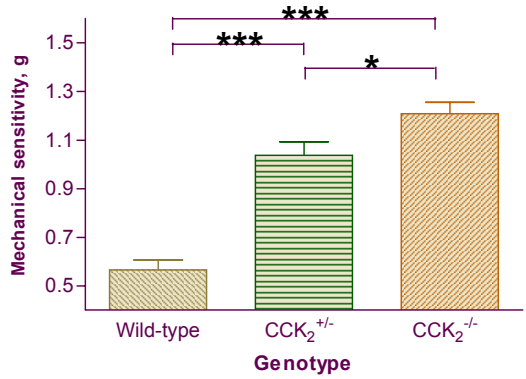

Figure 1. Baseline thresholds of mechanical sensitivity in wild-type, $CCK_2^{+/-}$ and $CCK_2^{-/-}$ mice. $CCK_2^{+/-}$ and $CCK_2^{-/-}$ mice displayed significant mechanical hyposensitivity, compared to the wild-type. In addition, the $CCK_2^{-/-}$ mice were slightly more hyposensitive than the $CCK_2^{+/-}$ mice. *$p<0.05$, ***$p<0.001$, Tukey *post-hoc*.

In addition, the mean scores of the homozygous $CCK_2^{-/-}$ and heterozygous $CCK_2^{+/-}$ animals were also statistically different, $CCK_2^{-/-}$ mice being a bit more hyposensitive than $CCK_2^{+/-}$ animals (Figure 1). However, this mean difference was small ($p<0.05$, Tukey *post-hoc*) and statistical significance reached only if quite large group sizes were used (N=16, in this case).

The effect of CCK_2 antagonist L-365260 to mechanical sensitivity

Administration of L-365260 (0.01, 0.1 and 1 mg/kg), an antagonist of CCK_2 receptors, induced mechanical hyposensitivity in wild-type mice (Figure 2), but had no effect on $CCK_2^{-/-}$ or $CCK_2^{+/-}$ animals (2-way ANOVA genotype effect $F(2, 225)=20.0$, $p<0.0001$; genotype x dose interaction $F(8, 225)=3.3$, $p<0.05$, N=16 in each group). The mean mechanical sensitivity scores and *post-hoc* p-values show that the effect is dose dependent; at higher doses the sensitivity score of wild-type mice reached the level of the $CCK_2^{-/-}$ and $CCK_2^{+/-}$ mice. Administration of L-365260 did not alter the mechanical sensitivity of the genetically modified mice.

We separately tested the effect of vehicle administration, compared to the situation where nothing was injected (Figure 2, comparison between the baseline and vehicle). The vehicle-treated and baseline (no injections) latencies were not different.

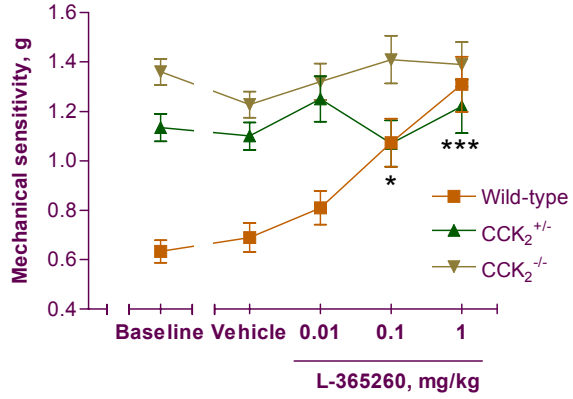

Figure 2. The effect of an antagonist of the CCK_2 receptors on the thresholds of mechanical sensitivity (right side of the figure) and baseline mechanical sensitivity values before the administration of drugs (left side of the figure). I.p. injection of L-365260 induced dose-dependently mechanical hyposensitivity in wild-type mice. L-365260 did not affect the sensitivity of the CCK_2 receptor deficient mice. Stars indicate comparisons between vehicle-treatment and L-365260 treatment in wild-type group (*p<0.01, ***p<0.001, Tukey *post-hoc*).

The effect of opioid antagonist naloxone to mechanical sensitivity

Administration of naloxone (0.1, 1 and 10 mg/kg) affected mechanical sensitivity of the $CCK_2^{-/-}$ and $CCK_2^{+/-}$ mice (Figure 3), inducing hypersensitivity (2-way ANOVA genotype effect $F(2,225)=20.4$, $p<0.0001$; genotype x dose

interaction F(8, 225)=8.2, p<0.0001, N=16 in each group). All doses of naloxone induced significant hypersensitivity in $CCK_2^{-/-}$ mice (p<0.001, Tukey *post-hoc*). In the case of $CCK_2^{+/-}$ mice, lower doses of naloxone (0.1 and 1 mg/kg) induced hypersensitivity (p<0.001 and p<0.01, respectively, Tukey *post-hoc*). The highest dose of naloxone (10 mg/kg) resulted in no effect on mechanical sensitivity in these animals.

We separately tested the effect of vehicle administration, compared to the situation where nothing was injected (Figure 3, comparison between the baseline and vehicle). The vehicle-treated and baseline (no injections) latencies were not different.

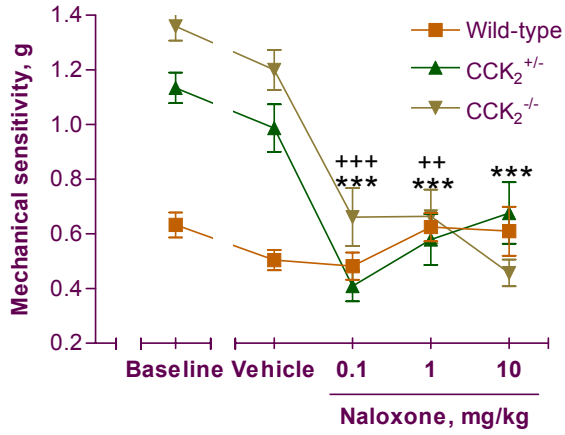

Figure 3. The effect of naloxone on thresholds of mechanical sensitivity (right side of the figure) and baseline mechanical sensitivity values before the administration of drugs (left side of the figure). I.p. injection of naloxone induced mechanical hypersensitivity in CCK_2 receptor deficient mice. Naloxone did not affect the sensitivity of wild-type mice. Stars indicate comparisons between vehicle-treatment and naloxone treatment in $CCK_2^{-/-}$ group (***p<0.001) and crosses indicate comparisons in $CCK_2^{+/-}$ group (+++p<0.001, ++p<0.01).

Neuropathic pain phenotype of the $CCK_2^{-/-}$ mice

Next, we used CCI, a model of neuropathic pain and studied the development of hypersensitivity in wild-type and $CCK_2^{-/-}$ animals. Ligation of the sciatic nerve resulted in typical chronic hypersensitivity in wild-type mice (Figure 4). Number of animals was 6...12 in each group. Hypersensitivity was present on the 2nd day post-operatively, and this persisted as long as tested (44 days). Interestingly, the $CCK_2^{-/-}$ mice did not develop chronic hypersensitivity.

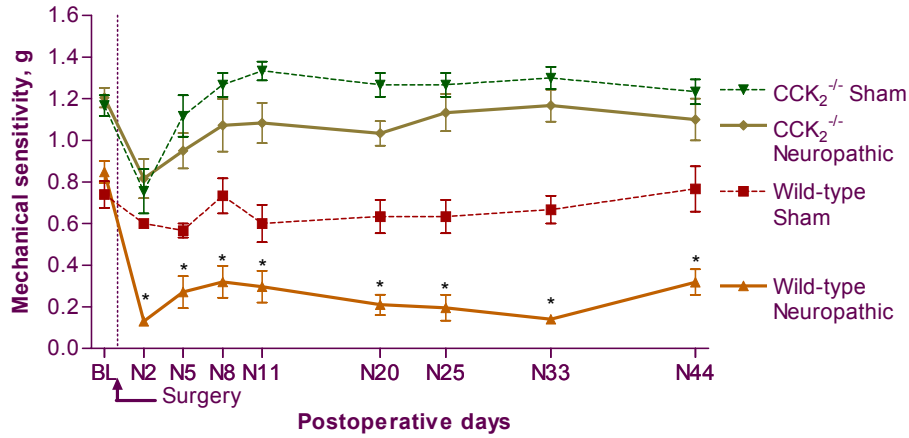

Figure 4. Development of mechanical hypersensitivity after ligation of the sciatic nerve. Chronic constriction injury (the neuropathic group) or sham-operation (the sham group) was conducted on day 0 (an arrow marked with "surgery"), postoperative days are indicated with the letter "N" (N2, N5, N8, etc). Wild-type mice displayed significant hypersensitivity starting from the 2nd day postoperatively (N2). This hypersensitivity persisted as long as tested. On the 2nd postoperative day, both the $CCK_2^{-/-}$ sham and neuropathic mice also displayed hypersensitivity. However, the hypersensitivity of the $CCK_2^{-/-}$ mice was not chronic; these animals displayed no hypersensitivity on subsequent days. Stars indicate comparisons with baseline (BL) mechanical sensitivity (*$p<0.01$, Tukey post-hoc).

Repeated-measures ANOVA showed a genotype effect $F(8, 280)=4.0$, $p<0.001$ (because the $CCK_2^{-/-}$ animals displayed constant lower mechanical sensitivity values) and surgery effect $F(8, 280)=2.6$, $p<0.01$ (because the mean mechanical sensitivity values were higher in neuropathic groups), but no significant interaction (because the $CCK_2^{-/-}$ neuropathic group was also somewhat more sensitive, compared to the Sham group). *Post-hoc* analysis confirmed significant hypersensitivity in wild-type mice (Tukey *post-hoc* wild-type sham compared to the neuropathic group p-values were all lower than 0.01). $CCK_2^{-/-}$ neuropathic and sham-operated mice displayed short-lived hypersensitivity on the 2nd day post-operatively ($p<0.01$, Tukey *post-hoc,* after repeated-measures ANOVA), but subsequent response did not differ from the baseline level. Interestingly, the $CCK_2^{-/-}$ sham group also displayed similar hypersensitivity on the 2nd day post-operatively ($p<0.01$, Tukey *post-hoc*), this hypersensitivity was not apparent on subsequent days. Wild-type sham-operated mice did not display hypersensitivity.

To make sure that we could state the $CCK_2^{-/-}$ animals to be less hypersensitive, we took a different statistical approach. One of the hindering aspects with using mechanical sensitivity mean-values as an input to ANOVA is that this way we might mask a genotype x surgery interaction. This is because a

few of the $CCK_2^{-/-}$ animals (as opposed to most of the wild-type animals) displayed hypersensitivity on each postoperative day. Although most of the animals did not exhibit hypersensitivity, the resulting mean values were still a bit lower and caused the same direction shift as in the wild-types and thus, the quantitative approach could not differentiate between large hypersensitivity of the wild-type animals and small hypersensitivity of the $CCK_2^{-/-}$ animals.

Therefore, to describe the postoperative behaviour qualitatively, we transformed our data into a binary form: 1="hypersensitive" or 0="not-hypersensitive". Now we could easily calculate cumulative numbers of hypersensitive postoperative days for each animal and test if there were differences between genotypes. According to this approach, using 2-way ANOVA (genotype x surgery) for the cumulative hypersensitive days, both main effects were significant (genotype main effect $F(1, 36)=26.8$, $p<0.001$, surgery main effect $F(1, 36)=51.3$, $p<0.001$). In addition, there was a significant interaction ($F(1, 36)=20.4$, $p<0.001$), showing that induction of neuropathy affected development of hypersensitivity differently for $CCK_2^{-/-}$ animals (Figure 5).

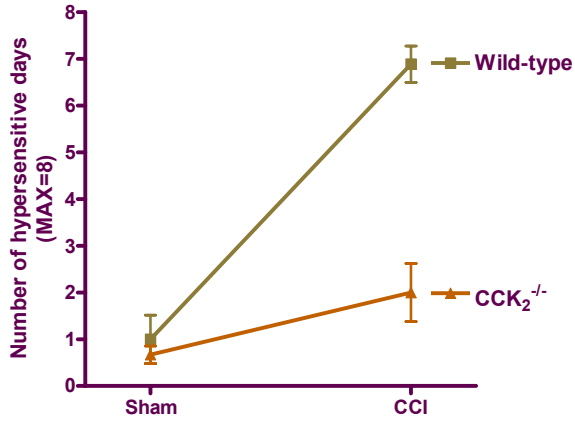

Figure 5. Development of postoperative hypersensitivity after the CCI surgery. For the hypersensitivity score, original data were transformed to a binary form (1="hypersensitive" or 0="not-hypersensitive") and cumulative hypersensitive days calculated. As the postoperative sensitivity was measured during 8 days, the maximum score of hypersensitivity is 8 in the current graph. Wild-type animals display near-maximum score, since on average, wild-type animals were hypersensitive during 7 days out of 8. $CCK_2^{-/-}$ animals display some hypersensitivity: these animals were hypersensitive during 2 days out of 8.

Gene expression analysis of the endogenous opioids and their receptors

We next performed gene expression analysis in lumbar spinal cord for the neuropathic wild-type and $CCK_2^{-/-}$ mice. Genotype x surgery 2-way ANOVA showed a significant interaction for the POMC mRNA expression ($F(1, 12)=26.7$, $p<0.001$, $N=4$). Tukey *post-hoc* analysis showed a reduction of POMC expression in wild-type mice and increase in $CCK_2^{-/-}$ mice as a result of the CCI surgery (Figure 6 A). PENK and PDYN gene expression levels were influenced neither by the genotype nor by the surgery (data not shown).

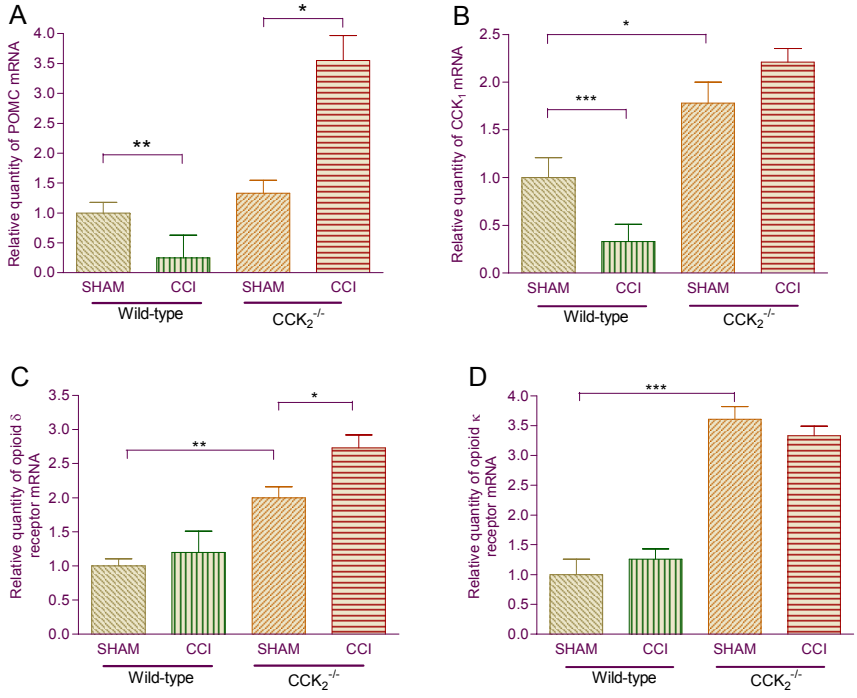

Figure 6. Relative gene expression levels in the lumbar spinal cord after induction of chronic constriction injury. mRNA levels are expressed relative to wild-type sham-operated (designated as SHAM) mice. Gene expression levels of POMC, CCK_1 receptor and opioid receptors δ and κ are presented. (A) Induction of CCI resulted in decrease of POMC expression in the wild-type mice, but increase in the $CCK_2^{-/-}$ mice. (B) Induction of CCI resulted in downregulation of CCK_1 in wild-type mice. Baseline CCK_1 receptor levels were higher in $CCK_2^{-/-}$ mice. (C and D) $CCK_2^{-/-}$ mice expressed higher levels of opioid receptors δ and κ. Induction of CCI did not alter the expression levels in wild-type mice. However, the expression of opioid δ receptor increased further in the $CCK_2^{-/-}$ mice after induction of CCI. Stars indicate Tukey *post-hoc* comparisons (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Opioid μ, δ and κ receptor gene expression

Genotype x surgery 2-way ANOVA showed a significant genotype effect for the Oprd mRNA expression in lumbar spinal cord ($F(1, 12)=48.4$, $p<0.001$), the $CCK_2^{-/-}$ mice natively expressing higher levels of the receptor δ (Figure 6 C). We also saw a surgery effect ($F(1, 12)=8.8$, $p<0.05$), accordingly, Oprd expression increased in response to the surgery.

In case of Oprk we saw a significant genotype effect ($F(1, 12)=83.6$, $p<0.001$), which indicates that $CCK_2^{-/-}$ animals natively express higher levels of receptor κ (Figure 6 D).

We did not detect any genotype or surgery effects or genotype x surgery interactions for the Oprm (data not shown).

CCK and CCK_1 receptor gene expression

We also measured the expression levels of CCK and CCK_1 receptors. Genotype x surgery 2-way ANOVA showed a significant interaction for the CCK_1 mRNA expression in lumbar spinal cord ($F(1, 12)=33.7$, $p<0.001$). Induction of neuropathy resulted in downregulation of CCK_1 receptor in wild-type mice (Figure 6 B). Similarly to the opioid δ and κ receptors, the native expression level of CCK_1 receptor was higher in $CCK_2^{-/-}$ mice, showing about 2-fold increase, compared to the wild-type littermates.

We did not see any genotype or surgery effects or genotype x surgery interactions for the CCK (data not shown).

Paper 2

In the second study we used mouse gene microarrays to obtain gene expression profile of the whole transcriptome and compared changes in the expression of all genes in midbrain and medulla oblongata in response to induction of neuropathy.

Analysis of gene expression data was divided into two parts. In the first part, ANOVA for each probeset combined with Benjamini and Hochberg's false-discovery-rate (Benjamini & Hochberg, 1995) (FDR, controlled at $q=0.05$) was used to find genes with the strongest evidence of either an effect of genotype, surgery, or genotype-surgery interaction across the two brain regions (different tissue samples from the same animals were treated as replicates in the analyses). In the second part of the analysis we applied functional annotation to discover which biochemical pathways or biological processes are influenced by experimental manipulations (genotype effect, surgery effect and genotype x surgery interaction), using a longer list of genes with an uncorrected ANOVA p value ≤ 0.01.

ANOVA with FDR correction: Genotype effects

There is strong evidence for a genotype effect in many probesets (the best twenty are shown in Table 4, 15 of them have their q<0.05). Aside from CCK_2 itself, almost all of these are from the region flanking the CCK_2 locus, and are most likely the result of a "congenic footprint" (this topic is covered in detail in Paper 4). This footprint reflects genetic background differences between the mouse lines used to generate the knockout (129S4 and C57BL/6BkL) in the region flanking the targeted locus (the CCK_2 receptor gene).

Table 4. The best 20 probesets of the genotype main effect testing in 2-way ANOVA combined from the tissues of medulla and midbrain. Gene symbols, as well as chromosomal locations, p- and q-values are presented for each test.

Probeset ID	Entrez ID	Gene	Chr	p values	q values
1454770_at	12426	cholecystokinin B receptor	7	4.74E-12	1.08E-07
1424614_at	233575	similar to FGF receptor activating protein 1	7	7.18E-07	5.27E-03
1421385_a_at	17921	myosin VIIa	7	8.16E-07	5.27E-03
1417379_at	78368	RIKEN cDNA 1810006G21 gene		9.43E-07	5.27E-03
1417379_at	29875	IQ motif containing GTPase activating protein 1	7	1.16E-06	5.27E-03
1415748_a_at	59288	dynactin 5	7	3.04E-06	1.15E-02
1417961_a_at	20128	tripartite motif protein 30	7	1.03E-05	3.34E-02
1423819_s_at	54208	ADP-ribosylation-like factor 6 interacting protein	7	1.23E-05	3.49E-02
1455036_s_at	68197	RIKEN cDNA 1810004I06 gene	7	1.85E-05	4.11E-02
1424415_s_at	101647	expressed sequence AI666765	7	2.07E-05	4.11E-02
1460335_at	80289	cDNA sequence BC003322	13	2.17E-05	4.11E-02
1416665_at	12850	demethyl-Q 7	7	2.18E-05	4.11E-02
1450154_at	53320	folate hydrolase	7	2.39E-05	4.11E-02
1424857_a_at	94094	tripartite motif protein 34	7	2.57E-05	4.11E-02
1417839_at	12741	claudin 5	16	2.72E-05	4.11E-02
1418163_at	21898	toll-like receptor 4	4	3.88E-05	5.48E-02
1449692_at	18762	protein kinase C, zeta	4	4.10E-05	5.48E-02
1421594_a_at	83671	synaptotagmin-like 2	7	5.04E-05	6.36E-02
1428069_at	66953	RIKEN cDNA 2310021G01 gene	2	6.26E-05	7.48E-02
1426838_at	67967	RIKEN cDNA 2410142G14 gene	7	7.02E-05	7.97E-02

We were also able to detect changes in the gene expression levels not linked to the "congenic footprint" region and are therefore probably more related to the specific deletion of the CCK_2 (as opposed to the genomic area of 129sv origin, flanking the CCK_2 receptor). These highly significant, functionally relevant and genetically unlinked transcripts include three genes of particular interest: toll-like receptor 4 (Tlr4, probe 1418163_at), protein kinase C zeta (Prkcz) and claudin 5 (Cldn5). These transcripts are interesting, because Tlr4, Prkcz and Cldn5 were all up-regulated in the $CCK_2^{-/-}$ animals, whereas genes expressed from the "congenic footprint" region were almost all in the knockout-low direction. This finding indicates that changes in Tlr4, Prkcz and Cldn5 genes are more likely caused by deletion of CCK_2. The other reason for the specific interest towards these genes is addressed subsequently and is concerned with the genes belonging to certain biochemical pathways.

The Tlr4 gene expression differences and its changes dependent on the factors (genotype and CCI) are illustrated below (Figure 7). We see a strong genotype effect, i.e. the $CCK_2^{-/-}$ animals display higher expression levels of the transcript (midbrain $F(1,12)=388.2$, $p<0.0001$ and medulla $F(1,12)=49.1$, $p<0.0001$). In addition, we also found a significant genotype x CCI interaction at the midbrain level ($F(1,12)=5.9$, $p<0.05$).

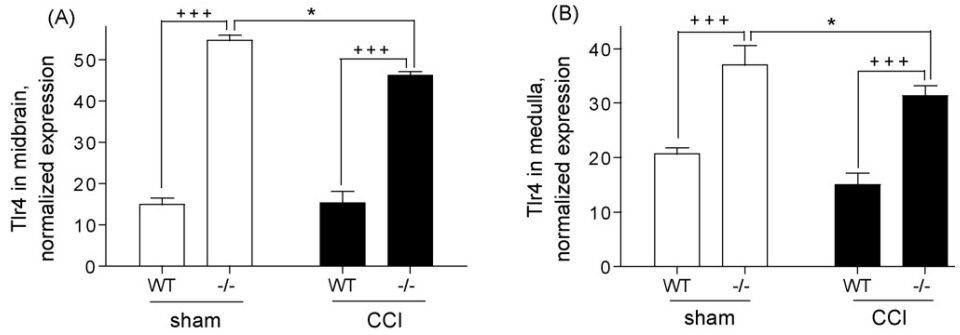

Figure 7. Genechip data of the expression of Tlr4 gene, arbitrary units. Raw data were normalized using dchip software. Two-way ANOVA was used to analyse the effects of genotype and CCI on the expression level. This figure visualizes elevated basal expression of the Tlr4 gene in the $CCK_2^{-/-}$ mice in the midbrain and medulla. (A) Midbrain data: +++$p<0.0001$ wild-type (WT) compared to $CCK_2^{-/-}$ (KO). *$p<0.05$ CCI compared to sham. (B) Medulla data: +++$p<0.0001$ wild-type compared to $CCK_2^{-/-}$. *$p<0.05$ CCI compared to sham.

ANOVA with FDR correction: Surgery effects

There are some substantial changes in the expression levels of genes (Table 5), induced by CCI (nominal p-values as low as 1.64E-05), but none of these satisfy a genomewide FDR criterion of $q=0.05$. Nonetheless, the q value for the

5 most different transcripts is 0.32, indicating that two or three of these five would represent real differences. Among these top 5 candidates, two – protein kinase C zeta (Prkcz) and the aryl-hydrocarbon receptor (Ahr) – are directly involved in the MAPK pathway, toll-like receptor pathway and in the regulation of the immune response (Martin *et al.*, 2005). Therefore, these two genes may well be of relevance in the context of CCI and an inflammatory response to the surgery.

Table 5. The best 20 probesets of the surgery main effect testing in 2-way ANOVA combined from the tissues of medulla and midbrain. Gene symbols, as well as chromosomal locations, p- and q-values are presented for each test.

Probeset ID	Entrez ID	Gene	Chr	p values	q values
1436845_at	12006	axin2	11	1.64E-05	0.32
1451087_at	225348	RIKEN cDNA 5730444A13 gene	18	4.31E-05	0.32
1422631_at	11622	aryl-hydrocarbon receptor	12	4.70E-05	0.32
1449692_at	18762	protein kinase C, zeta	4	6.77E-05	0.32
1426292_at	233056	RIKEN cDNA 6330581L23 gene	7	7.03E-05	0.32
1452246_at	20409	SH3 domain protein 3	19	1.15E-04	0.37
1424075_at	68115	RIKEN cDNA 9430016H08 gene	1	1.25E-04	0.37
1455405_at	19201	proline-serine-threonine phosphatase-interacting protein 2	18	1.29E-04	0.37
1449232_at	14460	GATA binding protein 1	X	1.79E-04	0.44
1455642_a_at	74257	RIKEN cDNA 2210021G21 gene	13	2.02E-04	0.44
1419230_at	268482	keratin complex 1, acidic, gene 12	11	2.14E-04	0.44
1427360_at	74706	RIKEN cDNA 4930507D05 gene		2.48E-04	0.47
1456583_x_at	70652	RIKEN cDNA 5730537D05 gene	3	3.27E-04	0.56
1451909_a_at	19134	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	13	3.79E-04	0.56
1429370_a_at	69077	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	11	4.13E-04	0.56
1435110_at		RIKEN cDNA D330016C04 UNC5H2 homolog [Rat]		4.25E-04	0.56
1416208_at	59025	ubiquitin specific protease 14	18	4.51E-04	0.56
1427676_a_at	14805	glutamate receptor, ionotropic, kainate 1	16	5.15E-04	0.56
1433573_x_at	22072	trypsin 2	6	5.22E-04	0.56
1420558_at	20344	selectin, platelet	1	5.43E-04	0.56

ANOVA with FDR correction: Surgery x Genotype interaction

As with surgery effects, there was no stringent evidence for any individual expression differences following correction for multiple testing (Table 6). However, the FDR for the top four genes is 0.23, so we can consider that out of these 4 genes only one would be a false positive. From these 4 genes we found one interesting candidate – Copps5. It is Jun co-activator and thus also directly connected to the MAPK pathway. Interestingly, MAPK activation was also evident when the effect of surgery was analyzed as the main factor. These results suggest that CCI may induce MAPK pathway activation in brain areas related to pain modulation.

Table 6. The best 20 probesets of the surgery x genotype interaction testing in 2-way ANOVA combined from the tissues of medulla and midbrain. Gene symbols, as well as chromosomal locations, p- and q-values are presented for each test.

Probeset ID	Entrez ID	Gene	Chr	p values	q values
1452218_at	104479	expressed sequence AU018638	11	2.09E-05	0.23
1421894_a_at	22019	tripeptidyl peptidase II	1	2.74E-05	0.23
1439243_x_at	26754	COP9 homolog, subunit 5 (Copps)	1	3.90E-05	0.23
1417755_at	106021	expressed sequence AW105885	5	4.03E-05	0.23
1451840_at	80334	calsenilin-like protein	5	1.32E-04	0.53
1451909_a_at	19134	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	13	1.49E-04	0.53
1422304_at	16821	lipocalin 4	2	1.63E-04	0.53
1452476_at	12296	calcium channel, voltage-dependent, beta 2 subunit	2	2.11E-04	0.60
1416208_at	59025	ubiquitin specific protease 14	18	3.75E-04	0.76
1423616_at	21357	TAR (HIV) RNA binding protein 2	15	3.92E-04	0.76
1419586_at	19889	retinitis pigmentosa 2 homolog (human)	X	4.66E-04	0.76
1422018_at	15273	human immunodeficiency virus type I enhancer binding protein 2		5.04E-04	0.76
1422702_at	54375	ornithine decarboxylase antizyme inhibitor	15	5.08E-04	0.76
1419329_at	20410	SH3 domain protein 4	14	5.17E-04	0.76
1417256_at	17386	matrix metalloproteinase 13	9	5.23E-04	0.76
1420446_at	69287	RIKEN cDNA 1700011O04 gene	7	5.90E-04	0.76

Probeset ID	Entrez ID	Gene	Chr	p values	q values
1425852_at		clone MGC:31405 IMAGE:4456622, mRNA		6.14E-04	0.76
1455019_x_at	216197	RIKEN cDNA 5630400A09 gene	10	6.17E-04	0.76
1448880_at	22195	ubiquitin-conjugating enzyme E2L 3	16	6.40E-04	0.76
1449687_at	52666	DNA segment, Chr 10, ERATO Doi 610, expressed	10	7.67E-04	0.87

Functional annotation and pathway analysis

For the purpose of functional annotation of the genechip data we used three lists of genes with nominal p-values less than 0.01 from ANOVA analysis. One list contained genes changed (up- or downregulated) by genotype (mutation effect), the second list contained genes changed by CCI (surgery) and the third list contained genes changed by CCI x genotype interaction. As we found the MAPK pathway activated by CCI, for descriptive purposes we uploaded expression data describing the MAPK pathway from our chips and mapped this expression data to this pathway. These results are illustrated in Appendix 2.

Functional analysis of the gene expression profiles to look for relationships between different transcripts was performed using Pathway Explorer (https://pathwayexplorer.genome.tugraz.at/) (Mlecnik *et al.*, 2005). Log2 ratio values for genes with uncorrected p-values less than 0.01, calculated separately according to the two main factors (genotype and surgery), and genotype by surgery interaction, were uploaded to Pathway Explorer. Gene expression log2 ratio data were then mapped to all the available pathway databases in the server (KEGG, Biocarta and GenMapp). The CCI surgery gene list gave the most significant enrichment of the MAPK signalling pathway (id 04010). Genes whose expression difference was related to genotype did not map to any particular pathway, supporting the "congenic footprint" (genetic) effect. Analysis of genes with a genotype by CCI surgery interaction resulted again in a significant mapping to the MAPK pathway. Therefore, activation of the MAPK signalling pathway appears to occur after induction of CCI in the midbrain and medulla and this is dependent on the genotype of animals. Statistical analysis for this pathway using a right-tailed Fisher's exact test confirmed that there was significant activation of the MAPK pathway ($p<0.05$) that was specific to surgical manipulation as the majority of the mapped genes were those altered in the CCI groups. The direction of changes in the gene expression profile (Appendix 2) also makes functional sense, with genes that activate the pathway increased and genes inhibiting the pathway decreased after CCI surgery. These results suggest that CCI activates the MAPK pathway in

supraspinal regions related to pain sensitivity and this activation does not occur after the knockout of the CCK_2 receptor.

Tlr4 and IL1b gene expression

The increased Tlr4 expression seen in the $CCK_2^{-/-}$ animals is most likely related to deletion of CCK_2, and could be related to the resistance of $CCK_2^{-/-}$ mice to CCI-induced allodynia. To assess whether Tlr4 expression was altered in another region implicated in neuropathic pain, we measured mRNA in the lumbar region of the spinal cord (taken from the same mice used for the microarray experiment, Figure 8). We saw no genotype effect, i.e. the Tlr4 expression was not significantly higher in the lumbar region of sham-operated $CCK_2^{-/-}$ mice compared to sham-operated wild-type controls. There was an increase in Tlr4 expression following surgery in the wild-type mice that was not seen in the $CCK_2^{-/-}$ mice (surgery effect, $F(1,8)=6.0$, $p<0.05$, Figure 8 A). We also measured the expression level of Interleukin-1 beta (IL1b) as it has been shown to play a central role in the generation of mechanical hyperalgesia and is functionally linked to Toll-like receptor pathway (Zelenka *et al.*, 2005). IL1b expression was significantly increased in wild type mice after the CCI, but not in $CCK_2^{-/-}$ mice (genotype by surgery interaction $F(1,16)=8.5$, $p<0.05$, Figure 8 B).

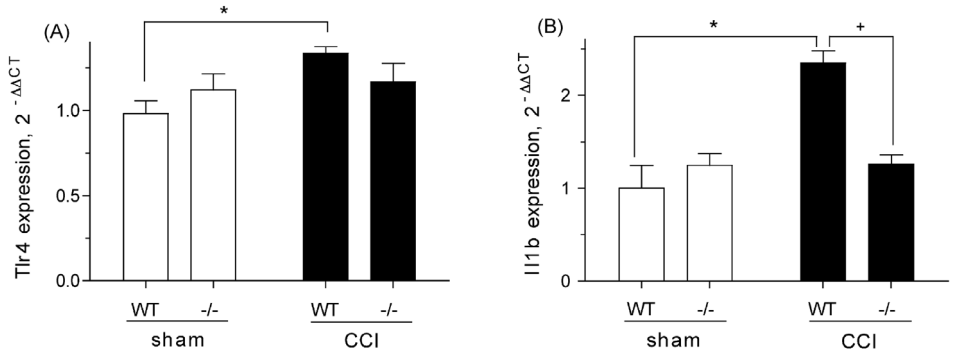

Figure 8. Real-time PCR based expression analysis of Tlr4 and IL1b in the lumbar spinal cord after induction of CCI in $CCK_2^{-/-}$ mice (–/–) and wild type littermates (WT). (A) We found a significant surgery effect in the expression of Tlr4. (B) We found a significant genotype by surgery interaction in the expression of IL1b. *$p<0.05$ compared to wild-type sham-operated mice. +$p<0.05$ compared to $CCK_2^{-/-}$ CCI mice.

Paper 3

Stress-induced analgesia phenotype of the $CCK_2^{-/-}$ mice

In the third study we analysed the SIA phenotype of the $CCK_2^{-/-}$ animals.

We applied different stressor intensities – 0.2, 0.4, 0.6, 0.9 mA – to the animals. In both wild-type and $CCK_2^{-/-}$ mice, greater intensities produced stronger analgesia (Figure 9 A), yielding a stress-dose curve. To verify the stress-dose curve for the $CCK_2^{-/-}$ animals, a small group of these animals (N=5) received 0.9 mA stress. Baseline tail flick (data not shown) and 0 mA tail flick (animals were placed on the metal grid, but no shocks were delivered) latencies were not different.

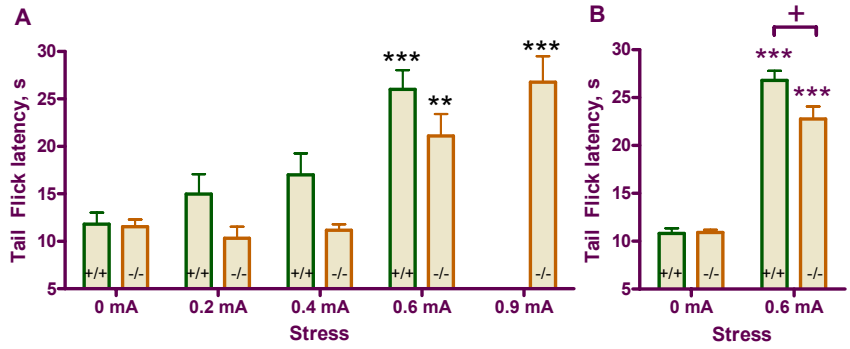

Figure 9. Development of SIA in response to different intensities of stress. (A) Stress was induced by delivering electric foot-shock. Stress induced dose dependently analgesia in both genotypes of mice. 0.6 mA stress intensity was sufficient to produce significant analgesia in both wild-type (bars marked with +/+) and $CCK_2^{-/-}$ (bars with –/–) mice. (B) $CCK_2^{-/-}$ mice develop smaller analgesia in response to 0.6 mA footshock when combining the tail flick latencies from 3 experiments. Stars represent Tukey *post-hoc* comparisons with 0 mA stress. **p<0.01, ***p<0.001. Cross represents a Tukey *post-hoc* genotype comparison. +p<0.05.

0.6 mA stress produced significant analgesia in both wild-type and $CCK_2^{-/-}$ animals. $CCK_2^{-/-}$ animals developed SIA at a slower rate, compared to wild-type littermates (excluding the 0.9 mA group, genotype main effect $F(1, 66)=16.7$, $p<0.001$, N=6–12), although the mean values of the wild-type and the $CCK_2^{-/-}$ group were not statistically different (according to Tukey *post-hoc*) at any single stress level (0.2, 0.4 and 0.6 mA). 0.6 mA stress was sufficient to produce SIA in both genotypes of mice and this stressor intensity was used in the following experiments.

To further analyse the question whether stress induced lower analgesia in $CCK_2^{-/-}$ mice we combined all the data from current experiment and from

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subsequent experiments (injection of CB_1 antagonist rimonabant and opioid antagonist naloxone, followed by measurement of SIA) for the comparison of tail flick responses in stressed (0.6 mA) and non-stressed animals (Figure 9 B). Two-way ANOVA showed a significant interaction (genotype x stress) $F(1, 112)=4.7$, $p<0.05$. *Post-hoc* analysis confirmed the genotype difference at the stress level of 0.6 mA ($p<0.05$, Tukey).

The effect of CB_1 antagonist rimonabant to the development of SIA

Administration of CB_1 antagonist rimonabant (0.1, 1, 3 mg/kg) dose-dependently blocked the development of analgesia in wild-type mice after application of stress (Figure 10). In contrast, rimonabant did not affect SIA in $CCK_2^{-/-}$ animals. Two-way ANOVA revealed a significant interaction between genotype and drug dose ($F(3, 77)=6.8$, $p<0.001$, N=8–12).

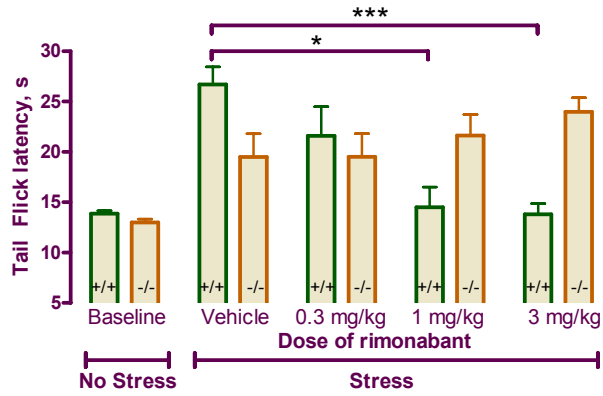

Figure 10. The effect of CB_1 antagonist rimonabant to the development of SIA. Development of SIA is depicted with bars marked with "Stress". Rimonabant blocked the development of SIA in wild-type animals (bars marked with +/+). The $CCK_2^{-/-}$ animals (bars with –/–) were insensitive to rimonabant treatment, because their SIA was not altered. Stars represent Tukey *post-hoc* comparisons with vehicle injection. *p<0.05, ***p<0.001.

The effect of opioid antagonist naloxone to the development of SIA

Administration of opioid antagonist naloxone (0.01, 0.1, 1, 10 mg/kg) antagonised the development of SIA (Figure 11) in both wild-type and $CCK_2^{-/-}$ animals (2-way ANOVA dose main effect $F(4, 87)=9.9$, $p<0.001$, N=8–10).

Administration of naloxone induced a U-shaped response curve in both genotypes, showing that both lower and higher doses of naloxone were ineffective against SIA. For wild-type mice, 0.1 mg/kg of naloxone exerted maximal anti-analgesic effect, but for $CCK_2^{-/-}$ animals, both 0.1 and 1 mg/kg of opioid antagonist significantly antagonised the development of SIA (2-way ANOVA genotype main effect $F(1, 87)=5.1$, $p<0.05$).

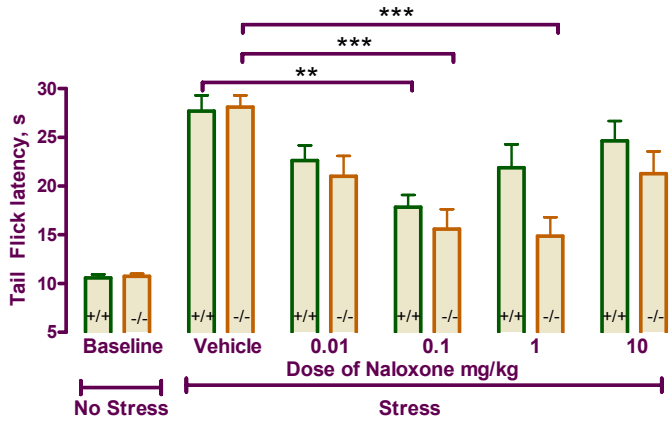

Figure 11. The effect of naloxone to the development of SIA. Naloxone antagonised the development of SIA in both wild-type (bars with +/+) and $CCK_2^{-/-}$ (bars with –/–) animals. Stars represent comparisons with vehicle group. **p<0.01; ***p<0.001, Tukey *post-hoc*.

CCK and CCK_2 gene expression after induction of SIA

We also performed gene expression study to evaluate gene expression changes in lumbar spinal cord, brainstem, midbrain, striatum and mesolimbic area after application of stress to the wild-type and $CCK_2^{-/-}$ animals. In wild-type animals, stress induced upregulation of CCK-related genes in two CNS areas: the lumbar spinal cord and mesolimbic area (Figure 12). In both of these structures, we established an increase of CCK_2 receptor mRNA ($p<0.001$ and $p<0.01$, Figure 12 A, B, respectively, T-test for independent samples). In addition, CCK mRNA was upregulated in the mesolimbic area (Figure 12 C). In $CCK_2^{-/-}$ animals, there was similar mesolimbic upregulation of CCK (ANOVA stress main effect $F(1, 20)=17.0$, $p<0.001$, $N=6$). See Appendix 1 for the exact gene expression numerical and SEM values.

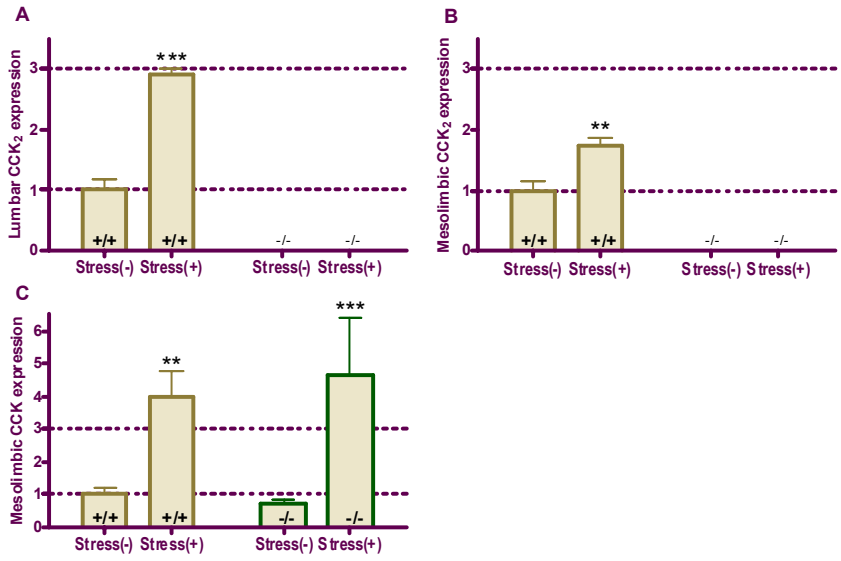

Figure 12. Stress-induced changes in gene expression levels for CCK and CCK_2 receptor in lumbar spinal cord and mesolimbic area. Relative expression levels were measured before (Stress(–)) and after (Stress(+)) application of stress in wild-type (bars designated with +/+) and $CCK_2^{-/-}$ (bars with –/–) mice. Stress resulted in upregulation of CCK_2 gene in wild-type mice in both lumbar (A) and mesolimbic (B) regions. In addition, stress induced strong upregulation of CCK in mesolimbic area in both wild-type and $CCK_2^{-/-}$ animals (C). Stars represent stress-induced effects, i.e. comparisons with respective non-stress (Stress(–)) group. **p<0.01, ***p<0.001, Tukey *post-hoc*.

Endocannabinoid peptide gene expression after induction of SIA

Stress induced a strong activation of endocannabinoid system in two CNS areas: the lumbar spinal cord and mesolimbic area (Figure 13).

First, in both of these structures we found a CB_1 receptor mRNA up-regulation in response to stress (ANOVA stress main effect $F(1, 20)=19.1$, $p<0.001$ for the lumbar (Figure 13 A) and $F(1, 20)=11.4$, $p<0.01$ for mesolimbic area (Figure 13 B)). However, the stress-induced upregulation was significant only in wild-type mice (wild-type non-stressed versus stressed *post-hoc* comparison $p<0.01$ for both structures).

Second, there was a stress-induced upregulation of 2-AG synthesising enzyme DAGLa in the lumbar section of spinal cord (Figure 13 C) in wild-type mice (interaction between genotype and stress $F(1, 20)=5.1$, $p<0.05$, *post-hoc* Tukey $p<0.001$). For the $CCK_2^{-/-}$ mice, the basal expression level of the DAGLa gene was higher (wild-type baseline versus $CCK_2^{-/-}$ baseline *post-hoc* comparison, $p<0.01$, Appendix 1), indicating higher basal 2-AG synthesis in these animals.

Third, anandamide synthesising enzyme NAPE-PLD was upregulated after induction of stress in the mesolimbic area (Figure 13 D, ANOVA stress main effect $F(1, 20)=15.5$, $p<0.001$). Again, as in the case of DAGLa, this stress related activation was evident only in case of wild-type mice (wild-type non-stressed versus stressed *post-hoc* comparison, $p<0.05$).

Finally, 2-AG degrading enzyme MAGL was upregulated after the induction of stress in the mesolimbic area (Figure 13 E, ANOVA stress main effect $F(1, 20)=12.1$, $p<0.01$). This effect was significant only in $CCK_2^{-/-}$ animals ($CCK_2^{-/-}$ non-stressed versus stressed *post-hoc* comparison, $p<0.05$).

See Appendix 1 for the exact gene expression numerical and SEM values.

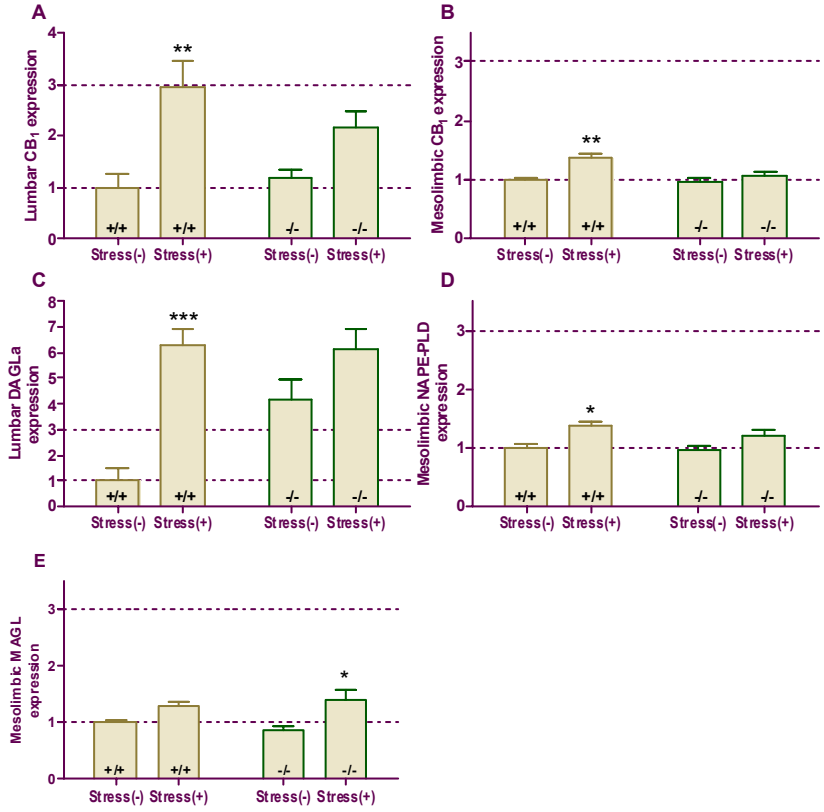

Figure 13. Stress-induced changes in gene expression levels for CB_1 receptor, 2-AG synthesizing enzyme DAGLa, anandamide synthesizing enzyme NAPE-PLD and 2-AG degrading enzyme MAGL in lumbar spinal cord and mesolimbic area. Relative expression levels were measured before (Stress(–)) and after (Stress(+)) application of stress in wild-type (bars designated with +/+) and $CCK_2^{-/-}$ (bars with –/–) mice. Induction of stress upregulated several genes in wild-type mice: CB_1 receptor in lumbar (A) and mesolimbic (B) areas, DAGLa in lumbar area (C) and NAPE-PLD in mesolimbic area (D). Stress induced mesolimbic upregulation of MAGL in $CCK_2^{-/-}$ mice (E). Stars represent stress-induced effects, i.e. comparisons with respective non-stress (Stress(–)) group. *p<0.05, **p<0.01, ***p<0.001, Tukey *post-hoc*.

Opioid peptide gene expression after induction of SIA

Stress induced upregulation of Oprm and Oprd receptor genes in the two above-mentioned CNS regions: the lumbar spinal cord and mesolimbic area. In addition, we established Oprm upregulation in the striatum in response to stress. These effects were mostly established in wild-type animals, but not in genetically modified mice. Oprm receptor was upregulated in both genotypes in the lumbar spinal cord in response to stress (Figure 14 A, ANOVA stress main effect $F(1, 20)=20.7$, $p<0.001$). $CCK_2^{-/-}$ mice expressed lower basal levels of Oprm mRNA in the lumbar spinal cord (*post-hoc* comparison between non-stressed wild-type and $CCK_2^{-/-}$, $p<0.05$, Appendix 1).

We established an upregulation of the Oprm receptor gene in the striatum (Figure 14 C) in response to stress (ANOVA stress main effect $F(1, 20)=11.6$, $p<0.01$), although the stress effect was significant only in wild-type mice (wild-type non-stressed versus stressed *post-hoc* comparison $p<0.05$).

Finally, we found an upregulation of the Oprd receptor mRNA in the mesolimbic area (Figure 14 B) in response to stress. In this case, there was a significant interaction between genotype and stress (ANOVA $F(1, 20)=5.4$, $p<0.05$), because only wild-type mice responded with gene upregulation.

See Appendix 1 for the exact gene expression numerical and SEM values.

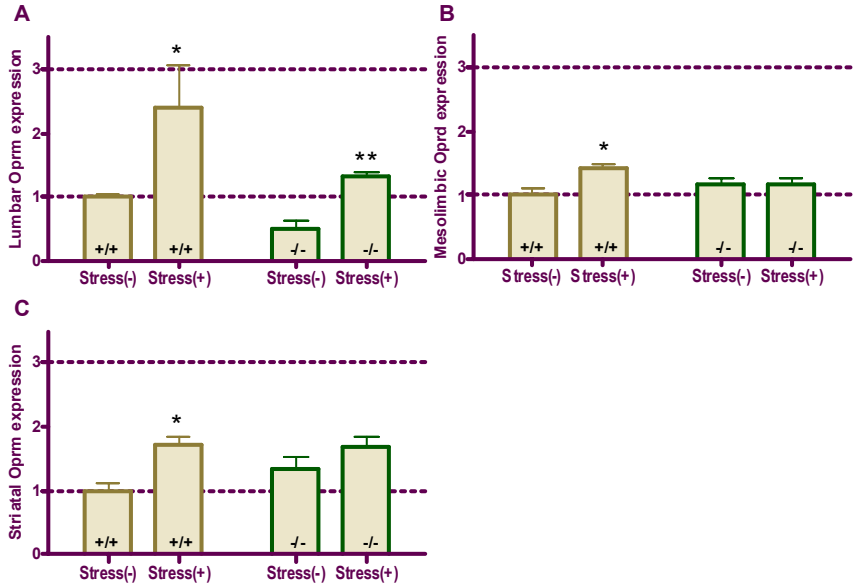

Figure 14. Stress-induced changes in expression of opioid receptors Oprm and Oprd. Stress induced increase in Oprm expression in lumbar (A) and striatal (C) regions and increase in Oprd in mesolimbic area (B) in wild-type mice (bars with +/+). Stress condition is depicted with Stress(+). Only one of these changes was also seen in $CCK_2^{-/-}$ animals (bars with –/–), Oprm increase in lumbar area (A). Stars represent stress-induced effects, i.e. comparisons with respective non-stress (Stress(–)) group. *p<0.05, **p<0.01, Tukey *post-hoc*.

Paper 4

In the final study we analysed the data from the Paper 2 (mouse gene microarray data) from entirely different perspective. We did not look at the neuropathic phenotype of the $CCK_2^{-/-}$ animals but focused only on the genotype main effects and analysed the genetic background of the mutant mouse line.

The data for the analysis are exactly the same as described in Paper 2. We did a conservative analysis of the samples (32 samples, divided to 8 different groups, N=4 per group: CCI wild-type midbrain, CCI knockout midbrain, sham wild-type midbrain, sham knockout midbrain, CCI wild-type medulla, CCI knockout medulla, sham wild-type medulla, sham knockout medulla). We treated the two tissues (midbrain and medulla) as replicate samples and every probeset as a separate test.

We found 15 probesets with very strong evidence for an expression difference between $CCK_2^{-/-}$ and wild-type mice, one of which represents CCK_2 itself. Across the entire array, the false discovery rate for these 15 is estimated at <0.05 (FDR, (Benjamini & Hochberg, 1995; Storey & Tibshirani, 2003; Storey *et al.*, 2004), these methods give the same result in this case). 12 of the probesets, representing 10 different genes in addition to CCK_2, map in a 40 Mbp region around the CCK_2 locus on chromosome 7 (Appendix 3, the figure was generated using Webgestalt (Zhang *et al.*, 2005)). All but one differed in the same direction, knockout-low (Appendix 4).

To further analyse the region around the CCK_2 locus we plotted the p-values for difference of chromosome 7 probesets against their position on the chromosome (Figure 15). We found a distinctive chromosome region of differing gene expression shows a cloud of values above 0.1 between 73 and 123 Mb (indicated in pale blue).

We next included only chromosome 7 into the analysis, again using FDR Q=0.05. Using this less conservative criterion we found 33 probesets in the 40 Mb region that differed significantly and only one in the remainder of the chromosome.

Hypothesizing that these expression differences were in ES-cell-derived sequences linked to the knockout allele (i.e. a differential chromosomal segment), we genotyped 6 informative SNPs outside of genes flanking CCK_2 (Figure 15, bottom). These confirmed that the region is indeed of non-C57BL/6 origin in the knockout strain. The three SNPs on the proximal side (black triangles in Figure 15) are all homozygous 129 genotype in knockout animals. For the distal three SNPs (grey in Figure 15), this is also true, except that three of the 16 knockout DNAs show a C57BL/6 genotype, indicating that there are different congenic breakpoints represented in our set of mice.

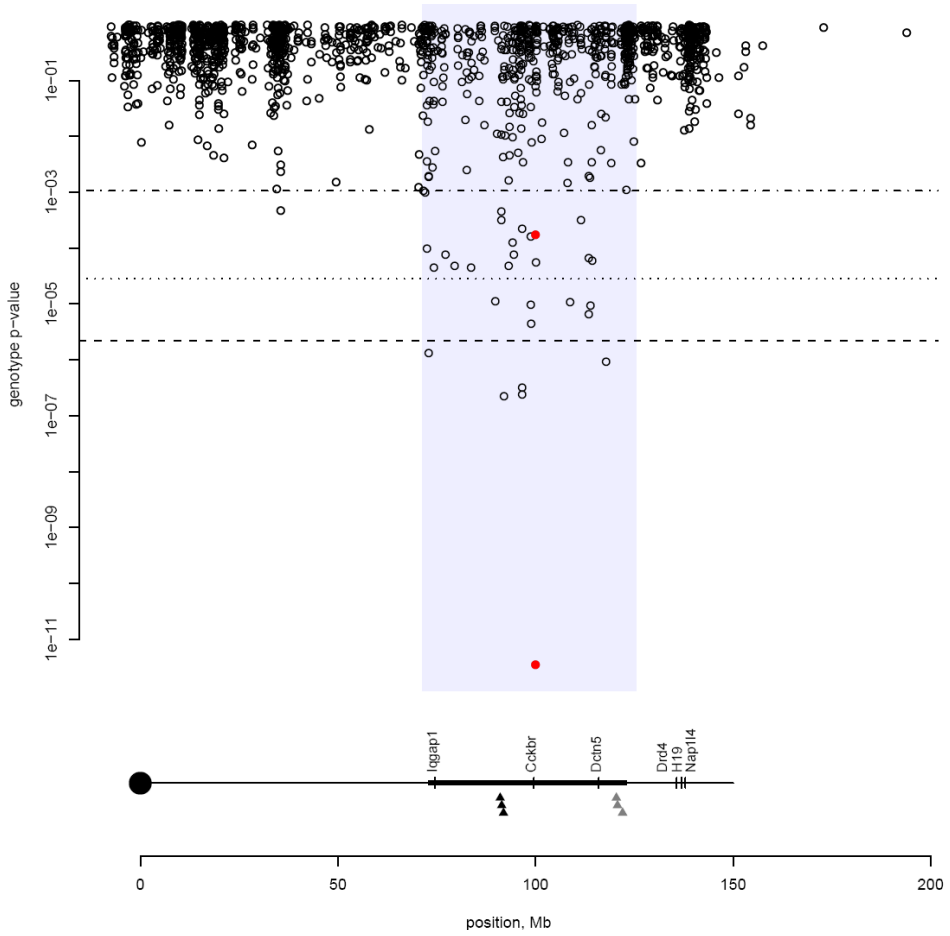

Figure 15. The congenic footprint: mouse chromosome 7. Upper: uncorrected p-values for genotype effect plotted by location (Mbp) on chromosome 7. The two red filled points are CCK_2 probesets. The three lines are significance thresholds with differing multiple-testing criteria (α=0.05). Dashed: Bonferroni corrected (whole genome); dotted: FDR (whole genome); dot-dashed: FDR (chromosome 7). The shaded area indicates the footprint region. Lower: map of chromosome 7 on the same scale. The heavier line represents the congenic footprint, and the filled triangles are the locations of the six SNP markers. The grey triangles indicate that there were a few $CCK_2^{-/-}$ animals with C57BL/6 alleles at these loci, indicating the presence of more than one breakpoint. All positions are from NCBI sequence build 34.

The ten genes that most strongly define the differentially expressed region around CCK_2 are Dctn5 (dynactin 5, actin-associated, 1415748_a_at), Coq7 (demethyl-Q 7, ubiquinone biosynthesis, 1416665_at), Trim30 (tripartite motif, T-lymphocyte regulatory, 1417961_a_at), Trim34(1424857_a_at), Myo7a (myosin VIIa, 1421385_a_at), Arl6ip1 (ADP-ribosylation factor-like 6 interacting protein 1, 1423819_s_at), Spon1 (spondin 1, extracellular matrix, 1424415_s_at), Ndufc2 (NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 1455036_s_at), Iqgap1 (IQ motif containing GTPase activating protein 1, 1417379_at) and Frag1 (FGF receptor activating 1, 1424615_at, 1424614_at).

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DISCUSSION

In general, the present thesis has described pain-related phenotype of the $CCK_2^{-/-}$ mice and studied endogenous analgesic mechanisms in relation to the neuropeptide CCK, using a transgenic tool to disrupt the transmission of neuronal CCK. Additionally, we draw attention to some methodological aspects of producing and using transgenic animals as a behavioural tool for revealing a function of a single gene.

Role of CCK in neuropathic pain

Mechanical sensitivity of the $CCK_2^{-/-}$ animals

In the first part we showed that the $CCK_2^{-/-}$ mice displayed reduced mechanical sensitivity compared to their wild-type littermates. This finding suggests that CCK may contribute to the regulation of mechanical sensitivity, as the antagonist of CCK_2 receptors elicited similar effect in the wild-type animals. The most plausible explanation to this phenotype is upregulation of opioid system in the $CCK_2^{-/-}$ animals (Pommier *et al.*, 2002) and resulting opioid tone which results in hypoalgesic phenotype in these animals. This is supported by the effect of naloxone which modified (i.e. counteracted hypoalgesia) the levels of mechanical sensitivity only in transgenic mice and not in wild-type animals. Interestingly, similar results have been noted before, although in different methodological settings, using model of chronic pain. In this work the authors differentially selected rats that did or did not develop hypersensitivity after spinal cord lesion (Xu *et al.*, 1994). Systemic naloxone, in rats that did not develop hypersensitivity, induced typical allodynia (i.e. modified their mechanical sensitivity), while it failed to modify the mechanical sensitivity in normal, not operated animals. It is thus likely that neuropathic pain induced tonic opioidergic transmission and blocking that tone modified behavioural response. The $CCK_2^{-/-}$ animas in our experiment resemble these animals in this regard that their opioid tone is also elevated.

Considering the opioid antagonist's pharmacological profile and selectivity towards each opioid receptor (Marchand *et al.*, 2003) we can not conclude which receptor subtype is involved. However, we can hypothesise that the baseline tonic activity of endogenous opioid transmission seems to be higher in $CCK_2^{-/-}$ mice than in wild-type littermates. Administering naloxone eliminated the tonic (higher) transmission of endogenous opioids in the $CCK_2^{-/-}$ animals.

Neuropathic pain phenotype of the $CCK_2^{-/-}$ animals

Our main goal was to study the involvement of CCK in pain mechanisms. Interaction of CCK with opioids and their contribution to chronic pain has been soundly confirmed (see Wiesenfeld-Hallin *et al.*, 1999 for a review). Our central result is establishment that the CCK_2 receptor deficient animals did not develop chronic hypersensitivity after ligation of sciatic nerve. This is in good agreement with previous works, regarding the established role of CCK in chronic pain mechanisms (Noble & Roques, 1999).

However, statistically, the absent or smaller-scale hypersensitivity of the $CCK_2^{-/-}$ animals is difficult to establish. Although one can easily see the difference between wild-type and knockout animals, there are two aspects that hinder statistical analysis. First, the existent difference in baseline level of mechanical sensitivity. From mathematical point of view, there is a clear hypersensitivity in the wild-type animals, as their scores drop from about 0.7 to 0.2 g. Yet, the mean sensitivity of the knockout animals also shifts somewhat (from about 1.2 to 1.0 g). We can not straightforwardly state that the change is larger for the wild-type animals, as the baseline starting points were also different for the groups. Secondly, as mentioned before, the postoperative change in mechanical sensitivity was in the same direction in both genotypes and thus, according to the repeated-measures ANOVA interaction (day x genotype – surgery) we can not state a significant difference. This is because a few of the $CCK_2^{-/-}$ animals (as opposed to most of the wild-type animals) displayed hypersensitivity on each postoperative day. Consequently, the resulting mean values were a bit lower from the preoperative levels and caused the same direction shift, as in the wild-type animals and thus, the quantitative approach could not differentiate between large hypersensitivity of the wild-type animals and smaller hypersensitivity of the $CCK_2^{-/-}$ animals.

Nevertheless, we observed one aspect of the behaviour that very clearly differentiated the genotypes – the number of animals that developed hyper-sensitivity. Accordingly, we decided to perform an additional statistical analysis, solely based on that parameter. Specifically, for each animal we quantified postoperative days when the sensitivity could be classified as "hypersensitive" (1="hypersensitive" or 0="not-hypersensitive"). Using this variable eliminated the two hindering aspects, explained previously; the preoperative baselines are no more used, only the change ("postoperative" – "preoperative") is assessed qualitatively.

Not surprisingly, using this analysis, the difference between the genotypes is clear. Wild-type animals displayed hypersensitivity in most of the postoperative days (on average, 7 days out of 8), as opposed to much lower number for the knockout animals (2 days out of 8).

These results are in good agreement with previous works, which have shown that CCK, through CCK_2 receptor inhibits endogenous opioid system (Noble & Roques, 1999), (Figure 16) and CCK_2 antagonist, in naloxone reversible

manner, effectively relieved allodynia-like symptoms in spinally injured rats (Xu *et al.*, 1994). The interaction between CCK and opioids is often referred to in the literature as "CCK being the functional antagonist of the opioids". First, it has been shown that activation of CCK_1 receptors potentiates the analgesic effects of opioids (Noble *et al.*, 1992), while CCK_2 receptor activation negatively modulates the opioidergic system (Noble & Roques, 1999). Second, the opioid system appears also to regulate the release of CCK peptides. Thus, the stimulation of μ opioid receptors has an inhibitory and stimulation of δ opioid receptors has excitatory influence on the K^+-evoked release of CCK-like material at spinal and supraspinal levels (Benoliel *et al.*, 1991; Benoliel *et al.*, 1992). These effects are summarised in Figure 16 (adapted from Noble & Roques, 1999). Numerous studies have shown that peripherally administered CCK_2 receptor antagonists potentiate opioid antinociceptive responses (see Roques & Noble, 1996 for a review). It is likely that neuropathic pain induces higher CCK transmission (and consequent inhibition of opioid tone) (Xu *et al.*, 1993) and dysfunction of this control by the disrupted endogenous CCK system also disrupts the development of painful sensations in these animals. In our case, the inhibitory element of the chain (CCK_2 receptor) is missing (Figure 17) and therefore, the baseline "opioid tone" is not inhibited or may even be escalated due to the remaining CCK_1 receptor. The higher opioid tone before the surgery in turn, has a protective effect against developing hypersensitivity postoperatively (Figure 17).

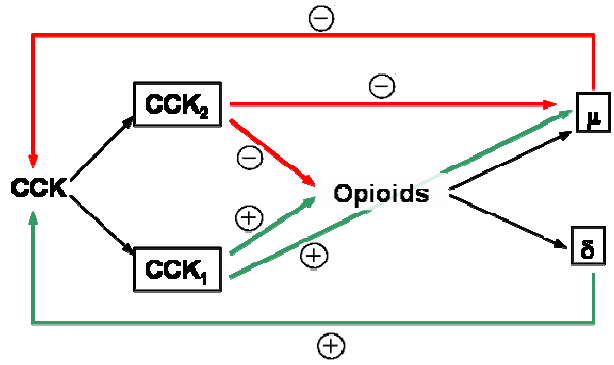

Figure 16. Interactions between CCK and opioids. Adapted from Noble F, et al. 1999. Hypothetical model, describing interactions between CCK and opioid systems. CCK exerts its action through its receptors CCK_1 and CCK_2. The activation of the CCK receptors modulates the opioid system directly (affecting the activity of opioid receptors) or indirectly, modifying the levels of opioid peptides. The opioid system in turn, modulates the activity of the CCK system, where μ-opioid activation inhibits and δ-receptor activation enhances the levels of CCK. Inhibiting modulation is presented with "minus"-signs and red lines; "plus"-signs and green lines depict enhancing modulation.

Changes in opioid levels in the $CCK_2^{-/-}$ animals have been shown also in previous studies. Increase in extracellular amount of endogenous opioids with *in vivo* binding and decreased binding affinity with membrane binding have been established in the $CCK_2^{-/-}$ animals (Pommier *et al.*, 2002; Veraksits *et al.*, 2003). However, these studies used whole brain (Pommier *et al.*, 2002) or parts of forebrain (cerebral cortex, hippocampus, striatum and mesolimbic area) (Veraksits *et al.*, 2003) in their binding studies and thus, these results may not be most informative from our perspective. Nevertheless, it is plausible to assume that changes in opioid levels have occurred also in other CNS areas, such as spinal cord, RVM or PAG, which would be most interesting in explaining pain mechanisms and pain modulation.

Therefore, we attempted to find changes in the expression of opioid receptors or endogenous μ-opioid ligand in spinal cord – the lowest-level modulation site, where changes in opioid and CCK transmission may regulate sensory ascension. This was studied in the first paper (Paper 1), using a specific set of candidate genes and real-time PCR. In the subsequent study we tremendously expanded our target and, as a separate experiment, measured gene expression levels in brainstem and medulla, using gene expression microarrays and scanning through the whole transcriptome (Paper 2).

Gene expression changes in lumbar spinal cord after induction of neuropathic pain

Gene expression analysis at lumbar level showed some surgery and genotype effects, surgery x genotype interactions and in some cases, no effects. The most important opioid-related gene effect was a genotype x surgery interaction for the POMC mRNA, where induction of neuropathy resulted in strikingly opposite-direction changes in POMC expression in wild-type and $CCK_2^{-/-}$ animals. Surgery induced reduction of the POMC levels in wild-type (lowering of the opioid tone), but increase in the $CCK_2^{-/-}$ mice (further increase in endopioid tone).

Opioid receptors Oprd and Oprk

In case of the opioid receptor transcripts Oprd and Oprk we saw a genotype effect, the $CCK_2^{-/-}$ animals expressing higher levels of the receptors, regardless of the surgery. The Oprd and Oprk genotype effect and the striking interaction of POMC expression fit well in view of the neuropathic pain phenotype of the $CCK_2^{-/-}$ animals and with previous results that the $CCK_2^{-/-}$ mice have an upregulation of opioid system (Pommier *et al.*, 2002). It is also in good agreement with the model proposed by Noble (1999), (Figure 16), where invalidation of the CCK_2 receptors results in disinhibition of the endogenous opioids (Figure 17). Downregulation of the POMC in wild-type animals with neuropathic pain may be one of the spinal mechanisms producing allodynia or

hyperalgesia, the symptoms also often seen in humans. Thus, increase of POMC expression in the $CCK_2^{-/-}$ animals in neuropathic pain conditions may underlie the markedly decreased development of hypersensitivity in these mice. Alternatively, preoperatively higher levels of opioid receptor δ and κ may counteract the development of chronic pain symptoms. Lack of the regulatory signal from the CCK, through CCK_2 receptors apparently induces radical changes in opioid receptor levels (as is in case of Oprd and Oprk) and natural opioid response (as is in case of POMC expression in response to neuropathy).

There is some discrepancy in previous works about opioid peptide levels after nerve injury. Some have shown that the level of opioid peptides is little changed in the dorsal horn following peripheral nerve injury (Hökfelt *et al.*, 1997). Our results are in good agreement with other works that have shown decrease of beta-endorphin, an opioid peptide product of POMC, following peripheral nerve injury in rats in several brain areas, bilaterally (Panerai *et al.*, 1988). In yet another study, rapid and sustained up-regulation of preprodynorphin mRNA was found, following CCI (Draisci *et al.*, 1991). It may be that the levels of the opioid peptides depend on the nerve injury model used.

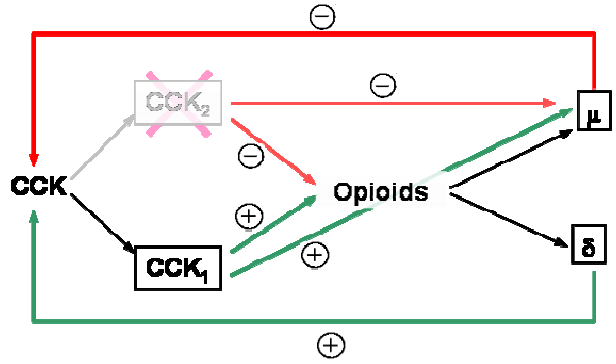

Figure 17. Interactions between CCK and opioids. The $CCK_2^{-/-}$ mice display upregulation of the opioid system. Current figure demonstrates that eliminating the signal from the CCK_2 receptor not only abolishes the inhibitory effect to the opioids, but enhances pro-opioid signal through the CCK_1 receptor. Inhibiting modulation is presented with "minus"-signs and red lines; "plus"-signs and green lines depict enhancing modulation.

Opioid receptor Oprm

Surprisingly, we did not find surgery or genotype effects for the μ receptors. One of the disadvantages of gene expression analysis via qRT-PCR is that this way we can not visualise regional distribution, as in case of in-situ hybridisation, immunohistochemistry or autoradiographic approach (although the qRT-PCR has excellent quantitative properties and allows to measure many genes). Thus we can not differentiate between ipsi- and contralateral expression

and this may also be the reason for different results, compared to the "histological" approach. Previously conducted autoradiographic binding studies have found that peripheral nerve injury induces changes in the level of opioid receptors in the dorsal horn of the spinal cord. Models based on axotomy have described decrease of the opioid receptors (possibly due to the direct decrease of nerve endings) (Fields *et al.*, 1980; Besse *et al.*, 1990; Besse *et al.*, 1992). Similar results have also been confirmed both by immunohistochemistry and in situ hybridization showing a downregulation of Oprm in rat and monkey DRG neurons and in the dorsal horn after complete or partial sciatic nerve injury (Zhang *et al.*, 1998).

Models involving similar Bennett's CCI, as was used in current study have described more complex changes (Stevens *et al.*, 1991). Thus, Oprm levels were increased bilaterally during the first postoperative days in laminae V and X and ipsilaterally in laminae I–II, but subsequently, over the first 10 postoperative days the binding declined towards control level in rats with CCI. Taking into account that we quantified over the whole lumbar spinal cord (not only the laminae I–X and not only the ipsilateral side) and this way diluted our signal from the region of interest, our signal might have been indistinguishable from the control level at the 9th postoperative day.

CCK, CCK_1 and CCK_2 receptors

We also did not find any surgery effects for the CCK. Upregulation of CCK transmission has been associated with chronic pain situations, although some controversy exists between different laboratories. The controversy may be due to the differences in chronic pain models (axotomy or spinal nerve ligation), methods of estimating the levels of CCK (ISH, IHC, and autoradiography) or in anatomical regions of interest (DRG or dorsal horn). Thus, peripheral nerve section has been associated with elevated CCK mRNA in the ipsilateral DRG (Verge *et al.*, 1993; Xu *et al.*, 1993). However no increase in peptide immuno-reactivity was observed, suggesting that increased mRNA might not be translated into peptide. In another study, the tissue levels and *in vitro* release of CCK were unchanged in the dorsal quadrant of the lumbar spinal cord on the ipsilateral side in axotomised rats (Antunes Bras *et al.*, 1999). In addition, measurements in DRG at L4-L6 levels revealed no significant changes in CCK mRNA after the lesion. Our results also did not find differences in expression of CCK in lumbar segments and therefore, it is likely that the enhancement of the CCK transmission is exerted through its receptors.

Upregulation of the CCK_2 receptor mRNA has been described in DRG neurons after peripheral nerve injury (Zhang *et al.*, 1993). This was supported by a marked increase in the autoradiographic labelling of CCK_2 receptors in the superficial layers of the lumbar dorsal horn (Antunes Bras *et al.*, 1999). Unfortunately, we do not have data about CCK_2 receptor expression levels in wild-type mice. Interestingly, our results showed significant decrease in expression of CCK_1 receptor in wild-type mice. In view of the model of

CCK-opioid interactions (Figure 16), downregulation of the CCK_1 receptors results in decrease in pro-opioid signals and may also be one of the factors enhancing development of hypersensitivity. To our knowledge, involvement of CCK_1 in neuropathic pain mechanisms has not been studied and possible biological significance of this can not be ruled out and has to be investigated in the future studies. In addition, the $CCK_2^{-/-}$ animals displayed higher basal expression of the CCK_1, compared to the wild-type animals. Again, this fits well with the upregulated opioid system in the transgenic mice (Pommier *et al.*, 2002), due to the higher pro-opioid signalling through the CCK_1 (Figure 17).

Taken together the lumbar gene expression results, when comparing the expression profiles of CCK_1 and POMC genes, both of them being opioid-enhancing elements, one can easily see similarities both in terms of the surgery effects (direction of changes in the gene expression after the CCI) and genotype effects (supporting previously risen hypothesis about the higher opioid tone in the $CCK_2^{-/-}$ animals). For both of the genes we saw similar statistical gene x surgery interaction, where the expression level decreases with the CCI in wild-type animals and the opposite is true for the $CCK_2^{-/-}$ animals. Thus, it seems that neuropathic pain due to peripheral nerve damage is accompanied with profound neurochemical changes at the spinal cord level. These changes may be caused by painful stimuli arising from the nerve damage or these changes themselves may be the cause of the well known symptoms of chronic pain and also may very well underlie the insensitivity to opioid treatment phenomenon of the neuropathic pain syndromes (Portenoy *et al.*, 1990).

Our next question was – does peripheral nerve damage also induce changes in higher CNS regions? Rostroventral medulla and periaqueductal gray are well known pain-modulating centres, sending descending inhibitory and facilitatory signals to the dorsal horn of the spinal cord. The activity of these regions has been numerously associated with development and persistence of chronic pain (Pertovaara *et al.*, 1996; Ossipov *et al.*, 2000; Mason, 2001; Zhang *et al.*, 2009). Therefore, we used the same model of peripheral nerve injury as in the first part of the study and assessed changes in gene expression in brainstem and midbrain due to the nerve injury, and again, in relation to the CCK system, using the $CCK_2^{-/-}$ animals as a tool to disrupt major part of CCK within CNS.

We also expanded our target in two ways. First, we did not use a predefined set of candidate target genes, but a mouse genechip, a tool to estimate a "gene expression profile" of the whole transcriptome. This way we had an opportunity to not only measure the expression levels of genes of interest (like opioid- and CCK-related genes), but acquire information about possible new targets of chronic pain related genes. Secondly, all gene expression levels were measured individually, i.e. tissue pooling was no longer used. This way we could also take into account individual variability and although the approach is much more expensive in terms of time and expendables, knowing individual variability for a given gene is the only way of learning if the measured effect is really biologically significant. For example, the gene expression analysis in lumbar

spinal cord (Paper 1) yielded several notable effects, like downregulation of POMC in response to the CCI in wild-type mice. Although similar effect has already been previously reported and the result fits well with the general picture (and probably represents a real effect), the noted effect in our result list may not actually be biologically significant, because we pooled the tissues of several animals and thus, acquired a mean expression level of several animals. We obtained knowledge about differences between mean values of expression of sham-operated and neuropathic animals but we do not know the actual variability of the POMC expression of the sham-operated and neuropathic animals. Therefore, individual approach in estimating gene expression levels was used in all subsequent works.

Gene expression changes in medulla oblongata and midbrain after induction of neuropathic pain

As mentioned, the importance of supraspinal modulation on the development of a neuropathic syndrome has been discovered previously (Vanegas & Schaible, 2004). Supraspinal descending facilitation may be responsible for the development and maintenance of hypersensitivity and the role of CCK in the control of descending facilitation has been proposed (Kovelowski *et al.*, 2000). CCK also exerts an anti-opioid action in both midbrain and medullary structures interacting with CCK_2 receptor (Heinricher *et al.*, 2001). Therefore, targeting the CCK_2 gene may induce gene expression changes in midbrain and medulla that are of relevance to the development of neuropathic pain.

Genotype effects

The strongest effects on gene expression observed in our study were due to genotype (changes induced by the deletion of CCK_2 gene). Evidence for a "congenic footprint" was found in genes flanking the CCK_2 locus (Paper 4). Most of the changed genes are located in this footprint area and probably reflect the flanking allele effect. This finding is fully addressed in the Paper 4 of current thesis. In short, it suggests that it is still important to consider flanking allele effects even after extensive backcrossing. Theoretically, it is possible that any of the genes within the footprint region could be responsible for aspects of the "knockout" phenotype (Gerlai, 1996).

In addition to the congenic footprint we found significant upregulation of Cldn5, Tlr4 and Prkcz in $CCK_2^{-/-}$ mice. Claudin 5 (Cldn5) is tight-junction protein involved in blood brain barrier formation (Nitta *et al.*, 2003; Amasheh *et al.*, 2005). A recent, family based association study suggested involvement of Cldn5 in the development of schizophrenia (Sun *et al.*, 2004). Interestingly, CCK and its receptor genes are also associated with vulnerability to schizophrenia (Wei & Hemmings, 1999; Sanjuan *et al.*, 2004). However, the

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functional significance of increased Cldn5 expression in $CCK_2^{-/-}$ mice needs further investigation.

The other gene up-regulated in the $CCK_2^{-/-}$ mice was toll-like receptor 4 (Tlr4). Tlr4 is a very interesting candidate in relation to the neuropathic pain and inflammatory response as it is a lipopolysaccharide (LPS)-recognizing gene (LPS-receptor) and plays a role in the regulation of innate immunity (Poltorak *et al.*, 1998). For example, a C57 substrain (C57BL/10ScCr, now designated as C57BL/10ScNJ) has a spontaneous deletion of Tlr4 gene and these mice do not respond to LPS stimulation. A recent study showed that Tlr4 triggers CNS microglial activation with subsequent expression of cytokines during induction of a neuropathic syndrome (Tanga *et al.*, 2005). Genetic deletion or antisense suppression of Tlr4 expression attenuates painful neuropathy (Tanga *et al.*, 2005). Thus, upregulation of Tlr4 in $CCK_2^{-/-}$ mice may have significant functional relevance in the development of allodynia and could potentially account for the lower hypersensitivity in the CCI model. To extend our findings from the microarray data in midbrain and medulla samples, we also analyzed Tlr4 expression in the lumbar region in the same animals using real-time PCR. We found that CCI induced Tlr4 upregulation in the lumbar region of wild-type mice (Figure 8), and this is in agreement with previously published data (Tanga *et al.*, 2005). CCI did not change the basal Tlr4 expression level in the lumbar region of the $CCK_2^{-/-}$ mice. However, the expression profile of Tlr4 in the lumbar region was different from the changes seen in the midbrain and medulla. Specifically, Tlr4 was significantly upregulated in the mutant mice and CCI induced downregulation of Tlr4 in mutant mice in the supraspinal regions. Therefore, there appears to be a significant interaction between CCK and Tlr4, but the direction of change depends on the CNS region. We hypothesize that deletion of CCK_2 results in resistance to the microglial activation induced by CCI. A crucial factor in this process is the interaction between CCK_2 and Tlr4, a key regulator of CNS innate immunity. To further study the changed immune response, we also analyzed the expression level of IL1b and found that similarly to the Tlr4, IL1b expression did not change in $CCK_2^{-/-}$ mice after the CCI (Figure 8). Therefore, the immune system of the mutant mice may be unresponsive to certain stimuli due to the deletion of the CCK_2 gene. This is further supported by a recent physiological study that describes lack of LPS-induced sickness behaviour and fever in $CCK_2^{-/-}$ mice (Weiland *et al.*, 2007).

Finally, the third gene altered by the deletion of CCK_2 is Prkcz and its function is directly related to the Tlr4 induced activation of immune response. This further supports the interaction between CCK_2 and Tlr4.

Surgery effects and surgery x genotype interaction

Surprisingly, there were not very strong results for the surgery effect. One might assume that the genotypes (wild-type and $CCK_2^{-/-}$ animals) respond so

differently to the surgery that no general effects can be seen. However, we also did not find any surgery x genotype interaction candidates.

Although we found some gene expression changes after the CCI, the number of altered genes was limited. One simple explanation for the small number of differentially expressed genes is that there may not be very many gene expression changes in higher brain structures induced by CCI.

Of course, the described gene expression profile also depends on the time of the tissue collection (in our case, postoperative 9th day). The expression level may gradually increase during postoperative days for some genes, or gradually decrease after initial strong postoperative activation for some other genes (some examples of such changes have been described above, in the opioid-related lumbar gene expression section). Development of neuropathic pain is related to the initial inflammatory response, due to direct tissue damage and acute reaction to that damage. Subsequently, the acute inflammatory reaction is gradually replaced by more profound, firm and long-lasting changes in gene expression and resulting change in the behaviour of subpopulation of neurons. Thus, some gene expression effects may be part of the initial inflammatory reaction and other due to chronic pain. We chose the 9th postoperative day because the initial inflammatory reaction should be minimal by that time and all changes in gene expression should reflect true chronic pain effects. Most investigators choose the time-point at around 1 week postoperatively.

Considering that we saw only few gene expression effects, one could argue that the surgery did not induce any effects at the brainstem or midbrain level. Although this is a possibility, it is not very plausible. More probable is that we were not able to detect the changes because of high inter-individual variability. There is a possibility that chronic pain syndrome is associated with different possible activation profiles or different activation degrees. We only included 4 animals to each group and thus, to capture statistically significant changes, the expression levels must be quite monotone both pre- and postoperatively. Our experience with gene expression profiling (Paper 3) of the stressed mice clearly demonstrated that experimental manipulation (like inducing neuropathic pain) resulted in quite monotone changes in some cases, but inter-individual variability increased tremendously in case of some genes. In addition, regardless of the variability, number of individuals in group should be more than 4. Therefore, the effects found in the current study are probably the ones that had most clear expression profile (lowest variability) and most clear difference (greatest magnitude) between pre- and postoperative situations.

On the other hand, there are also good points in having fewer false negative results, as the candidates that turned out statistically significant may really be strong mediators in the chronic pain processes.

Genetic networks

We next moved on from single gene effects and looked at the functional groups of genes and analysed how these behaved after the CCI surgery. We used

stringent statistical analysis of the microarray data to minimize false positive findings, assuming that the changes in gene expression are totally independent. However, it is likely that many interactions between genes occur and this conservative approach would be biased against detecting such interactions. Therefore, to explore the effect of CCI and gene deletion on genetic networks, we took an unbiased approach and separately mapped the log2 ratio data for all the genes on the microarray to the functional annotation database. As a result, the most significant transcriptional enrichment induced by surgery was found in the MAPK pathway.

Previous studies have also shown that the MAPK pathway is activated after CCI at the site of injury or at the segmental level of spinal cord, implicating the MAPK pathway in the development of neuropathic pain (Jin *et al.*, 2003; Marchand *et al.*, 2005; Crown *et al.*, 2006). Our results extend these findings to the central nervous system as we found evidence for activation of the MAPK pathway following surgery in both the midbrain and medulla in wild-type mice. The expression of several genes (Appendix 2) involved in MAPK pathway changed following surgery, and the expression level of several of these genes was also dependent on genotype (i.e. had a significant genotype x surgery interaction). Furthermore, the direction of gene expression changes was in the correct functional direction – MAPK pathway activating genes were upregulated and pathway inhibitory genes were downregulated (Appendix 2), thus, supporting the activational role of the MAPK pathway in chronic pain model. Finally, several genes with the most significant difference in expression level have been linked to the activation of the MAPK pathway in other studies. For example, aryl-hydrocarbon receptor (*Ahr*) is a dioxin receptor related to the regulation of immune response and MAPK pathway activation (Rodriguez-Sosa *et al.*, 2005; Weiss *et al.*, 2005). Protein kinase C zeta (Prkcz) is directly involved in the MAPK pathway and in regulation of the immune response. Specifically, Prkcz activation is required for LPS-induced Tlr4-mediated MAPK pathway activation and cytokine production (Cuschieri *et al.*, 2004). Prkcz is particularly interesting as Tlr4 is required for microglial activation and both of the genes were changed in our study. Therefore, we conclude that a model of neuropathic pain, CCI, activates the MAPK pathway and immune response in higher brain structures such as the medulla and midbrain.

Taken together, we saw that induction of neuropathy resulted in specific pattern of gene expression changes in lumbar spinal cord. The general picture of the pattern strongly supports interaction between CCK and opioids in chronic pain mechanisms. We also conducted extensive gene expression profiling in brainstem and midbrain and saw that the CCK_2 receptor may be necessary for the activation of Tlr4, a key regulator of CNS innate immunity, suggesting that the $CCK_2^{-/-}$ mice might have disturbances in inflammatory system.

Role of CCK in endogenous antinociceptive mechanisms

The second part of the thesis concerns with the role of neuropeptide CCK in endogenous analgesic mechanisms, stress-induced analgesia. Investigating SIA is important, because SIA involves basic mechanisms which are able to modify pain input; achieving analgesia selectively via medical means is possible only by using these mechanisms. Thus knowing more about these mechanisms is important for better clinical pain control.

Although involvement of CCK has been shown in different pain conditions, its involvement in SIA has not been studied before. Our hypothesis of possible CCK involvement came from previous histological results, showing extensive cellular colocalisation of CCK and cannabinoid receptor CB_1 in several CNS regions and from other recent results showing involvement of cannabinoids in SIA mechanisms.

Again, we used our tool for studying the CCK system – the CCK_2 receptor deficient mice. We combined the CCK_2 deficiency with pharmacological antagonism of CB_1 and opioid receptors to test whether the impairment of CCK-ergic transmission modified the effect of two SIA components – endocannabinoids and opioid peptides.

SIA phenotype of the $CCK_2^{-/-}$ animals

First, we studied the relation between the intensity of stress and resulting analgesia. We found that stress induced intensity-dependent analgesia in both wild-type and $CCK_2^{-/-}$ mice, but that the $CCK_2^{-/-}$ animals responded with weaker analgesia compared to the wild-type animals, although this effect was small in magnitude. When comparing the analgesia levels in all three experiments (SIA baseline, administration of rimonabant and administration of naloxone) we saw that the smaller analgesia in $CCK_2^{-/-}$ animals was also observed in the subsequent rimonabant experiment, but not in naloxone experiment. In this latter case both genotypes exerted equally near-ceiling analgesia in response to stress. Combining the tail flick latencies from all the three experiments revealed significantly smaller analgesia in $CCK_2^{-/-}$ animals in response to stress.

It should be noted that the genotype effect in the development of SIA can probably not be attributed to changes in pain sensitivity in $CCK_2^{-/-}$ mice. There is controversial evidence from previous studies showing that $CCK_2^{-/-}$ mice display reduced nociceptive sensitivity in plantar analgesia test (Veraksits *et al.*, 2003), but shorter latency for jumping response in the hot-plate test (Pommier *et al.*, 2002; Veraksits *et al.*, 2003). Both of these effects are interpreted by the authors as a change in the opioid system in $CCK_2^{-/-}$ animals. However, we did not establish any differences in the baseline sensitivity of wild-type and mutant mice in the tail-flick test. Accordingly, under the current circumstances the

mutant mice do not display differences in the baseline nociceptive sensitivity, but they have somewhat different response to stress. Stress intensity 0.6 mA was strong enough to induce significant analgesia in both genotypes and this intensity of foot-shocks was selected for the further studies.

Interestingly, we saw that identical foot-shock intensities produced different magnitudes of analgesia in different experiments. Our interpretation is that this deviance or variability is a part of "biological variation", because the experimental stress parameters were easily controlled (i.e. 3 min 0.6 mA) and could not be attributable as the source of variance.

The effect of naloxone to the development of SIA

Second, we administered an opioid antagonist naloxone prior to exposure to stress. Involvement of opioid peptides in SIA mechanisms has been established long ago (Basbaum & Fields, 1984). Therefore, we already expected naloxone to antagonise the development of SIA. Previous studies have described the transition from opioid-dependent SIA to non-opioid as related to the severity of stress (duration and intensity of foot-shock) (Terman *et al.*, 1986a). It has been shown previously in rats that 3 min, 0.9 mA foot-shock induced analgesia that was insensitive to naltrexone, an antagonist of μ-opioid receptor (Terman *et al.*, 1986a). In our case, using mice and somewhat milder, 3 min, 0.6 mA stress, we were able to establish opioid-mediated stress-induced analgesia in wild-type mice.

On the other hand, it has been shown that invalidation of CCK_2 receptors results in changes in the opioid system. Namely, increase in opioid receptor binding sites and change in binding affinity in the striatum and cerebral cortex (Runkorg *et al.*, 2003) and increase in gene expression of several opioid receptors and ligand precursors (Paper 1) have been established. Systemic administration of CCK-8 has been shown to reduce opioid-mediated SIA (Faris *et al.*, 1983). Thus, we had assumed that blocking the opioid receptors affected the development of analgesia differently in wild-type and $CCK_2^{-/-}$ mice. Nevertheless, naloxone antagonised SIA in both wild-type and $CCK_2^{-/-}$ animals, although it did not exert a dose-dependent effect. Instead, we established a U-shaped response in both genotypes. This suggests that there may be several opioid dependent mechanisms involved. Naloxone at low doses is rather μ-opioid specific, while in higher doses gradually binds also to δ– and κ–opioid receptors (Marchand *et al.*, 2003). Therefore, the U-shape effect of naloxone is possibly caused by the unspecific binding profile of drug to opioid receptors.

The effect of rimonabant to the development of SIA

Third, we tested the involvement of endocannabinoids in stress-induced analgesia. For that, we administered rimonabant, an antagonist of CB_1 receptors, before exposure to stress. Endogenous cannabinoids have also been shown to be mediators of SIA (Valverde *et al.*, 2000; Hohmann *et al.*, 2005). Rapid

modulation of stress response was demonstrated by Hohmann (2005), where increase of 2-AG in the dorsal midbrain peaked 2 min after termination of stress and returned to the baseline level already 15 min after termination of stress. As expected, we found that rimonabant dose-dependently blocked the development of SIA in wild-type mice. Surprisingly, rimonabant did not exert any effect on the development of SIA in $CCK_2^{-/-}$ mice. Thus, the stress-induced analgesia scores were identical regardless of the administration of rimonabant or vehicle. From this we can conclude that the development of SIA in $CCK_2^{-/-}$ animals is probably not an endocannabinoid-related phenomenon. Elimination of neurotransmission through CCK_2 receptor seems to disrupt at least a part of the stress-related endocannabinoid activation.

Gene expression changes after application of stress

Fourth, we performed gene expression studies in order to find molecular support for established differences in pharmacological experiments. In general, our model of stress induced a clear pattern of gene expression changes in certain CNS regions in wild-type mice. Stress reaction activated the endocannabinoid and CCK systems in two CNS areas: the lumbar spinal cord and mesolimbic area (nucleus accumbens, olfactory tubercle). Upregulation of the CCK system included both CCK and CCK_2 receptor genes in the mesolimbic area and CCK_2 receptor mRNA in the lumbar region. Upregulation of the endocannabinoid system included CB_1 receptor and ligand synthesising enzymes. The lumbar upregulation of DAGLa after stress is in good accordance with previous studies (Suplita *et al.*, 2006), where increased 2-AG levels were measured via LC/MS in rat lumbar spinal cord. Therefore, stress seems to upregulate endocannabinoid transmission in wild-type animals. In contrast and in good agreement with our behavioural results, CCK_2 deficient animals did not display many of these stress-induced changes. All the stress-induced cannabinoid transmission enhancements were absent in CCK_2 receptor deficient mice. Altogether, we found that application of stress induced a clear pattern of expression changes in wild-type mice, indicating enhanced CCK and endocannabinoid transmission in discrete CNS areas.

An interesting question is why the elimination of the CCK tone abolishes activation of endocannabinoid transmission. This goes beyond the scope of this paper, but there is a possibility for interaction between CCK and endocannabinoids at a cellular level. We established that stress induced both CCK- and cannabinoid-related gene expression changes in the same CNS regions. Beinfeld (2001) showed that CB_1 receptor activation could inhibit the release of CCK in rat hippocampal neurons, indicating that endocannabinoids modulate to some extent the activity of CCK. On the other hand, current study suggests that CCK is able to modulate the endocannabinoid tone. It is well established that CCK and CB_1 are located in the same neurons. Accordingly, adjacent neurons should contain CCK_2 receptors (the main effector pathway of CCK in CNS) and endocannabinoid synthesising enzymes (the source of endogenous cannabinoid

ligands). Activation of CCK_2 receptors results in PLC-related signal cascade (see Noble & Roques, 1999 for a review), which results in the generation of IP_3 and DAG and an elevation of intracellular Ca^{2+} levels (Figure 18). DAG is hydrolysed to 2-AG by DAG lipases. The activation of the PLC cascade may be essential for the generation of 2-AG in the rat brain (Sugiura & Waku, 2000). Thus, at least theoretically, activation of CCK_2 receptors could lead to elevation of endocannabinoid levels.

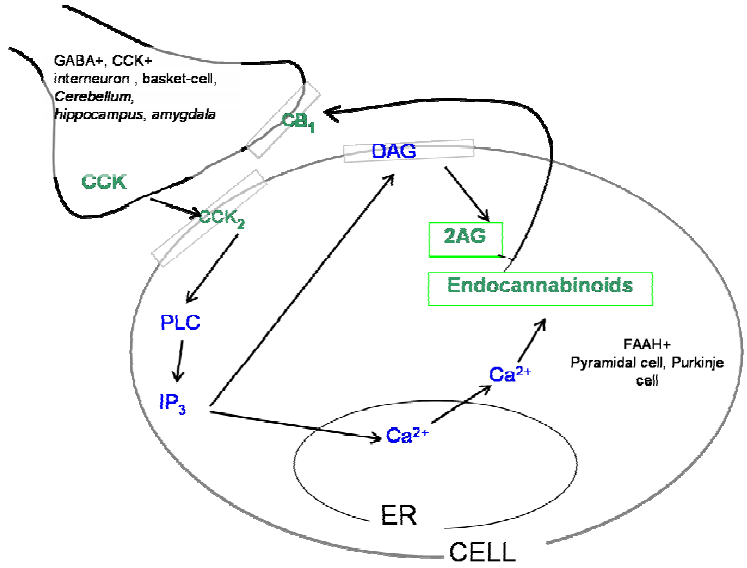

Figure 18. Hypothetical model of intracellular CCK-endocannabinoid interactions. The presynaptic neuron is a classical example of GABA+, CCK+ interneuron in cerebellum, hippocampus or amygdala that makes synaptic connection to a pyramidal cell. The activation of CCK_2 receptor activates intracellular PLC cascade, involving elevation of intracellular Ca^{2+} levels and thus leading to the biosynthesis of endocannabinoids. Activation of PLC, in conjunction with elevation of Ca^{2+}, through DAG again lead to the biosynthesis of endocannabinoids.

We also measured the expression levels of opioid genes. In general, stress-related opioid changes were less pronounced than endocannabinoid changes. Fewer opioid genes changed their expression in response to stress and the magnitudes of expression changes were smaller. Of course, we can not rule out the importance of endogenous opioids only because of lower magnitude gene expression changes after the application of stress. In wild-type mice, stress reaction included an increase in the opioid receptor gene expression in several CNS regions. Thus, the Oprm gene upregulation in the lumbar spinal cord in stressed animals may be the link to opioid-mediated SIA. Wild-type animals also had Oprm and Oprd genes up-regulated in the striatum and mesolimbic

area, respectively, after the induction of stress. It is known that stimulation of opioid receptors modifies dopamine release in these brain areas (Spanagel *et al.*, 1992). Mesolimbic dopamine, in turn, is implicated in behavioural activation, motility and motivational processes, which all are important in coping with stress. Therefore, stress-related changes in the Oprm and Oprd receptor gene expression in the mesolimbic area are probably a part of a physiological stress response. Interestingly, $CCK_2^{-/-}$ animals again had smaller changes in response to stress. The lumbar upregulation of the Oprm gene in the mutant mice was similar to wild-type animals and this presumably may be related to stress-induced analgesia.

The question why there are several different neurochemical systems for pain modulation is unknown. Considering what is known about the opioids and cannabinoids and differences in their biosynthesis and degradation, some hypothesis can be drawn. One possibility is that endocannabinoids provide a rapid modification of nociceptive processing, because the ligands are synthesized on-demand. In contrast, the opioid peptides are synthesised as gene product precursors and subsequent enzymatic cleavage yields the final products. The rapid reaction hypothesis is supported by results showing that the levels of 2-AG in dorsal midbrain exhibited a clear peak only 2 minutes after the termination of stress and returned to baseline already 15 minutes after stress termination (Hohmann *et al.*, 2005). The involvement of endocannabinoid versus opioid antinociceptive mechanisms seems to be influenced by the type of stressor used and stressor parameters (Akil & Mayer, 1972; Terman *et al.*, 1986a). The synergistic analgesic effect has also been shown between endocannabinoid and opioid systems, where tetrahydrocannabinol enhanced the effect of morphine analgesia (Welch & Stevens, 1992).

Concluding remarks and aims for the future

We found that genetic invalidation of the CCK_2 receptor gene also disrupted endocannabinoid-induced analgesia. Considering our results about the impaired stress mechanisms in these animals, and assuming that coping with stress is important in evolutionary perspective, one would expect to see serious disturbances in adaptational mechanisms or how the animals cope with stressful environment. Indeed, earlier results have confirmed that mice, lacking CCK_2 receptors, display deficient adaptation, at least to some degree. For example, it has been shown that exposure of male mice to the motility boxes for 3 consecutive days induced less inhibition of locomotor activity in CCK_2 deficient mice than in wild-type animals (Runkorg *et al.*, 2003). Reduction of motility is a measure of adaptation in familiar environment. Veraksits (2003) showed that CCK_2 receptor deficient mice displayed longer hindpaw withdrawal latencies in the hot-plate test. The longer latency was reversed to wild-type level with both single and repeated injections of saline in these mice. It seems that stronger stimuli are needed to evoke adaptational responses in $CCK_2^{-/-}$ mice, compared to wild-type animals. This can also be seen from the current experiment with

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development of SIA in response to different stress intensities (Figure 9). A stronger stimulus (higher stress) is needed to elicit the same analgesia in the $CCK_2^{-/-}$ animals as in the wild-type mice.

Current thesis raised a hypothesis that the extensive colocalisation of CCK and CB_1 receptors in many areas of the CNS is not coincidence but has a physiological importance and sought to find evidence for the crosstalk between CCK and endocannabinoids. To the date, when studying endocannabinoid functions, CCK has only been a marker for identifying the cannabinoid receptor CB_1 containing (inter)neurons in the brain. The involvement of CCK itself has been ignored in these studies. Current work is a first step towards characterising the interactions between these neurochemical systems. It provides evidence that activation of the CCK_2 receptors is necessary for the development of endocannabinoid-induced analgesia. Very recently, the functional role of the colocalising CCK and CB_1 has been addressed in two other publications. A possible role in extinction learning (Chhatwal *et al.*, 2009) and anxiety (Antonelli *et al.*, 2009) has been established. Considering that endocannabinoids have been associated with many physiological roles (as is the case with CCK) and in view of the extensive colocalisation, the interaction of the two may also be extensive and involve other functions besides antinociception, anxiety and learning mechanisms. Future aims are to describe the interactions in more detail, including more specific histological methods for the characterisation of the localisation of CCK and cannabinoid systems and including strains of animals with different predisposition to stress.

Methodological aspects when using transgenic animal lines

The genechip experiment (Paper 2) provided us with huge amount of data and information that could be used for more than analysis of pain phenotype of the $CCK_2^{-/-}$ mice. By studying only the genotype main effect of 2-way ANOVA (the study design is explained in the methods section of the Paper 2) we found some interesting effects that can be used to analyse genetic manipulation of the mouse genome, including "knocking-out" individual genes and backcrossing one mouse line into another.

When analysing the genechip data we noticed a surprising feature of the genotype main effect results: 12 out of the 15 most significant probesets (with strong evidence for an expression difference between CCK_2 knockout and wild-type mice) mapped in close region around the CCK_2 locus on chromosome 7 (Appendix 3). Another unexpected feature was that all but one of these differed in the same direction, knockout-low. The probability that these findings are due to the chance is very small. We hypothesized that the $CCK_2^{-/-}$ animals had a "residual" region of 129 origin in their genome, because the knockout construct with deficient CCK_2 was originally inserted into the 129 substrain and

subsequently backcrossed to the C57/BL6 strain (as is the usual practice when producing knockout mouse strains).

Closer inspection, using lower statistical criteria (i.e. including only the chromosome 7 into the analysis) indicated that there were many more differences in the same region. The 40 Mb region around the CCK_2 had 33 probesets that differed significantly between genotypes. This is a very striking and specific pattern. The genes within the region are diverse in function and do not have an obvious common functional theme. The pattern of expression differences that we see must be a consequence of the differences between sequences with an alternate strain of origin. We call the pattern a *congenic footprint*.

The identified 40 Mbp congenic footprint region contains 573 genes. The importance the congenic footprint finding is that all genes with demonstrated expression differences must logically be considered candidates for causing the so-called "$CCK_2^{-/-}$ phenotype", in addition to CCK_2 deficiency. Especially, considering that almost all of the expression differences are low in the knockout. The point here is not to demonstrate that these differences actually do cause the phenotype in this case, but rather to point out that there is every reason to believe that the situation, in which multiple expression differences must be considered, is typical. A recent paper reports a similar congenic footprint observation with a Rab3a knockout (chromosome 8) (Yang *et al.*, 2007). Gene knockouts are extremely valuable, but are not as clean a system for testing function as is commonly assumed.

When using gene knockout models, additional control means whenever possible should be used to control for the possible "carryover" effect from other parent strain. For example, pharmacological manipulation should be used whenever possible (i.e. when investigating signal transmitter systems). Of course, the targeted mutations should be made directly in strains of interest as methods are refined (Ware *et al.*, 2003).

To sum up, when characterising single gene deficient animals obtained by genetic manipulation, additional factors should be considered before attributing behavioural effects to a specific phenotype. This applies to the situations when a knockout has been created in one substrain and subsequently backcrossed into another. As this is common methodological route for producing knockout strains up to current time, this caution extends to most of the knockout mouse lines. Fortunately, more and more genetic manipulations are recently being made into several different background strains and methods have been developed to obtain knockouts directly to the C57BL/6 strain, without the need of backcrossing.

CONCLUSIONS

1. CCK_2 receptor deficient mice developed less evident hypersensitivity in the model of neuropathic pain than wild-type control mice. Gene expression analysis showed that CCK modulated opioid levels and opioid response to the nerve injury, because $CCK_2^{-/-}$ mice expressed basal higher levels of opioid receptor transcripts Oprd and Oprk and the $CCK_2^{-/-}$ mice displayed differential opioid peptide response to the induction of chronic pain syndrome. From this we conclude that the neuropathic pain phenotype of the $CCK_2^{-/-}$ mice may be due to the preoperative higher opioid tone (from the opioid receptors Oprd and Oprk) or due to the abnormal opioid response to the nerve injury.
2. Following peripheral nerve injury, we established activation of genes belonging to MAPK pathway in the brainstem and midbrain, using mouse gene expression microarray, indicating that this pathway is important in neuropathic pain mechanisms. We also established basal higher expression levels of Tlr4 in the $CCK_2^{-/-}$ mice in the brainstem and midbrain, suggesting that these mice may exhibit deficient immune system activation.
3. $CCK_2^{-/-}$ mice developed slightly weaker stress-induced analgesia, compared to the wild-type mice. Differently from wild-type mice antagonist of CB_1 receptors did not modify stress-induced analgesia in the CCK_2 receptor deficient mice. We conclude that deletion of CCK_2 receptor disrupts cannabinoid component of the SIA. This was further supported by the gene expression results showing that the extensive endocannabinoid activation in response to stress established in wild-type mice was largely absent in the $CCK_2^{-/-}$ mice. Thus, activation of the CCK_2 receptors is necessary for the development of endocannabinoid-induced analgesia.
4. CCK_2 receptor deficient mice display wide variety of neurochemical alterations. Despite of the extensive backcrossing of the initial mixed 129/B6 genotype to the C57BL/6 strain, a part of the genome flanking the CCK_2 receptor gene remains of 129sv origin. Any of the genes from this region – a "congenic footprint" region – may, at least partially be responsible for the phenotype of the CCK_2 receptor deficient mice. Mouse gene knockouts generated to the mixed background is not as clean model as previously suggested and the observed phenotype of these mice may not be the result of only disruption of a single target gene.

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Appendix 1.

Relative gene expression mean values for the wild-type (+/+) and $CCK_2^{-/-}$ (–/–) animals in the absence of stress (Stress(–) or after application of stress Stress(+), respectively). Gene expression levels were measured in lumbar spinal cord (A), brainstem (medulla, pons) (B), midbrain (C), striatum (D) and mesolimbic area (nucleus accumbens, olfactory tubercle) (E). Animal number was 6 in each group. Stars represent stress-induced effect Tukey *post-hoc* values, i.e. comparisons with the respective non-stress (Stress(–)) group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Crosses represent genotype related basal gene expression difference Tukey *post-hoc* values (comparison with wild-type Stress(–) group). + $p<0.05$, ++ $p<0.01$.

A Lumbar spinal cord

	+/+			–/–				+/+				–/–			
	Stress (–)			Stress (–)				Stress (+)				Stress (+)			
	MEAN	±	SEM	MEAN	±	SEM		MEAN	±	SEM		MEAN	±	SEM	
CB1	1.00	±	0.25	1.19	±	0.16		2.95	±	0.49	**	2.16	±	0.32	
CCK	1.00	±	0.04	0.90	±	0.06		0.93	±	0.09		0.91	±	0.13	
CCK2	1.00	±	0.16					2.91	±	0.10		***			
DAGLa	1.00	±	0.47	4.14	±	0.84	++	6.26	±	0.66	***	6.15	±	0.74	
DAGLb	1.00	±	0.33	1.00	±	0.31		0.79	±	0.14		0.88	±	0.09	
FAAH	1.00	±	0.09	0.88	±	0.14		1.25	±	0.06		0.95	±	0.06	
MAGL	1.00	±	0.28	0.53	±	0.06		0.94	±	0.19		0.73	±	0.12	
NAPE	1.00	±	0.14	3.21	±	1.43		1.07	±	0.08		1.46	±	0.33	
Oprd	1.00	±	0.24	0.84	±	0.11		1.52	±	0.23		1.15	±	0.16	
Oprk	1.00	±	0.50	0.41	±	0.03		0.67	±	0.11		0.48	±	0.08	
Oprm	1.00	±	0.05	0.51	±	0.12	+	2.41	±	0.64	*	1.33	±	0.05	**
PDYN	1.00	±	0.15	1.00	±	0.12		1.27	±	0.18		1.07	±	0.23	
PENK	1.00	±	0.35	0.58	±	0.12		0.76	±	0.21		0.58	±	0.08	
POMC	1.00	±	0.45	0.47	±	0.17		0.68	±	0.21		0.43	±	0.10	

B Brainstem (medulla, pons)

	+/+ Stress (–)			–/– Stress (–)			+/+ Stress (+)			–/– Stress (+)		
	MEAN	±	SEM	MEAN	±	SEM	MEAN	±	SEM	MEAN	±	SEM
CB1	1.00	±	0.09	0.92	±	0.12	0.92	±	0.13	0.97	±	0.16
CCK	1.00	±	0.18	0.97	±	0.21	0.79	±	0.15	0.90	±	0.12
CCK2	1.00	±	0.19				1.08	±	0.05			
DAGLa	1.00	±	0.13	0.82	±	0.21	0.80	±	0.19	1.08	±	0.23
DAGLb	1.00	±	0.21	0.72	±	0.14	0.66	±	0.06	1.08	±	0.20
FAAH	1.00	±	0.12	1.09	±	0.08	0.88	±	0.17	0.73	±	0.04
MAGL	1.00	±	0.13	1.35	±	0.24	3.32	±	1.76	0.92	±	0.18
NAPE	1.00	±	0.12	1.06	±	0.21	0.77	±	0.15	1.23	±	0.31
Oprd	1.00	±	0.13	1.30	±	0.08	1.13	±	0.15	1.05	±	0.12
Oprk	1.00	±	0.16	4.25	±	2.43	1.38	±	0.34	1.30	±	0.13
Oprm	1.00	±	0.15	1.04	±	0.14	1.16	±	0.44	0.73	±	0.09
PDYN	1.00	±	0.28	0.95	±	0.30	0.55	±	0.07	0.81	±	0.07
PENK	1.00	±	0.09	2.36	±	0.95	1.80	±	0.40	0.89	±	0.13
POMC	1.00	±	0.17	2.32	±	0.76	2.28	±	0.57	1.25	±	0.31

C Midbrain

	+/+ Stress (–)			–/– Stress (–)			+/+ Stress (+)			–/– Stress (+)		
	MEAN	±	SEM	MEAN	±	SEM	MEAN	±	SEM	MEAN	±	SEM
CB1	1.00	±	0.13	1.30	±	0.16	1.29	±	0.15	1.19	±	0.10
CCK	1.00	±	0.05	0.89	±	0.08	0.99	±	0.07	0.86	±	0.09
CCK2	1.00	±	0.09				1.20	±	0.10			
DAGLa	1.00	±	0.16	1.34	±	0.14	1.29	±	0.18	1.21	±	0.22
DAGLb	1.00	±	0.05	0.97	±	0.07	1.25	±	0.04	1.07	±	0.09
FAAH	1.00	±	0.09	1.09	±	0.08	1.20	±	0.11	1.04	±	0.10
MAGL	1.00	±	0.08	1.04	±	0.11	1.00	±	0.12	1.00	±	0.10
NAPE	1.00	±	0.09	1.13	±	0.10	1.22	±	0.11	1.23	±	0.09
Oprd	1.00	±	0.11	1.22	±	0.12	0.71	±	0.09	0.77	±	0.11
Oprk	1.00	±	0.15	1.18	±	0.18	1.13	±	0.17	1.39	±	0.15
Oprm	1.00	±	0.14	1.18	±	0.12	1.24	±	0.10	1.47	±	0.12
PDYN	1.00	±	0.16	1.28	±	0.17	1.52	±	0.18	1.68	±	0.20
PENK	1.00	±	0.19	0.87	±	0.15	0.83	±	0.21	0.80	±	0.14
POMC	1.00	±	0.18	0.75	±	0.19	0.57	±	0.15	0.47	±	0.17

D Striatum

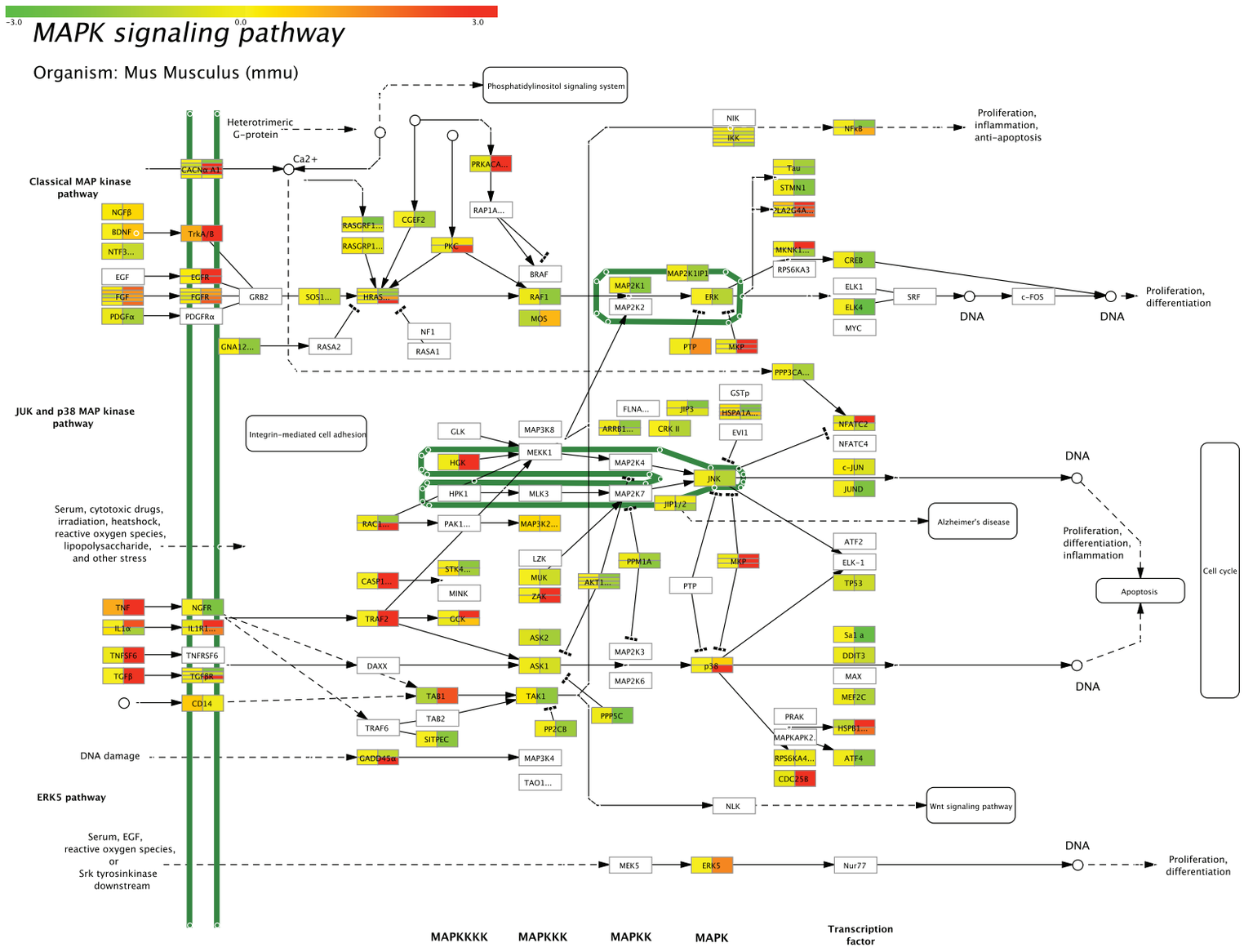
	+/+ Stress (–)			–/– Stress (–)			+/+ Stress (+)				–/– Stress (+)		
	MEAN	±	SEM	MEAN	±	SEM	MEAN	±	SEM		MEAN	±	SEM
CB1	1.00	±	0.24	0.90	±	0.18	0.77	±	0.17		1.12	±	0.16
CCK	1.00	±	0.07	1.01	±	0.06	0.77	±	0.19		0.57	±	0.10
CCK2	1.00	±	0.07				0.66	±	0.17				
DAGLa	1.00	±	0.17	1.11	±	0.21	0.99	±	0.25		1.03	±	0.17
DAGLb	1.00	±	0.06	0.98	±	0.04	0.92	±	0.09		1.04	±	0.10
FAAH	1.00	±	0.08	1.07	±	0.04	0.70	±	0.15		0.64	±	0.10
MAGL	1.00	±	0.08	0.97	±	0.11	1.10	±	0.11		1.02	±	0.09
NAPE	1.00	±	0.12	1.11	±	0.05	1.10	±	0.10		0.94	±	0.05
Oprd	1.00	±	0.08	1.33	±	0.12	1.12	±	0.13		1.00	±	0.09
Oprk	1.00	±	0.07	0.98	±	0.11	0.96	±	0.14		1.00	±	0.14
Oprm	1.00	±	0.11	1.34	±	0.19	1.71	±	0.13	*	1.70	±	0.15
PDYN	1.00	±	0.08	0.82	±	0.08	1.48	±	0.20		1.35	±	0.22
PENK	1.00	±	0.10	0.77	±	0.03	1.10	±	0.22		1.15	±	0.23
POMC	1.00	±	0.08	0.97	±	0.07	1.51	±	0.48		1.20	±	0.17

E Mesolimbic area (nucleus accumbens, olfactory tubercle)

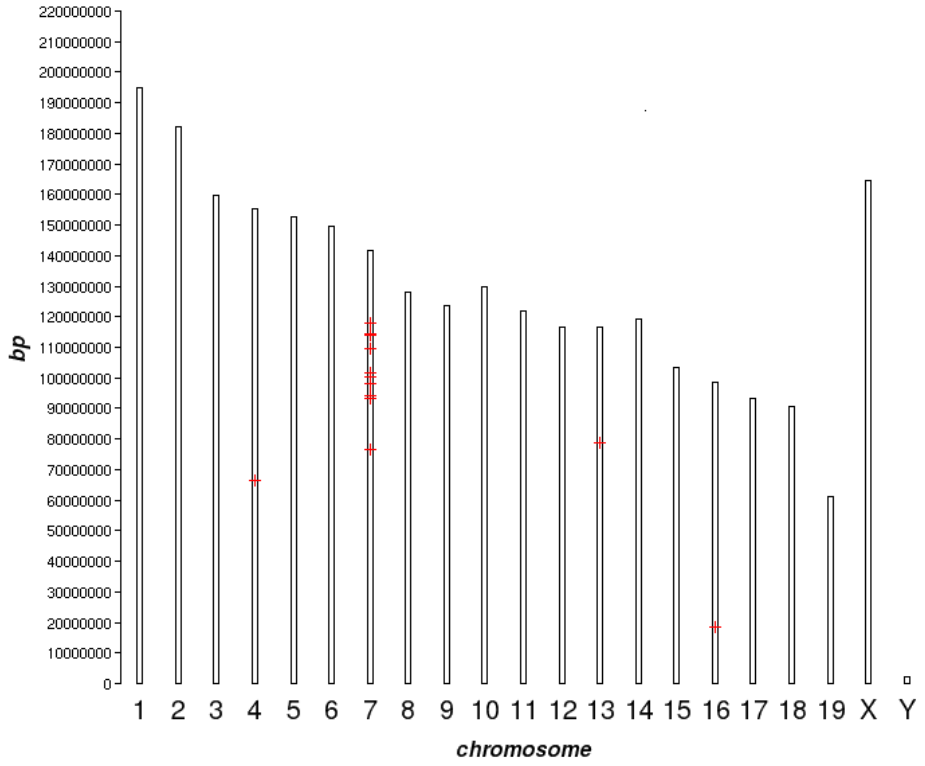
	+/+ Stress (–)			–/– Stress (–)			+/+ Stress (+)				–/– Stress (+)			
	MEAN	±	SEM	MEAN	±	SEM	MEAN	±	SEM		MEAN	±	SEM	
CB1	1.00	±	0.04	0.97	±	0.07	1.37	±	0.08	**	1.08	±	0.07	
CCK	1.00	±	0.19	0.70	±	0.13	3.98	±	0.77	**	4.66	±	1.76	***
CCK2	1.00	±	0.16				1.72	±	0.15	**				
DAGLa	1.00	±	0.05	0.93	±	0.08	1.16	±	0.09		1.26	±	0.14	
DAGLb	1.00	±	0.07	0.89	±	0.06	1.15	±	0.04		1.02	±	0.09	
FAAH	1.00	±	0.08	0.91	±	0.09	1.16	±	0.10		1.20	±	0.16	
MAGL	1.00	±	0.04	0.86	±	0.09	1.30	±	0.08		1.39	±	0.21	*
NAPE	1.00	±	0.08	0.97	±	0.06	1.38	±	0.07	*	1.21	±	0.10	
Oprd	1.00	±	0.10	1.18	±	0.10	1.42	±	0.06	*	1.18	±	0.08	
Oprk	1.00	±	0.06	0.98	±	0.08	1.25	±	0.08		1.00	±	0.12	
Oprm	1.00	±	0.09	0.92	±	0.08	1.00	±	0.11		0.99	±	0.07	
PDYN	1.00	±	0.09	0.97	±	0.11	1.24	±	0.13		1.02	±	0.14	
PENK	1.00	±	0.05	0.94	±	0.13	1.06	±	0.13		0.86	±	0.16	
POMC	1.00	±	0.14	0.89	±	0.05	1.15	±	0.15		1.01	±	0.08	

Appendix 2.

MAPK pathway activation in supraspinal structures as detected in our study according to the analysis using Pathway Explorer. This web-tool mapped the differentially expressed gene profiles (log2 ratio values) to all available biological databases. Each colour-coded box represents mapped genes with two datasets – the left side of each box is coloured according to the genotype effect (genes affected by CCK_2 mutation), the right side of each box is coloured according to the CCI effect (genes affected by CCI). Upregulated genes are coloured in green; downregulated genes are coloured in red. Horizontally split boxes represent genes where more than one gene ID (Genebank ID) was matched. The expressions of many of the genes in this pathway were altered following CCI surgery and are widely distributed, suggesting that the MAPK pathway has been activated at multiple points following CCI. The strongest activation (coloured in red) is in case of several inflammatory mediators (TNF, IL1a, TGF) and their receptors, which activate other elements in the pathway (e.g. TRAF2, TNF receptor-associated factor 2, Mapk14 or mitogen activated protein kinase 14). This figure also illustrates significant involvement and activation of classical parts of the MAPK pathway (e.g. EGF, BDNF and NGF).

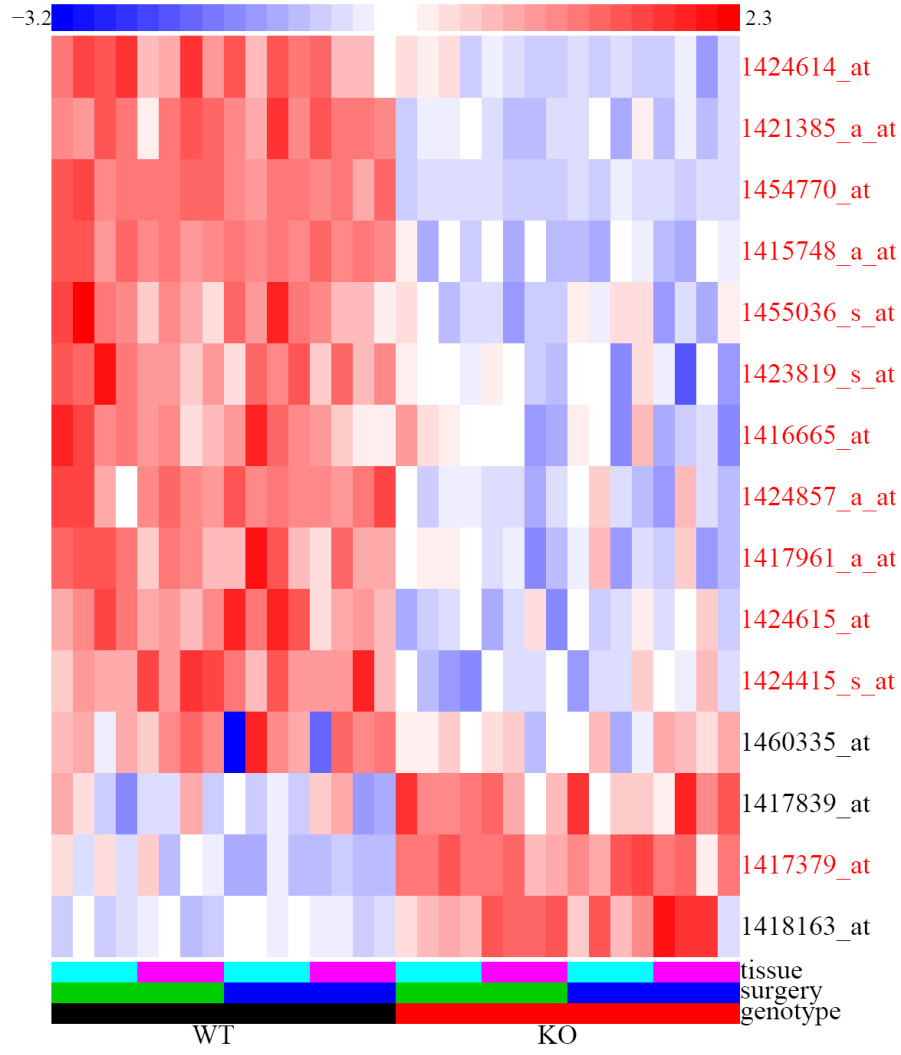

-3.0
0.0
3.0
MAPK signaling pathway
Organism: Mus Musculus (mmu)
Phosphatidylinositol signaling system
Heterotrimeric G-protein
Ca2+
Classical MAP kinase pathway
JUK and p38 MAP kinase pathway
ERK5 pathway
Integrin-mediated cell adhesion
Serum, cytotoxic drugs, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
DNA damage
Serum, EGF, reactive oxygen species, or Srk tyrosinkinase downstream
Proliferation, inflammation, anti-apoptosis
Proliferation, differentiation
Alzheimer's disease
Wnt signaling pathway
Cell cycle
Apoptosis
DNA
CACNA1...
NGFβ
BDNF
NTF3...
TrkA/B
EGF
EGFR
FGF
FGFR
PDGFRα
GRB2
SOS1...
HRAS...
RASGRF1...
RASGRP1...
CGEF2
PKC
PRKACA...
RAP1A...
BRAF
RAF1
MOS
NF1
RASA1
RASA2
GNA12...
MAP2K1
MAP2K2
MAP2K1IP1
ERK
PTP
MKP
NIK
IKK
NFkB
Tau
STMN1
LA2G4A...
MKNK1...
RPS6KA3
CREB
ELK1
ELK4
MYC
SRF
c-FOS
PPP3CA...
GSTp
FLNA...
JIP3
HSPA1A...
ARRB1...
CRK II
EVI1
NFATC2
NFATC4
c-JUN
JUND
GLK
MAP3K8
MEKK1
HGK
HPK1
MLK3
MAP2K4
MAP2K7
JNK
JIP1/2
RAC1...
PAK1...
MAP3K2...
CASP1...
STK4...
MINK
LZK
MUK
ZAK
AKT1...
PPM1A
MKP
PTP
ATF2
ELK-1
TP53
TNF
IL1α
TNFSF6
TGFβ
CD14
NGFR
IL1R1...
TNFRSF6
TGFβR
TRAF2
GCK
DAXX
TAB1
TAB2
SITPEC
TRAF6
GADD45α
ASK2
ASK1
TAK1
PP2CB
PPP5C
MAP2K3
MAP2K6
p38
Sa1 a
DDIT3
MAX
MEF2C
PRAK
MAPKAPK2
RPS6KA4...
CDC25B
HSPB1...
ATF4
MAP3K4
TAO1...
NLK
MEK5
ERK5
Nur77
MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor
Experiments: (Mus Musculus|mm0504_cci_short.txt)
log2CENOT
log2CCI

Appendix 3



Genomic locations of the 15 probesets (marked as red crosses) most significantly differing between wild-type and $CCK_2^{-/-}$ mice. The figure was generated using Webgestalt (Zhang et al., 2005).

Appendix 4



Heatmap of the probesets most significantly differing between wild-type (left, designated as WT) and $CCK_2^{-/-}$ (right, designated as KO) mice. Each column represents one of the 32 Affymetrix MOE430A 2.0 arrays, and each row represents a probeset (transcript). The probeset names on the right are colour coded red for chromosome 7 and black for others. Each row is scaled and the colour scale at the top is calibrated in standard deviations, red for high values, white near the mean and blue for low values. The rows have been hierarchically clustered using |1-R| (where R is the Pearson correlation coefficient) and a complete linkage criterion. The colour bar at the bottom shows the identities of the samples applied to each array. Tissue: pale blue- medulla; pink- midbrain. Surgery: blue- sham; green- CCI. Genotype: black- wild-type; red- $CCK_2^{-/-}$.

SUMMARY IN ESTONIAN

Koletsüstokiniini seotus kroonilise valu mehhanismidega ja endogeense valuvastase süsteemiga

Käesoleva dissertatsiooni käigus uuriti CCK_2 retseptori puudulike ($CCK_2^{-/-}$) hiirte kroonilise valu fenotüüpi ja üritati sellele fenotüübile leida ka neurokeemilisi korrelaate. Esiteks leidsime, et $CCK_2^{-/-}$ hiirtel esines von Frey mehaanilise tundlikkuse testimise mudelis hüposensitiivsus. Selle leiu põhjuseks leiti olevat CCK ja opioidide vastastikune regulatsioon, sest hüposensitiivsuse fenotüübi sai blokeerida opioidi retseptorite antagonisti naloksooni (0.1…10 mg/kg) manustamisega ja teisalt sai metsiktüüpi loomadel esile kutsuda hüposensitiivsuse CCK_2 antagonisti L-365260 (0.01…1 mg/kg) manustamisega. Teiseks kirjeldasime $CCK_2^{-/-}$ hiirtel tundetust neuropaatilise valu suhtes, sest pärast kroonilise kompressiooni vigastust (*chronic constriction injury*) tekkis neil loomadel ainult minimaalne ülitundlikkus, vastandina metsiktüüpi loomadele, kellel tekkis väga tugev ülitundlikkus. Geenieksпressioonanalüüs näitas mutantsetel hiirtel häireid opioidsete peptiidide ja opioidiretseptorite geenieksпressioonis. Sellest järeldame, et CCK signaali häirimine läbi CCK_2 retseptori võimendab opioidisüsteemi sellisel määral, et nähtavaid neuropaatilise valu sümptomeid ei teki.

Käesoleva töö teises osas uurisime kroonilise kompressiooni vigastuse tekitamise järgselt $CCK_2^{-/-}$ hiirte geenieksпressiooni muutusi ajutüves ja keskajus, kasutades Affymetrix'i hiire geenieksпressiooni kiipe. Leidsime, et vastuseks närvivigastusele, sõltumata genotüübist tekib tugev MAPK raja aktivatsioon mõlemas KNS piirkonnas. Me ei leidnud tugevaid genotüübi x perifeerse närvi vigastuse interaktsioone. Kõige huvitavam genotüübist sõltuv (genotüübi ANOVA peaefekt) kandidaatgeen oli Tlr4, lipopolüsahhariidile reageeriv geen. Ka see tulemus näitab perifeerse närvi vigastuse järgset immuunaktivatsiooni ajutüves ja keskajus ja lubab oletada, et CCK_2 retseptor on seotud immuunsüsteemi aktivatsiooni ja KNS vaheliste interaktsioonidega.

Kolmandaks uurisime stress-analgeesiat ja CCK ja endokannabinoidide rolli selles. Stress-analgeesia on oluline adaptatsioonimehhanismide näitaja. Lisaks sellele võimaldab stress-analgeesia uurimine endogeensetest analgeetilistest mehhanismidest rohkem teada saada ja seda teadmist kliinilises praktikas kasutada. Stressimudelina kasutasime elektrišokki jalgadele (0.6 mA, 3 min) ja analgeesia tasemeid enne ja pärast stressi mõõtsime *tail flick* meetodiga. Leidsime, et $CCK_2^{-/-}$ hiirtel tekkis samale stressile vastuseks väiksem analgeesia. See lubab oletada, et CCK_2 signaali elimineerimine häirib mõningaid endogeense analgeesia mehhanisme. Järgmiseks manustasime loomadele opioidiretseptorite antagonisti naloksooni (0.01…10 mg/kg) ja kannabinoidi CB_1 retseptori antagonisti rimonabanti (0.1…3 mg/kg) ja uurisime nende toimet

stress-analgeesiale. Naloksoon pärssis analgeesiat mõlemal genotüübil, nii $CCK_2^{-/-}$ kui ka metsiktüüpi hiirtel. Erinevalt naloksoonist pärssis rimonabant stress-analgeesiat ainult metsiktüüpi loomadel ja ei mõjutanud *knockout* hiirte vastust. See tulemus sobib hästi kokku järgnevalt teostatud geenieksрressiooni analüüsiga, milles leiti metsiktüüpi hiirtel vastuseks stressile ulatuslik kannabinoidisüsteemi aktivatsioon. See stressiga seotud aktivatsioon suuresti puudus mutantsetel loomadel. Kokkuvõtteks, CCK toime läbi CCK_2 retseptorite on vajalik stressiga seotud endokannabinoidide aktivatsiooni jaoks, sest $CCK_2^{-/-}$ hiirtel puudub stress-analgeesia kannabinoidne komponent.

Viimaseks analüüsisime ülalmainitud Affymetrix'i geenieksрressiooni kiibi neuropaatiliste loomade andmeid teise nurga alt. Uurides ainult genotüübi efekti (st jättes närvivigastuse faktori analüüsist välja) nägime, et enamus genotüübi erinevusi (st erinevalt ekspresseerunud geene $CCK_2^{-/-}$ hiirtel) paiknes genoomis ühel konkreetsel regioonil, moodustades 40 Mbp ala CCK_2 geeni ümber. Peaaegu kõik genotüübi efektina muutunud ekspressiooniga geenid olid *knockout*-madala ekspressioonisuunaga. Need geenid klasterdusid genoomse asukoha, mitte aga geeni funktsiooni järgi. Me tegime kindlaks, et need geenid pärinevad 129sv vanemliinist ning need ei olnud kadunud isegi pärast paljukordset tagasiristamist C57BL/6 tausta. Sellest järeldame, et segatausta tekitatud geenipuudulikkused ei võimalda geenifunktsiooni uurida nii puhtalt nagu varasemalt arvatud ja et sellistel hiirtel kirjeldatud fenotüüp ei pruugi olla ainult ühe geeni väljalülitamise tagajärg.

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20 000 EEK (1282 EUR) for self-education in foreign countries

List of publications

Luuk H, Plaas M, Raud S, Innos J, Sütt S, Lasner H, Abramov U, **Kurrikoff K**, Kõks S, Vasar E. Wfs1-deficient mice display impaired behavioural adaptation in stressful environment.
Behav Brain Res. 2009 Mar 17;198(2):334–345.

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2005, 3 nädalat, Hiire kroonilise valu seisundiga seotud geenikspressiooni profiil Affymetrix geenikspressioonikiipidega. London King's College, Social, Genetic & Developmental Psychiatry Centre, Institute of Psychiatry.

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2004, 1 nädal, Kursus "Real-time PCR", Kliinilise ja molekulaarse meditsiini keskus, Tartu

2003, 1 nädal, Kursus "Behavioural Studies In Drug Discovery", University of Kuopio, Finland

2002, 1 nädal, Kursus "Hanseatic Student Days of Biomedicine", University of Rostock, Germany

Tunnustused

2004, Arstiteaduskonna päevad, Parima ettekane auhind: “Deletion of the CCK2 Receptor Gene Reduces Mechanical Sensitivity and Abolishes the Development of Hyperalgesia in Mononeuropathic Mice”, 20 000 EEK enesetäienduseks välismaa laborites.

Teadustööde loetelu

Luuk H, Plaas M, Raud S, Innos J, Sütt S, Lasner H, Abramov U, **Kurrikoff K**, Kõks S, Vasar E. Wfs1-deficient mice display impaired behavioural adaptation in stressful environment.

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1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
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5. **Aleksei Nelovkov.** Behavioural and neurogenetic study of molecular mechanisms involved in regulation of exploratory behaviour in rodents. Tartu, 2006.
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8. **Hendrik Luuk.** Distribution and behavioral effects of WFS1 protein in the central nervous system. Tartu, 2009.
9. **Anne Must.** Studies on molecular genetics of male completed suicide in Estonian population. Tartu, 2009.