Modulation of pain sensitivity by endogenous and exogenous ligands and by social isolation

Ph.D. thesis

Gábor Tuboly M.D.

University of Szeged

Albert Szent-Györgyi Medical and Pharmaceutical Centre

Faculty of Medicine

Department of Physiology

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Publications

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1. Horvath G., Kekesi G., Szikszay M., **Tuboly G.**, Benedek G. The enhancement of the effectivity of endomorphin-1 by other endogenous ligands. *6th European Opioid Conference.* Salamanca, Spain, 19-21 April, 2006.
2. Horvath G., **Tuboly G.**, Benedek G. Az anandamide és az endomorphin-1 interakciója gerincvelői szinten. *MÉT LXXI. Vándorgyűlése*. Pécs, 2007. June 6-8.

3. **Tuboly G.**, Horvath G., Benedek G. A fájdalomérzékenység változása ketamin és szociális izoláció együttes alkalmazásával. *MÉT LXXI. Vándorgyűlése*. Pécs, 2007. June 6-8. Acta Physiol Hung. 94:353-354. 2007
4. **Tuboly G.**, Horvath G., Benedek G. The effect of subchronic ketamine treatment and social isolation on pain sensitivity. *World conference of Stress.* Budapest, Hungary, August 23-26, 2007. Cell Stress & Chaperon 12(E1):396
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8. **Tuboly G.**, Vezér T., Horvath G., Benedek G. Complex model for schizophrenia in rats for assessment of pain sensitivity. *IBRO2008*. Debrecen, 2008, january 24-26
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Introduction

Pain pathways

Peripheral fibers and spinal center

The sensation of pain or nociception is the most distinctive of all the sensory modalities. By the definition of the International Association for the Study of Pain, “Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage”. Unlike other sensory modalities, it doesn’t have a specialized sensory organ, and has an urgent and primitive quality, a quality responsible for the affective and emotional aspect of pain perception (Kandel et al. 2000). It serves an important function, namely to warn the body and prevent of further injury that should be avoided or treated. However, the reduction of pain is often necessary in the clinical practice, which requires an overall knowledge about the systems responsible for the mediation of this sensation. Nociception is defined as "the neural processes of encoding and processing noxious stimuli”, triggers a variety of autonomic responses, however it does not necessarily lead to the experience of pain (Loeser and Treede 2008). Perception is a product of the brain’s abstraction and elaboration of sensory input (Fenton 2007).

The nociceptive system can be divided into peripheral and central components. Most peripherally are the specialized sensory receptors, called nociceptors. A nociceptor is defined as “A (sensory) receptor preferentially sensitive to a noxious stimulus or to a stimulus which would become noxious if prolonged” (Merskey 1986). Nociceptors are free nerve endings and are widely found in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments and articular capsules, periosteum, muscles, tendons, and arterial vessels, and may respond to three types of stimuli: mechanical, thermal (extremes of hot and cold), and chemical substances. Based upon the

connecting axon, there are two major peripheral pathways: the A-delta, and C-fiber mediated nociception.

- **A-delta nociceptors** respond to noxious thermal or mechanical stimuli and form small diameter (2.0 to 6.0 μm) myelinated afferents which propagate action potentials into the central nervous system (CNS) at a fast speed (15-30 m/s). Activity in these afferents is associated with 'first' or 'fast' pain sensations, which are often described as 'severe' and 'sharp'.

- **C-fiber afferents** can respond to all types of noxious stimuli (noxious mechanical, thermal or chemical). They form 0.4 to 1.2 μm wide unmyelinated, slow-conducting (0.5-2 m/s) afferent fibers. Activity in the C-fibers is associated with the 'second' or 'slow' pain sensations which are often described as 'dull' and 'aching'. These afferents are particularly sensitive to endogenous algesic chemicals resulting from cell damage, including potassium, serotonin (5-HT), bradykinin, histamine, prostaglandins, leukotrienes and substance P. (Table 1.) (Johnson 1997)

Substance	**Source**	**Effect on primary afferent fibers**
K+	Damaged cells	Activation
5-HT	Platelets	Activation
Bradykinin	Plasma kininogen	Activation
Histamine	Mast cells	Activation
Prostaglandins	Arachidonic acid – damaged cells	Sensitization
Leukotrienes	Arachidonic acid – damaged cells	Sensitization
Substance P	Primary afferents	Sensitization

Table 1. – Naturally occurring agents that activate or sensitize nociceptors (Fields 1987)

The cell bodies of the peripheral nociceptive afferents are found in the dorsal root ganglia (DRG) of the spinal cord and in sensory ganglia of some cranial nerves (V, IX, X). Neurons in the DRG can be classified as A- and B-cells. A-cells in general are larger and seem to equal the number of myelinated fibers, while smaller B-cells probably transmit noxious information to the CNS via unmyelinated axons (Tandrup 2004). Three subtypes of

B-cells were identified. Sugiura et al. studied the different sensory modalities of morphologically defined subtypes of B-cells in the DRG. High threshold mechano- and mechanical cold nociception are linked to B1, polymodal nociception to B2 and cooling reception to the B3 subtypes (Sugiura et al. 1988).

The central afferents of these ganglion cells enter the CNS by way of dorsal root to terminate in the spinal cord or by way of cranial nerves to end in the brainstem, where the initial stages of central processing occur. Recent evidence from human and animal studies has significantly expanded the understanding of pain perception and has demonstrated that a complex series of spinal and supraspinal structures are involved in pain (Fenton 2007). Intrinsic neurons of the dorsal horn (DH) promote the interaction of the afferent and efferent fibers, and are also responsible for their transfer to supraspinal structures.

The dorsal horn of the spinal cord has been described as a layered structure, based on histological sections stained for Nissl substance. The grey matter of the spinal cord consists of 10 laminae, including 6 in the DH (Szentágothai and Réthelyi 2002). The laminae of the DH can be grouped into the superficial layers (laminae I and II or the marginal zone and substantia gelatinosa, respectively) and the deep layers (laminae III-IV-V or the nucleus proprius, lamina VI or the base of the DH, the lateral spinal nucleus, nucleus caudalis, and some regions around the central medullary canal - lamina X) (Willis, Jr. 1988).

Nociceptive pathways in the DH spread out to different directions through various excitatory and inhibitory interneurons. In view of the reception and integration of the afferent stimulus, neurons in the DH can be classified as interneurons that can be divided into interlaminar and intrasegmental intralaminar types, having inhibitory or excitatory characteristics; and projecting neurons that directly transmit the information to supraspinal centers. There are two distinct types of projecting neurons, which respond to nociceptive information:

- Nociceptive specific (NS) cells are predominantly found in lamina I, II (external), V and VI of the DH (Willis, Jr. 1988). The sources of input for these neurons are A-delta and C fibers. These neurons respond only to noxious input. When activated, NS cells rapidly transmit information onward to the brain.

- Wide dynamic range (WDR) cells are found in laminae I, II (external), IV, V, VI, X and receive noxious input from A-delta and C nociceptors and also non-noxious

input from large diameter A-beta (touch) fibers. These A-beta fibers normally transmit information about non-noxious stimuli to produce touch sensations. The main characteristic of WDR cells is the capacity of coding for the stimulus intensity because they show increasing frequencies of response from innocuous to noxious stimulation. Because of the convergence of noxious and non-noxious fibers, this group also plays a fundamental role in the mechanisms of segmental suppression of pain.

After the direct or indirect connections with the projection neurons, the axons of these central nociceptive transmission cells ascend to supraspinal centers at least along five central pathways.

Central pathways

- **The spinothalamic** and **trigeminothalamic tracts** are the most prominent, direct nociceptive pathways, connecting the spinal cord and trigeminal nuclei with the thalamus. These axons project nociceptive information from the body and the head respectively, primarily from the NS and WDR neurons in laminae I and V of the DH (but also from laminae II, IV, VI, VII, VIII and X). These fibers cross the midline, and ascend in the anterolateral white matter of the spinal cord. Nociceptive information is projected from the thalamus onward to the somatosensory cortex where the sensory dimensions of pain are processed. This will provide information relating to the intensity, quality and location of the noxious stimuli.

Based on the origin and the model of projection of these fibers, three forms of afferences of the spinothalamic tract can be identified. One is the neospinothalamic pathway or ventral spinothalamic tract, which directly projects to nuclei of the lateral complex of the thalamus, involved in the sensory–discriminative component of pain.

Another is the paleospinothalamic pathway, or dorsal spinothalamic tract, which projects to nuclei of the posterior medial and intralaminar complex of the thalamus, involved in the motivational–affective aspects of pain.

Finally, a monosynaptic spinothalamic pathway projecting directly to the medial central nucleus of the thalamus is involved in the affective component of the painful experience.

- **The spino-reticulo-thalamic** or **spinoreticular tract** comprises the axons of neurons in laminae V, VII and VIII and also in laminae I and X. In contrast to the spinothalamic tract, many of the axons do not cross the midline. It ascends in the anterolateral quadrant of the spinal cord and terminates in the reticular formation of medulla and pons, and after synapses it projects to the hypothalamus and the thalamus. This tract is involved in the motivational-affective characteristics, as well as the neuro-vegetative responses to pain. The real functional importance of this tract is believed to be due to the connections established in the brainstem because the projections to the intralaminar nuclei of the thalamus are sparse and probably occur by means of collateral branches of the spinothalamic tract. This tract is also an important pathway for the modulation of nociceptive information by activating brain stem structures responsible for descending suppression.

- **The spinomesencephalic tract** comprises the axons of neurons in laminae I, II IV, V, VI, VII, and X. It projects in the spinotectal bundle to the deep layers of the superior colliculus and to the periaqueductal grey matter (PAG). The activity of this tract as well as the spinothalamic tract, suffers inhibitory or excitatory influences from interneurons activated by collateral neurons of the spinocervical tract. The spinomesencephalic tract together with the sacral parasympathetic nucleus and collaterals of the spinoreticular tract also sends projections to the parabrachial nucleus (PBN) of the pons. Since neurons of the PBN project to the amygdala, the major component of the limbic system, this track is thought to contribute to the autonomic, cardiovascular, motivational and affective responses to pain. However, the afferences to the amygdala and other limbic structures do not occur exclusively through the PBN. Direct tracts from the spinal cord to the amygdala, lenticular nucleus, nucleus accumbens, septum, cingular, frontal and infralimbic cortex have also been described. For this reason, they are considered spinal–limbic pathways by some authors (Gauriau and Bernard 2002, Schaible and Grubb 1993, Willis and Westlund 1997).

- **The spino-cervico-thalamic** tract arises from neurons in the lateral cervical nucleus, located in the lateral white matter of the upper two cervical segments. This nucleus receives input from the nociceptive neurons in laminae III and IV. Most axons in the tract cross the midline and ascend in the medial lemniscus of the brainstem to nuclei in the midbrain and to the ventroposterior lateral (VPL) and posteromedial nuclei (PM) of the thalamus. Some axons from the nociceptive neurons project through the dorsal columns of the spinal cord and terminate in the cuneate and gracile nuclei of the medulla.

- **The spinohypothalamic** and **trigeminal-hypothalamic tracts** comprise axons of neurons in laminae I, V, (VIII) and X. They project supraspinally to the autonomic control centers in the hypothalamus and are thought to activate complex neuroendocrine and cardiovascular responses (Kandel et al. 2000).

Brainstem

Brainstem sites previously thought to be primarily involved in cardiovascular function and autonomic regulation also have been demonstrated to play a role in the modulation of spinal nociceptive transmission (Jones 1992, Kwiat and Basbaum 1992).

The concept of nociceptive gating or descending control of pain has arisen more than 30 years ago (Melzack and Wall 1965). The authors have phrased that nociceptive information impinging upon the DH of the spinal cord from the skin, viscera and other tissues, is not automatically transferred to higher centers. According to our recent knowledge, this system can either inhibit or facilitate the activity of the ascending pain pathways. In this respect, mechanisms of both "descending inhibition" (DI) and "descending facilitation" (DF) must be recognized.

Terminals of descending pathways originating in the rostral ventromedial medulla (RVM), locus coeruleus (LC), the nucleus tractus solitarius (NTS), the PBN, the dorsal reticular nucleus (DRT) interact with afferent fibers, interneurons and projection neurons in the DH. Actions at these sites, as a function of the influence of individual receptors upon cellular excitability, either suppress or enhance passage of nociceptive information to the above mentioned higher centers (Millan 2002).

The **PAG** is a key relay station in the processing of nociceptive and antinociceptive information in the CNS. Its ventral and ventrolateral regions are widely known as key stations in descending pathways that act to control nociceptive inputs in the DH (Pelegrini-da-Silva et al. 2005). GABAergic antagonists, cannabinoids, and μ-opioid agonists all initiate brainstem-integrated, monoaminergic mechanisms of DI via actions in this structure. It receives sensory information from the spinoreticular tract, afferentation from pain-related cortical and subcortical areas, and is thought to represent the mechanisms whereby cortical and other inputs act to control the nociceptive 'gate' in the DH (Anuradha et al. 2004, Rainville 2002). Anatomical and physiological studies conducted throughout the 1970s elucidated a major pathway from the PAG to the raphe magnus and adjacent reticular formation of the RVM and in turn from RVM to the DH. Direct links from the PAG to serotonergic and nonserotonergic (e.g. opioid) neurones of the RVM, as well as to the noradrenergic nucleus of the medulla, are important pathways for expression of its role in the modulation of descending controls. Also, a small population of fibers directly projects from the PAG to the trigeminal nucleus and the DH.

Therefore the termination zone of many **RVM** axons within the superficial and deep layers of the DH matched the region where nociceptors terminated, suggesting that PAG and RVM modulate nociception, although they do not do so specifically or exclusively. Studies reveal that PAG and RVM are capable of altering numerous reactions and responses in addition to those associated with noxious stimulation (Mason 2005, Starowicz et al. 2007). Although the RVM receives direct sensory input, the activity of descending pathways originating therein is primarily modified by afferents from the PAG, PBN and NTS (Fields and Basbaum 1984, Fields and Basbaum 1999, Millan 1999). Based on functional characteristics, several contrasting classes of neurons have been recognized in the RVM. First, "OFF" cells are excited by opioids and inhibited by nociceptive input. They display a transient interruption in their discharge immediately prior to a nociceptive reflex and are thought to participate in the induction of DI. Second, "ON" cells are inhibited by opioids and excited by nociceptive input: they are thought to trigger DF (Fields et al. 1991, Fields and Basbaum 1999, Mason 1999, Zhuo and Gebhart 1992).

The **locus coeruleus (LC)** is considered the main noradrenergic nucleus involved in the ascending and descending control of pain (Stamford 1995, Zhang et al. 1997). It

receives its main inputs from the nucleus prepositus hypoglossi and from the PAG via the paragigantocellular nucleus of the ventromedial medulla. The LC sends a major pathway to the spinal cord, mainly to the DH. Acute impositions of high intensity noxious stimuli have been shown to increase the activity in the LC and the noradrenaline level in the DH (Chiang and Aston-Jones 1993, Crawley et al. 1979, Hong et al. 1993, Men and Matsui 1994, Szot et al. 1993), while electrical stimulation here produced a selective inhibitory action on the discharge evoked by nociceptive cutaneous or visceral stimuli (Guo and Zhao 2000, Liu et al. 2008, Margalit and Segal 1979, West et al. 1993). Under conditions of persistent noxious input, the potentiation of descending noradrenergic input to the DH is pronounced and plays a major role in the moderation of pain (Milne et al. 2001).

Thalamus

The thalamus has been long regarded as the key relay structure for the supraspinal receipt, integration and onward transfer of nociceptive information. The different projections to its nuclei and from them to the cortex define the functional circuitry of pain processing. It encodes information concerning the type, temporal pattern, intensity and topographic localization of pain. Further, it interlinks with cortical and limbic structures responsible for both the sensory-discriminative and emotional dimensions of pain (Millan 1999). In the thalamus, two groups of nuclei are particularly important in the processing of nociceptive information: the lateral and medial nuclear groups.

The lateral nuclear group comprises the ventroposteromedial nuclei (VPM), receiving input from the head through the trigemino-thalamic pathway, the ventroposterolateral (VPL) and the ventroposteroinferior (VPI) nuclei comprising afferentation from the body and limbs via the spinothalamic tract. Neurons in these nuclei respond to both thermal and mechanical stimuli and show a somatotopic organization. The receptor fields in VPI are larger than those in VPL and VPM, and connections of neurons here with the secondary somatosensory cortex (SII) suggest different forms of processing with respect to the sensory-discriminative and affective-cognitive aspects of pain. The VPL and the VPM as the main somatosensory relays process noxious and innocuous information of cutaneous,

muscular, articular and visceral origin. Through the interconnections of primary somatosensory cortex (SI), these nuclei are responsible for the localization and intensity of pain. Neurons of the VPL and VPM are predominantly of the WDR type, they contribute to the sensory-discriminative aspects of thermal, mechanical and tactile information, but through its interconnections with the prefrontal cortex, parabrachial region, amygdala, hypothalamus and PAG, they are also involved in the emotional and autonomic responses of pain.

The medial nuclear group of the thalamus comprises the posterior complex, consisting of the pulvinar oral nucleus, posterior nucleus and the posterior ventromedial nucleus, as well as the dorsal medial, central lateral and the intralaminar nuclei of the thalamus (medial complex). These nuclei receive input mostly from neurons in laminae I and V of the DH through the spinothalamic tract, and laminae VII and VIII. These afferentations indicate that nuclei here play a central role in the integration of painful information. Interconnections of this group with the insular and cingulate cortex suggest that it contributes to the emotional and affective-cognitive components of pain. The posterior region of the thalamus receives nociceptive information from the spinal cord by both the spinothalamic and spinomesencephalic tracts and its output is to the anterior cingulate cortex, an area with a signal processing role in nociception. The medial complex has similar projections to these limbic centers, but also includes structures, such as the striatum and cerebellum, responsible for arousal and motor responses.

Higher pain-related centers in the brain

Along with the linearly organized pathway model, the idea of parallel and bidirectional (down-up and up-down) processing of nociception appears to be more in line with the recently acquired information by recent imaging techniques (PET, SPECT, fMRI) on the pain network in the brain (Treede and Lenz 2006). These techniques can provide indirect measures of local brain activity, and made it possible to produce maps representing changes in the cortex, during painful stimulation.

Neurons in several regions of the **cerebral cortex** respond selectively to nociceptive input. Although some experimenters have different results when comparing painful and non-painful conditions, some regions are widely agreed to contribute to nociception. They include principally the contralateral primary and secondary somatosensory cortices (SI and SII), the anterior cingulate cortex (ACC), the insular cortex (IC) and regions of the frontal cortex (Berman 1995). Considering afferences to thalamic nuclei and their cortical projections, two systems of nociceptive projections are distinguished, i.e., the lateral and medial systems.

The lateral system participates directly in the sensory-discriminative attribution of nociception and involves thalamic nuclei projecting to NS and WDR neurons in SI and SII. Cell types are considered to code different modalities of nociception. Although they are both able to code the intensity of stimuli, this function seems to be related more to WDR neurons, whereas NS cells mainly act on the topographic localization of peripheral stimuli. In addition, nociceptive neurons located in the SII have been reported to code the painful stimulus in temporal terms (Timmermann et al. 2001, Treede et al. 1999). Since SI and SII cortices are interconnected with the posteroparietal area and the insular cortex through a cortico-limbic pathway, somatosensory information is associated with other sensory modalities and also with learning and memory.

The medial nociceptive system has less defined projections from the medial region of the thalamus to SI, SII and also to limbic structures such as the IC and the ACC (Picard and Strick 1996). This system is considered to contribute to the motivational-affective component of pain, but participates in the sensory-discriminative circuitry as well.

The **ACC** is part of the limbic system and is thought to be involved in processing the emotional component of pain. It receives anatomical projections from several sources, including the IC. The ACC is the most agreeable brain region activated in brain imaging studies of pain and pain-related activation is reported most consistently in the ventral part of the supracallosal ACC, in the dorso-caudal ACC, and occasionally in the perigenual area (Hsieh et al. 1996, Peyron et al. 2000, Price 2000). The supracallosal area may be involved more specifically when the afferent input is of somatic origin and has an intrinsic affective value, whereas the dorsal sector of the ACC may be involved when extrinsic, secondary affective value is attributed to stimuli, such as in cognitive studies (Rainville 2002). These

studies show that ACC is more directly involved in pain affect than in appreciating the sensory qualities of pain. Cingulotomy in patients suffering from intractable pain modifies emotional and behavioral reactions to pain, without impairing the ability to localize a painful stimulus (Ballantine et al. 1967, Foltz and White 1968).

The **IC** receives direct projections from the the ventral and posterior medial thalamic nuclei, and processes information on the internal state of the body, thus contributing to the autonomic component to the overall pain response. Damage to large parts of IC has been found among patients with pain asymbolia (Berthier et al. 1988, Weinstein et al. 1955). Patients with this condition do not display behavior indicative of threat or intrusion in response to painful stimuli despite their capacity to still appreciate the sensory qualities of painful stimuli. The IC may therefore integrate the sensory, affective and cognitive components of nociception.

The **amygdala** complex is a medial temporal lobe brain structure, which, as a part of the limbic system is generally believed to be involved in the neural substrates of emotion. The amygdala is directly linked to nociceptive centers in the spinal cord and brainstem through the spino-ponto-amygdaloid pathway from the pontine parabrachial area to the central nucleus of the amygdala (Bernard et al. 1996, Bernard and Besson 1990, Neugebauer and Li 2002). Apart from concerning the emotional components of pain, the amygdala might also be involved in learning the association between painful and neutral stimuli, to be able to avoid previously met aversive conditions (Buchel et al. 1999). Its central nucleus has direct connections with the PAG, the PBN, the thalamic nuclei and the IC: structures which play an important part in pain regulation (Xu et al. 2003).

The **hypothalamus** is another key structure involved in pain modulation and transmission. Its various nuclei have been strongly implicated in fear, emotional memory and behavior, and autonomic and somatomotor responses to threatening stimuli. Descending pathways extend from hypothalamus to the brain stem. Hypothalamic fibers, modulating afferent noxious stimuli, project to the thalamus, medulla oblongata (including nuclei of the trigeminal nerve), PAG and the substantia gelatinosa of the DH of the spinal cord (Sawchenko and Swanson 1982).

The **hippocampus**, as another part of the limbic system, participates in important brain functions like learning and memory, attention and arousal, and is also involved in

stress- and pain-related behavioral responses. Analgesia produced by intrahippocampal lidocaine (injected into the dentate gyrus) provides evidence of the involvement of the hippocampal formation in pain perception (McKenna and Melzack 1992). Nociceptive information is processed in distributed fashion by the hippocampus, and at least the ventral CA1 is implicated in nociceptive intensity-dependent integrative functions (Khanna et al. 2004). Two kinds of pain-related neurons were found here: pain-excited neurons (PEN) and pain-inhibited neurons (PIN). Experimental data suggest that muscarinerg acetylcholine receptors (mAchRs) play an important role in the modulation of nociceptive information in the hippocampal formation (Jiao et al. 2009, Yang et al. 2008).

The involvement of the **basal ganglia** in motor functions has been well studied. Evidence from neuroanatomical, neurochemical, and electrophysiological studies suggests that the basal ganglia are also involved in nociception. The basal ganglia circuitry plays role in the sensory, affective and cognitive dimensions of pain, and may also be involved in the modulation and sensory gating of nociceptive information (Chudler and Dong 1995). Some patients with basal ganglia disease (e.g., Parkinson's disease, Huntington's disease) have alterations in pain sensation in addition to motor abnormalities. Frequently, these patients have intermittent pain that is difficult to localize. Rats with a unilateral nigrostriatal lesion show enhanced sensitivity to a wide range of painful stimuli, of both thermal and mechanical nature (Saade et al. 1997, Takeda et al. 2005); the opposite effect – decreased pain sensitivity – can be obtained by electrical stimulation of the substantia nigra pars compacta or by activating striatal dopaminergic receptors (Jurna et al. 1978, Lin et al. 1981, Sandberg and Segal 1978).

Endogenous antinociceptive ligands

Pain is a dynamic phenomenon resulting from the activity of both excitatory and inhibitory endogenous modulation systems. It is well known that a multitude of substances and receptors are involved in the nociceptive system, some of them increase, and others inhibit the pain sensation both peripherally and centrally (Furst 1999, Sandkuhler 1996). Virtually no ligands/receptors are to be found that have not been investigated in this respect. These substances, which include neurotransmitters, neuromodulators, hormones, cytokines, etc., can modify the activity of nerves involved in the pain pathways. One of the physiological functions of the endogenous system is to tonically regulate nociceptive transmission; therefore the ratio of the pronociceptive and antinociceptive ligands determines the pain sensitivity. A very exciting and rapidly developing field of pain research relates to the roles of different endogenous ligands, acting on different receptor mechanisms. These substances have potentially advantageous features: their synthesizing and breakdown enzymes are available in the body; therefore, they have shorter half-lives and lower toxicity (Kristensen et al. 1993). On the other hand, certain endogenous ligands have lower specificity and affinity for their receptors compared with exogenous drugs, and they exert their effects at several types of receptors at different parts of the body (Fields et al. 1991). Therefore, the net effect depends on the localization of the ligands/receptors, and on which receptors and where they will be influenced by a ligand.

Anandamide

Both natural and synthetic cannabinoids (CBs) potently reduce pain-related behavior (Hohmann 2002, Pertwee 2001, Walker et al. 2002). Thus, CBs are highly effective against thermal, mechanical and chemical pain and are comparable to opiates in both potency and efficacy (Walker et al. 2002). A major limitation to the potential use of CB agonists as therapeutic agents is the profile of side effects, which include dysphoria, effects on motor coordination, memory, and abuse potential (Carlini 2004). An alternative approach, which may avoid such side effects, is to manipulate the endogenous CB system. The

endocannabinoid system consists of endogenous cannabinoids, cannabinoid receptors and the degrading enzymes responsible for synthesis and degradation of endocannabinoids. Soon after the identification of the CB receptors, it was discovered that the brain produces endogenous cannabinoids, which are capable of activating these receptors (Devane et al. 1992).

CB_1 receptors are present in the central nervous system and also in some peripheral tissues including pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, sympathetic ganglia, heart, lung, urinary bladder and adrenal gland (Guindon and Hohmann 2007, Szabo 2008). Centrally the cerebral cortex, hippocampus, lateral caudate putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus and the molecular layer of the cerebellum are all populated with particularly high concentrations of CB_1 receptors, a distribution pattern that is consistent with the well-established ability of cannabinoids to alter locomotor activity and produce catalepsy, particularly in rodents, and to impair cognition and memory. Additionally, CB_1 receptors are found on pain pathways in the brain and spinal cord and probably also at the peripheral terminals of primary afferent neurons and these receptors presumably mediate cannabinoid induced analgesia (Pertwee 2001). CB_2 receptors, on the other hand, are expressed mainly by immune cells, particularly those derived from macrophages, such as B-cells, natural killer cells, microglia, osteoclasts and osteoblasts, but it has also been identified on neurons, under certain conditions particularly (Pertwee 1997). A common property of CB_1 and CB_2 receptors appears to be the ability to modulate spontaneous or evoked release of chemical messengers, generally to suppress neuronal excitability and inhibit neurotransmission. These actions seem to be signaled through the inhibitory G_i and G_o proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase, and also to various ion channels, (positively to A-type and inwardly rectifying potassium channels and negatively to N-type and P/Q type calcium channels and to D-type and postsynaptic M-type potassium channels) (Howlett et al. 2004, Pertwee 1997, Pertwee 2001). Additionally CB_1 receptors can also couple to G_s proteins to activate adenylate cyclase and/or to reduce outward potassium current, possibly through arachidonic acid-mediated stimulation of protein kinase C (Demuth and Molleman 2006). However evidences exist, that cannabinoid

receptor signaling and G-protein coupling efficiency is not the same in all brain areas (Devane et al. 1992).

The endogenous cannabinoids are lipid derivatives and a feature that distinguishes them from many other neuromodulators is that they are not synthesized in advance and stored in vesicles. Rather, their precursors exist in cell membranes and are cleaved by specific enzymes on demand. Endocannabinoids are released generally postsynaptically, and act presynaptically (Walker et al. 2005). The first endocannabinoid identified was arachidonoyl-ethanolamine (anandamide: AEA), isolated from porcine brain and characterized as an endogenous eicosanoid with moderate affinity for the CB_1 and CB_2 receptors (Devane et al. 1992). Several lines of evidence suggest that AEA also activates other G protein-coupled receptors (GPCRs) and ion channels. The best known and characterised of these ion channel interactions is the activation of the transient receptor potential vanilloid 1 (TRPV1) by AEA (Hajos et al. 2001, Olah et al. 2001, Oz 2006, Tognetto et al. 2001, van der Stelt et al. 2005, Zygmunt et al. 1999). TRPV1 is a ligand-gated nonselective cation channel that is considered to be an important integrator of various pain stimuli such as capsaicin, heat and low pH (Jancsó et al. 1977, Jancsó and Lawson 1987, Kau et al. 1991, Szekely et al. 1997, Yu et al. 2003). Since CBs and TRPV1 receptors show co-expression in brain neurons, and AEA represents "chimeric" ligand acting on both cannabinoid and TRPV1 receptors, their co-activations can lead to a cross-talk between them (Starowicz et al. 2008). Some of its effects, including antinociception, may be at least partially due to TRPV1 activation (Di Marzo et al. 2002, Jancsó et al. 1985, Jancsó and Király 1980, van der Stelt and Di Marzo 2004, Zygmunt et al. 1999). Previous results in our laboratory have shown that spinal AEA significantly decreased inflammatory thermal pain sensitivity, and its effects were modified by TRPV1 antagonist capsazepine (CAPZ), suggesting that the effective doses of AEA influence not only cannabinoid but also TRPV1 receptors (Horvath et al. 2008).

Endomorphin

Since centuries, morphine has been the gold standard for the treatment of pain, against which all analgesics are compared. Due to several side effects and strong abuse potential, it has been desirable to find a similarly efficient but possibly safer way of antinociception. Early efforts to understand the endogenous targets of opiate drugs led to the identification of receptor sites. Binding studies suggested four main classes of opioid receptors, named μ-, δ-, κ- and opioid receptor-like receptors. Opioid receptors comprise a subfamily of structurally homologous GPCRs. Activation of these receptors inhibits the formation of cyclic adenosine 3',5'-monophosphate (cAMP), close voltage-gated Ca^{2+}-channels and opens inwardly rectifying potassium channels (Dhawan et al. 1996, Jordan et al. 2000, Lambert 2008). The net effect of these cellular actions is to reduce neuronal excitability and neurotransmitter release.

Opioid receptors and their endogenous ligands are widely distributed in the organism, thus the activation of this system might lead to effective antinociception (Akil et al. 1984, Bach 1997, Basbaum and Fields 1984, Bodnar and Klein 2004, Bodnar 2008, Horvath 2000, Menetrey and Basbaum 1987, Palkovits 2000, Pan et al. 2008, Rittner et al. 2008, Vaccarino et al. 2000). The antinociceptive effects are produced by peripheral, spinal and supraspinal levels as well (Przewlocki et al. 1999). During inflammation of the peripheral tissues, numerous mediators are produced by endothelial cells, resident cells, and leucocytes that are recruited to the site of injury. Leukocytes are the important source of the endogenous opioid peptides, and in peripheral inflamed tissue β-endorphin, Met-enkephalin, dynorphins and endomorphins are produced and released by these cells (Labuz et al. 2006, Mousa et al. 2002, Rittner et al. 2008). Opioid receptors located within the superficial DH, in lamina I. and particularly in lamina II, and in the DRG of sensory neurons undergo axonal transport to reach peripheral nerve terminals, and inflammation induces increases in μ-opioid receptor binding within DRG leading to an improved antinociceptive potency in these circumstances (Endres-Becker et al. 2007, Mousa et al. 2007, Zollner et al. 2003). Some of the analgesic actions of opioids may be due to modulation of the descending pathways (originating in RVM, PAG, LC, ACC, prefrontal

cortex and thalamic nuclei) to reduce nociceptive transmission in the DH (Anderson et al. 1977, Basbaum and Fields 1984).

Twelve years ago a novel group of specific μ-opioid receptor agonist tetrapeptides was discovered and named endomorphins (EMs). Endomorphin-1 (EM1) and endomorphin-2 have been isolated first from bovine and then from human brain cortex by Zadina et al. (Zadina et al. 1997). Compared to morphine, they possess partial, rather than full agonist properties at μ-opioid receptor sites, their effects are temporary and there is also an evidence suggesting a plateau effect, although the potencies of the drug and the duration of the effects seemed to depend on the species, on the applied pain tests, and on the route of administration (Horvath et al. 1999, Stone et al. 1997). Intraplantar administration of EM1 dose-dependently decreased the mechanical allodynia and the thermal hypersensitivity in neuropathic and inflammatory pain models (Labuz et al. 2006, Obara et al. 2004). Intracerebrovascular or intrathalamic administration of EMs produced antinociception in both acute and chronic pain models (Zadina et al. 1997, Zhao et al. 2007, Zubrzycka et al. 2005, Zubrzycka and Janecka 2008). Our previous results have demonstrated that intrathecal administration of EM1 is an effective method of inhibiting thermal hyperalgesia in rats (Csullog et al. 2001, Horvath et al. 1999).

Adenosine

Adenosine (ADE), originating from adenosine 5-triphosphate (ATP), is recognized to be an important modulator of neurotransmission in many physiological functions, such as regulation of arousal and sleep, anxiety, cognition and memory (Dunwiddie and Masino 2001, Haas and Selbach 2000, Sawynok and Liu 2003). It is well known that the stimulation of its GPCR receptors (A_1, A_{2A}, A_{2B} and A_3) modifies pain signaling, and a variety of molecules have been developed to provide analgesia through this non-opioid mechanism (Poon and Sawynok 1998, Sawynok 1998). A_1 receptors are present on the cell body of dorsal root ganglion cells and on the central terminals of primary afferent neurons (Macdonald et al. 1986, Santicioli et al. 1993). The action of A_1 receptor agonists appears to be directly on the sensory nerve terminal itself and results from inhibition of adenylate

cyclase and a decreased production of cAMP (Fredholm et al. 1990, Khasar et al. 1995a). The role of the A_1 adenosine receptor in inhibiting spinal sensory transmission has been confirmed by the inhibitory effect of A_1 analogues on the C-fiber-evoked responses of wind-up and postdischarge of dorsal horn neurons, associated with nociceptive information (Reeve and Dickenson 1995). Actions due to adenosine A_2 receptor activation have been proposed to result from stimulation of adenylate cyclase resulting in an increase in cAMP levels in the sensory nerve terminal (Khasar et al. 1995b, Taiwo and Levine 1991). The pronociceptive actions of A_3 receptor activation are mediated by an effect on mast cells to release histamine and 5-hydroxytryptamine (5-HT), an action likely mediated by increased inositol 1,4,5-triphosphate (IP_3) production and enhanced intracellular Ca^{2+} (Ramkumar et al. 1993, Sawynok et al. 1997). A study revealed that ADE directly inhibits the TRPV1 channel in vitro, which might influence its antinociceptive potential (Puntambekar et al. 2004).

However ADE analogs cause a number of side-effects and therefore cannot be used for pain therapy, and ADE is only slightly effective in neuropathic and inflammatory pain states, without influencing the normal pain sensitivity (Chiari and Eisenach 1999, Kekesi et al. 2004a).

Interactions of ligands in pain modulation

Accordingly, their effectivity might be lower than that of synthetic drugs, suggesting that these ligands alone would not be ideal drugs for pain therapy. Therefore, a good possibility for overcoming these problems might well be a combination of different drugs (Horvath et al. 2001, Horvath and Kekesi 2006, Kekesi et al. 2002, Kekesi et al. 2004a). Within the endogenous ligands, endomorphins, adenosine and anandamide have been investigated by several authors, but their interactions have not been characterized.

The antinociceptive interactions of ADE receptor and CB agonists with opioids have been widely investigated (Lavand'homme and Eisenach 1999, Welch and Eads 1999). It is well known that synthetic and plant-originated CBs and opioids show synergistic antinociceptive interactions, however the interaction of the endogenous ligands acting at

these receptors were not investigated. Furthermore, only a small number of studies have been made of the interactions of ADE or AEA with drugs acting at other receptors or systems, and a few data are available concerning the effects of coactivation of the ADE and CB receptors (Begg et al. 2002, Dar 2000, Guindon et al. 2006, Horvath and Kekesi 2006, Kekesi et al. 2004b, Kekesi et al. 2004a, Murillo-Rodriguez et al. 2003, Welch and Eads 1999).

Pain sensation in schizophrenia

Schizophrenia is one of the most severe and debilitating psychiatric disorders and a major public health problem. It is a devastating neuropsychiatric syndrome that typically strikes in late adolescence or early adulthood resulting in lifelong disability. Positive or psychotic symptoms, including delusions and hallucinations, are the most apparent manifestation of the disorder. These emerge episodically and usually trigger the first hospitalization in early adulthood. Chronic aspects of the disorder include negative symptoms such as social withdrawal, flattened affect, and anhedonia as well as pervasive cognitive deficits (Schmidt et al. 2008). The lifetime prevalence worldwide is between 0.5 and 1%, accounting for around 20% of all persons treated for mental illness and it appears to be relatively independent of geographic, cultural and socioeconomic variables.

Clinical reports suggest that many patients with schizophrenia are less sensitive to pain than other individuals; this is associated with increased morbidity and mortality (Dworkin 1994, Jochum et al. 2006). The absence of pain report was confirmed by clinical studies of pain reactivity conducted in large samples of individuals with schizophrenia in different medically painful conditions such as acute perforated peptic ulcer, acute appendicitis, ruptured appendix, peritonitis, compartment syndrome, fractures or myocardial infraction (El Mallakh et al. 2005, Lautenbacher and Krieg 1994, Murthy et al. 2004, Rosenthal et al. 1990, Singh et al. 2006, Torrey 1979). Clinically, diminished pain sensitivity in schizophrenia has been linked to key symptoms of the disorder, such as positive symptoms, active flattening, and/or attention deficits. On neurobiological grounds, disturbances in dopamine, serotonin, glutamate and opioids have been proposed to account

for hypoalgesia in schizophrenia. Some authors suggest that it can be related to abnormal excitatory mechanisms, a different mode of pain expression due to cognitive impairments and disturbances of body schema, a decrease in social communication or, as described in most cases, any observed increase in pain perception threshold was the result of "attitude", but not alteration in brain function (Kuritzky et al. 1999). Thus, the current state of science does not provide an unequivocal description of diminished pain sensitivity in schizophrenia therefore, a satisfactory explanation for hypoalgesia in schizophrenia is lacking (Potvin et al.).

There are several tests to examine pain perception in schizophrenia. Most of these are mainly based on a psychophysical method (self measurement of pain perception using a scale) or a method using the signal detection theory (the pain response is measured by the individual's ability to discriminate the sensory stimuli and by response criteria reflecting their attitude after painful stimuli). One experimental study deserves special attention because it has used a neurophysiologic measure of pain reactivity, the nociceptive RIII reflex threshold. The RIII reflex is studied by applying percutaneous electrical stimulation on the sural nerve and recording the reflex motor response from the biceps femoris muscle (a flexor muscle). Studies conducted on healthy participants have shown that the amplitude of the RIII reflex is correlated proportionally with the participant's self-reported pain threshold (Guieu et al. 1994).

However to investigate the pathophysiology and the possible medication of schizophrenia over the years, several effort have been made to generate a possible animal model that could mimic this human disease. There are three main methodical procedures applied mainly on rodents: neurodevelopmental, neurochemical and genetic models.

Encompassing the neurodevelopmental hypothesis of schizophrenia, manipulation of the environment, triggering chronic stress, can modify young animals' nervous system and therefore produce schizophrenia like alterations. As rats represent a social species, interaction with other rats is essential to ensure a normal neurological and physiological maturation; therefore, isolation causes behavioral changes, including decreased pain sensitivity, increased spontaneous locomotor activity, deficits in learning and memory, and increased aggression to altered reactivity to external stimuli (Gentsch et al. 1988, Paulus et al. 2000, Varty et al. 2006, Weiss and Feldon 2001). Social isolation of animals results in

altered neurochemical systems such as enhanced presynaptic dopaminergic function in the nucleus accumbens and the prefrontal cortex, a decrease in presynaptic serotonergic function, and an imbalance in dopamine and 5-HT in the frontal cortex (Crespi et al. 1992, Fone et al. 1996, Jones et al. 1992). These behavioral and neurochemical changes have been suggested to be similar to the changes seen in patients with schizophrenia, thus the postweaning isolation housing paradigm may provide a nonpharmacological neurodevelopmental method of inducing schizophrenia-like behavioral deficits and has potential utility in the screening of novel antipsychotic drugs (Geyer et al. 1993, Muchimapura et al. 2003, Paulus et al. 2000, Roberts and Greene 2003, Varty et al. 1999).

Neurochemical substances applied systemically or locally over different regions of the CNS are often used, either to create a psychotic state, or to study the role of different nuclei of the brain, through their degeneration, in the pathophysiology of schizophrenia respectively. The same neurodegenerative effect can be targeted when surgically destroying the corresponding areas of the brain. There is mounting evidence that the glutamate neurotransmitter system, and in particular N-methyl-D-aspartic acid (NMDA) receptor hypofunction, might be a contributing factor leading to symptoms of this illness (Brenner et al. 2007, Kristiansen et al. 2007, Muller and Schwarz 2006, Stone et al. 2007). NMDA receptor expression and localization is disrupted in patients with schizophrenia, and exposing rodents to NMDA receptor antagonists causes certain schizophrenia-like behaviors (Becker and Grecksch 2004, Guo et al. 2009, Kristiansen et al. 2007).

Lately different genetic models have been used to study the pathophysiology of schizophrenia mainly on knock-out mice lacking enzimes or regulating proteins like dopamine transporter, neuregulin1, reelin or NR1 subunit of the NMDA receptor, etc. (Tuboly and Horvath 2009). Although these models are promising, some of them cause changes not found in humans, and they usually can not reproduce all of the sypmtoms of the disease by themselves. Therefore the combination of the above mentioned different methods might be a more reasonable concept.

Aim of the studies

Earlier studies proved that EM1, AEA and ADE can produce antinociceptive effects at spinal level. The goal of the **first** part of the thesis was to determine the interactions of AEA with EM1 and ADE in an inflammatory thermal pain model at spinal level. We also investigated the effects of the ADE receptor antagonist caffeine (CAFF) on the antinociceptive potency of AEA and ADE. Therefore, the main objectives of the first part of the Thesis were:

1. To determine the dose–response and time course of intrathecally administered AEA and EM1.
2. To characterize the interaction of EM1 and AEA.
3. To test the antinociceptive interaction of ADE and/or CAFF with AEA.

A recent study has shown that subchronic ketamine treatment and subsequent social isolation produces changes in pain sensitivity in adult rats (Becker et al. 2006). These data suggest that these manipulations in adult animals cause only slight changes in pain threshold, and the changes are mainly due to the isolation. Since postweaning social isolation is a more striking stress for juvenile animals, we supposed that combination of experimental approaches, that is, social isolation and treatment with the noncompetitive NMDA receptor antagonist ketamine in young animals might produce an animal model that bears more resemblance to the disease state of patients suffering from schizophrenia, at least in terms of pain sensitivity.

As described above, the nociceptive pathway is now understood to be a dual system at each level, and the sensation of pain is considered to arrive in the CNS with the discriminative component of pain (“first pain”) carried separately by myelinated Aδ-fibers from the effective-motivational component of pain (“second pain”) by unmyelinated C-fibers. Rapid heating of the skin preferentially activates Aδ-nociceptors, whereas a slower rate of heating preferentially activates C-fiber nociceptors (Yeomans et al. 1996, Zachariou et al. 1997).

Therefore, the goals of the **second** part of the Thesis were:

4. To characterize the effects of postweaning ketamine treatment, postweaning social isolation, and the combination of these treatments on acute heat pain sensitivity at low and high temperature.

5. To determine the effect of these manipulations on heat hyperalgesia and

6. To investigate the antihyperalgesic potency of morphine in carrageenan-induced inflammatory model.

Methods

Intrathecal catheterization

The procedures involved in animal surgery and testing were approved by the Institutional Animal Care Committee of the University of Szeged, Faculty of Medicine. Adult, male Wistar rats (224 ± 1.9 g) were anesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg intraperitoneally: i.p., respectively). An intrathecal catheter (PE-10 tubing) was inserted through the cisterna magna and passed 8.5 cm caudally into the subarachnoid space (Yaksh and Rudy 1976), which served to place the catheter tip between Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws (Dobos et al. 2003). After surgery, the rats were housed individually, and they had free access to food and water. Rats exhibiting postoperative neurologic deficits (about 10%) or those that did not show paralysis of one of the hindpaws after 100 μg lidocaine were excluded (Dobos et al. 2003). The rats were allowed to recover for at least four days before the testing and were assigned randomly to the treatment groups (6–15 rats/group).

Social Isolation and Ketamine Treatment

In the 2nd part of our work, after weaning (on day 21–23 of age: 1st day) male Wistar rats were either housed individually or grouped for 21 days in cages measuring 42 × 15 × 12 cm and 42 × 30 × 12 cm, respectively (l × w × h). The animals were treated daily from day 7 to day 20 with either ketamine (30 mg/kg) or saline intraperitoneally. In total, the rats received 14 injections. Duration of treatment was adapted from the study by Becker et al. (Becker et al. 2006). Four experimental groups were studied (n=9–11 rats/housing/treatment condition): saline + non-isolated (sal-niso), ketamine + non-isolated (ket-niso), saline + isolated (sal-iso), and ketamine + isolated (ket-iso). Groups were matched according to body weight (55 ± 0.6 g). Rats were kept in a temperature controlled

room (22 ± 1 ºC); food and water were available ad libitum. The cages were located together in racks so that auditory and olfactory contact was maintained.

The test schedule of the experimental paradigm is presented in Fig.1. A total of three tail-flick test series were performed after the treatment period. The first one was performed 24 h after the last injection, and then all rats were rehoused in groups of 4–6 with similar housing and treatment conditions and remained housed for the next 5 weeks. Two and four weeks later, tail-flick tests were repeated. By this time, ketamine was expected to have been cleared since ketamine administered by i.p. injection has been reported to have a terminal half-time of 5.4–5 h and does not significantly accumulate in the brain (Hijazi et al. 2003). Paw withdrawal test was carried out on the 5th week. The body weights of all experimental groups were measured throughout the investigation period.

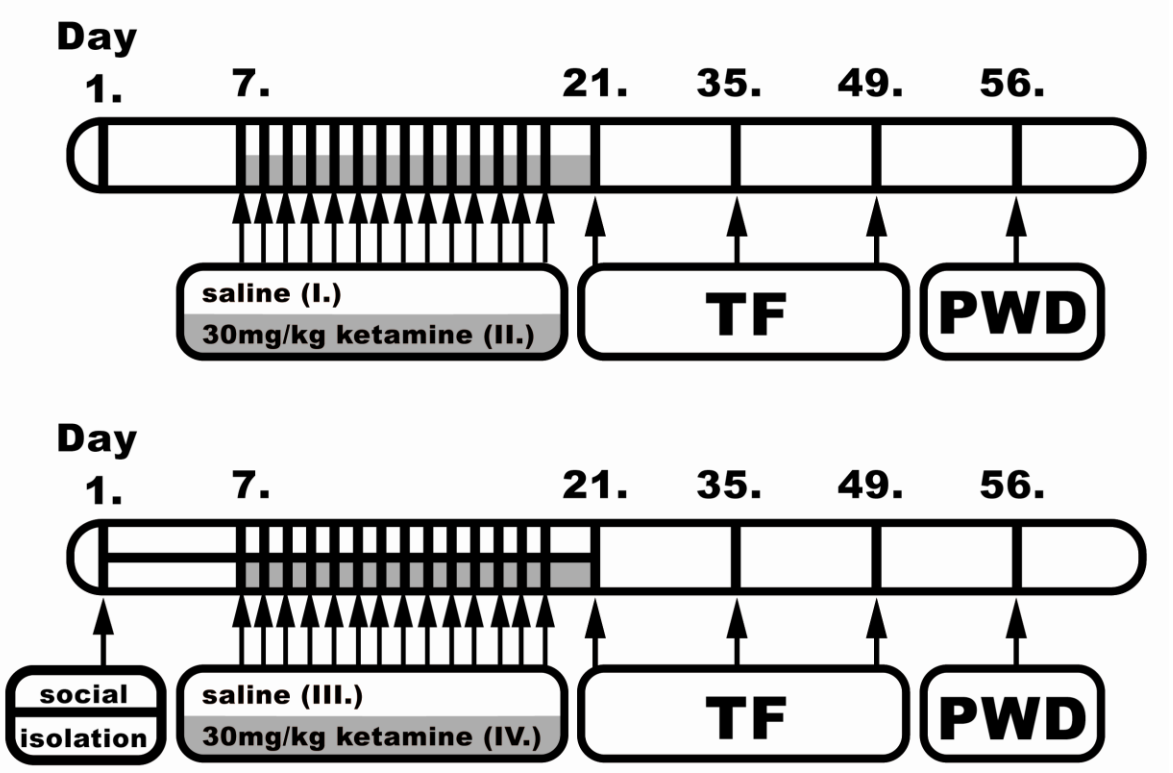


Figure 1. Experimental paradigm for schizophrenia model. TF: tail-flick, PWD: paw-withdrawal test.

Drugs

The following drugs were used: ketamine hydrochloride (Calypsol, Richter Gedeon RT, Budapest, Hungary) morphine hydrochloride (Hungaropharma, Budapest, Hungary), and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany). ADE, CAFF, EM1 and AEA were purchased from Sigma-Aldrich (Budapest, Hungary). AEA (MW: 348 kDa) was dissolved in ethanol: Tween = 2:1, CAFF in ethanol, ADE and EM1 (MW: 610.7 kDa) in saline. Stock solution of AEA and CAFF was diluted with saline to a final concentration of 10% (20 μM) ethanol (and 5% Tween in the AEA solution). Intrathecally administered drugs were injected over 120 s in a volume of 10 μl, followed by a 10 μl flush of physiological saline, and vehicle (Veh) -treated animals formed the control group. In the schizophrenia model experiment physiological saline served as a control against ketamine treatment, and freshly prepared solutions were injected i.p. at a volume of 4 ml/100 g body weight.

Nociceptive testing

The paw-withdrawal test (PWD) was used to measure the antinociceptive effects of the applied substances on carrageenan-induced inflammation (Hargreaves et al. 1988). Rats were placed on a glass surface in a plastic chamber and were allowed to acclimatize to their environment for 15–30 min before testing. The baseline hindpaw withdrawal latencies (pre-carrageenan baseline values at −180 min) were then obtained. A heat stimulus was directed onto the plantar surface of each hindpaw and the intensity of the thermal stimulus was adjusted to derive an average baseline latency of approximately 10.0 s. The cut-off time was set at 20 s to avoid tissue damage.

Unilateral inflammation was induced by intraplantar injection of 2 mg carrageenan in 0.1 ml physiological saline into one of the hindpaws (on the paralyzed side during the lidocaine test; see above (Dobos et al. 2003)). This induced hyperalgesia peaking at 3-4 h after the injection. PWD latencies were obtained again 3 h after carrageenan injection (post-carrageenan baseline values at 0 min). In the case of the first part of the study, AEA, EM1, or their combinations (for the doses applied and the number of animals see Table 2) were

injected after the determination of the post-carrageenan baseline value. PWD latencies were registered 5 min after the injection, and then every 10 min up to 70 min. As regards the experiments with AEA and drugs acting on ADE receptors, 400 μg CAFF or its Veh was injected after determination of the post-carrageenan baseline value (-20 min), the second injection (100 μg ADE or its Veh) 10 min later (-10 min), and the third one (100 μg AEA or its Veh) at 0 min. Since our earlier study showed low potency of ADE, we applied pretreatment with a single high dose of ADE (100 μg) (Kekesi et al. 2004a, Kekesi et al. 2004b). The dose of CAFF administered (400 μg) was based on an earlier study (Esser and Sawynok 2000). The paw withdrawal latencies were registered twice between the injections (at -15 and – 5 min), at 5 min after the third injection, and then every 10 min until 70 min. In the second part of the study, the antihyperalgesic potency of morphine (1, 2, and 3 mg/kg subcutaneously) was determined in the same model for 120 min.

Acute nociceptive threshold was assessed by the tail-flick test. The reaction time in the tail-flick test was determined by immersing the lower 5 cm portion of the tail in hot water (48 and 52 °C) until a tail-withdrawal response was observed (cut-off time: 20 s). The tail-flick latencies were obtained three times (at both temperatures consecutively) at 0, 30, and 60 min and, since they did not differ significantly, they were averaged to establish the pain threshold for each group at both temperatures. There was a 30 min resting period between the measurements. We started the experiments at 48 ^{o}C then proceeded at 52 ^{o}C.

	Endomorphin-1 (μg)				
	0	0.01	0.1	1	10
Anandamide (μg)					
0	11	8	10	11	10
1.5	8	9	9	10	9
10	12	8	8	8	7
30	10	9	8	11	10
100	7				

Table 2. Experimental paradigm, showing the doses of EM1 and AEA, and the number of animals used in each group.

Statistical analysis

Data are presented as means ± SEM. As regards the first series of experiments (interaction of EM and AEA), treatments generally resulted in a short-lasting effect, with the peak occurring at 5–10 min, therefore, their mean values (5 and 10 min) at the inflamed side were used for dose–effect curves and the linear regression analysis. For this analysis PWD latencies were transformed to % maximum possible effect (%MPE) by using the following formula:

%MPE = ([observed latency – post-carrageenan baseline latency]/[cut off time – post-carrageenan baseline latency]) × 100

Dose–effect curves were constructed for both drugs and their combinations. The 35% effective dose (ED_{35}) was defined as the dose that yielded 35% MPE, which means perfect antihyperalgesic effect. Because a higher level of the effect might also be important for therapeutic practice, we also determined ED_{60} for EM1 and the combination with AEA. The ED_{35} and ED_{60} values with 95% confidence intervals (CI) were calculated by linear regression.

Data sets were examined by one-way and two-way analyses of variance and repeated measures ANOVA, while the results of social isolation/ketamine treatment were analyzed using three-way ANOVA (factors: housing, treatment, and time with repeated measurements). The significance of differences between experimental and control values was calculated using the Fisher LSD test for post hoc comparison. A P value less than 0.05 was considered significant. Data were analyzed using STATISTICA 7.1. (Statsoft Inc., Tulsa, OK) and GraphPad Prism (GraphPad software Inc. La Jolla, California, USA) softwares.

Results

Antinociceptive potency of endogenous ligands by themselves

Basal thermal withdrawal latency was 9.1 ± 0.08 s. Carrageenan caused a significant decrease in PWD latency at the inflamed side (3.1 ± 0.08 s), while it did not significantly influence that at the noninflamed side. Because administration of Veh caused a slight decrease in the hyperalgesia, the treated groups were compared to the vehicle-treated one. Anandamide caused dose-dependent antihyperalgesia (Fig. 2.). The ED_{35} value was 67.65 μg (CI: 44.92–90.37), therefore we could not calculate with ED_{60} value. In terms of its time course, the lowest dose was ineffective, but both 30 and 100 μg AEA caused prolonged antihyperalgesic effect through the whole investigated period. However, it should be mentioned that 100 μg AEA also caused temporary vocalization and excitation, suggesting a pain-inducing potential of AEA; therefore, the highest dose of AEA in the combination was 30 μg.

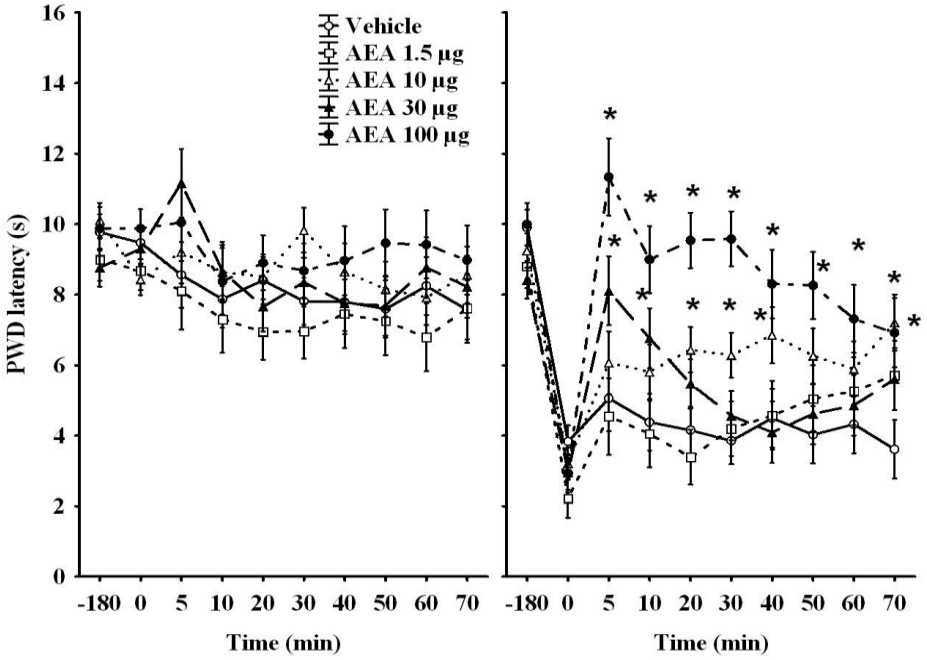


Figure 2. Time course of the effects of AEA on the noninflamed (left) and inflamed (right) sides. The arrows show the injections. Each point denotes the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the vehicle-treated group.

Endomorphin-1 alone caused dose-dependent antinociception at both inflamed and noninflamed sides, that is, 0.01 μg EM1 was ineffective, while 10 μg caused not only a perfect relieve of hyperalgesia, but also a significant antinociception at both sides (Fig. 3.). Regarding the time-course effect of EM1, only 10 μg produced long-lasting antihyperalgesia, whereas its antinociceptive effect was short-lived (5–20 min). The ED_{35} and ED_{60} values were 1.95 μg (CI: 0.6–3.3) and 6.95 μg (CI: 4–8.4), respectively, therefore, its potency was higher compared with AEA.

As regards the effects of ADE (100 μg) and CAFF (400 μg) alone, the comparisons with the control group by two-way ANOVA did not reveal significant differences by treatments (Fig. 4).

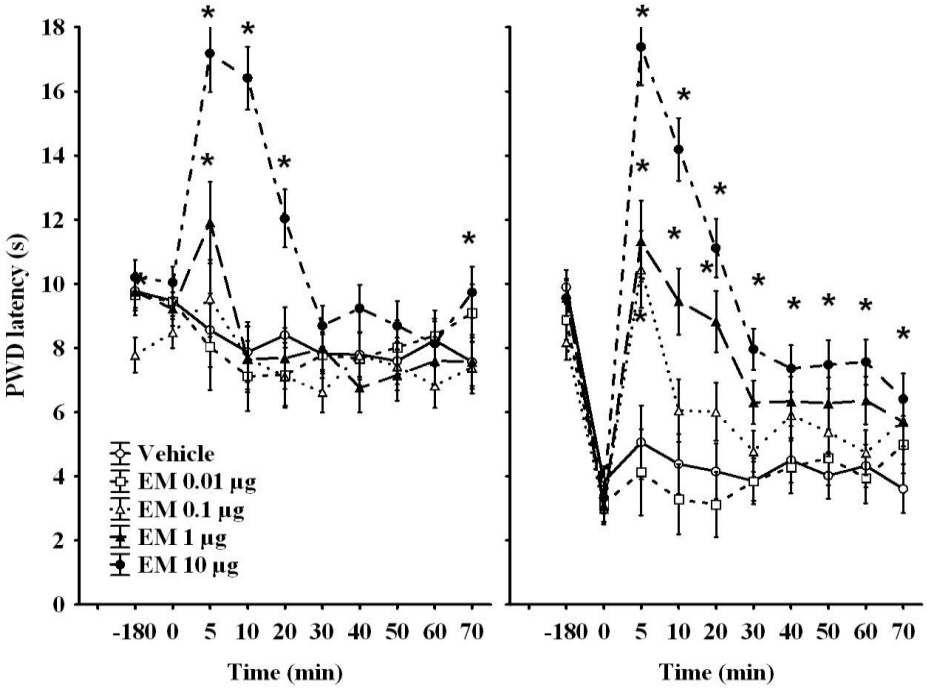


Figure 3. Time course of the effects of EM on the noninflamed (left) and inflamed (right) sides. The arrows show the injections. Each point denotes the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the vehicle-treated group.

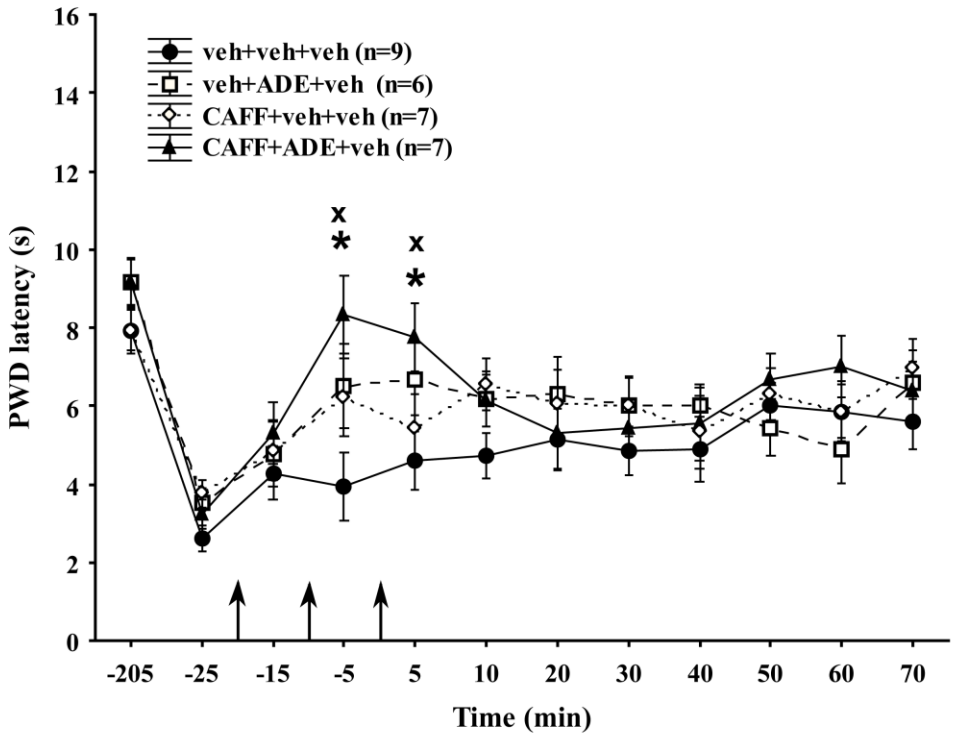


Figure 4. Time course of the effects of ADE (100 μg), CAFF (400 μg) and their combination on the thermal hyperalgesia. The 1st, 2nd and 3rd arrows show the injection of CAFF/vehicle, ADE/vehicle and AEA/vehicle, respectively. The symbol * denotes a significant ($p<0.05$) difference as compared with the vehicle-treated group. The symbol x indicates a non-significant difference between the data point and the pre-carrageenan baseline value.

Interaction of Anandamide and Endomorphin-1

Regarding the interaction of these ligands, the effect of EM1 was not influenced by AEA at the noninflamed side; therefore results were analyzed only at the inflamed paws. 1.5 μg AEA did not change the effects of EM1 in any doses, which is in accordance to its low efficacy (data not shown). The effect of 0.01 μg EM1 was increased by 10 and 30 μg AEA, and the combination of 0.1 μg EM1 and 30 μg AEA was more effective, than by themselves at 5 and 10 min after the administration of the drugs (Figs. 5., 6., 7.). Further combinations (Table 2.) were not more effective than EM1 by itself (data are not shown).

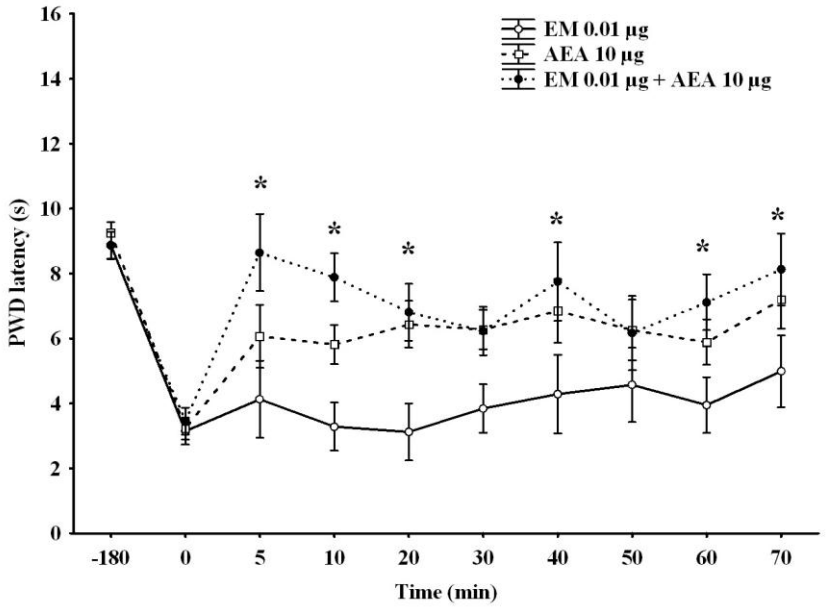


Figure 5. Time course of the antinociceptive effects of 0.01 µg EM in combination with 10 µg AEA. Each point denotes the mean ± SEM. * indicates a significant ($p < 0.05$) difference compared to the EM-treated group. # denotes a significant difference compared to the AEA-treated treated groups.

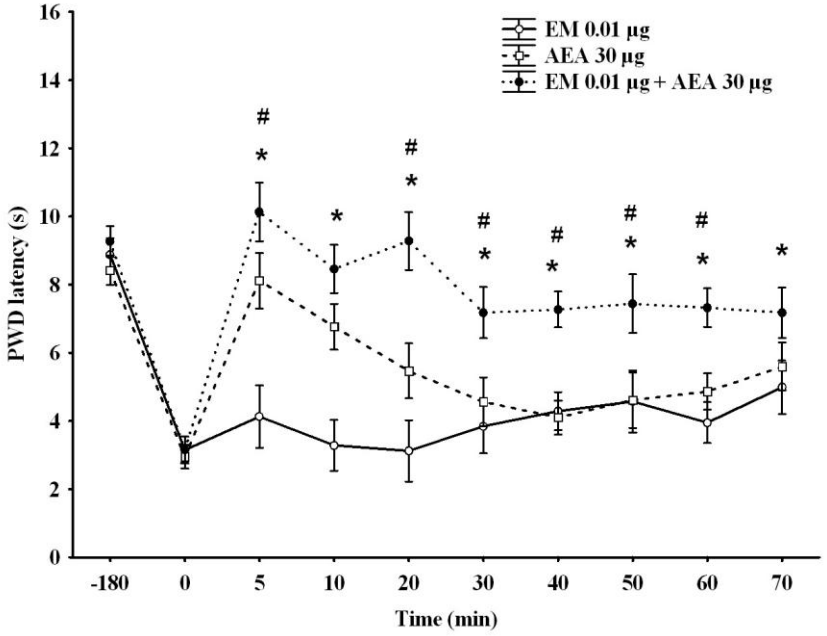


Figure 6. The antinociceptive effects of 0.01 µg EM in combination with 30 µg AEA. Each point denotes the mean ± SEM. * indicates a significant ($p < 0.05$) difference compared to the EM-treated group. # denotes a significant difference compared to the AEA-treated treated groups.

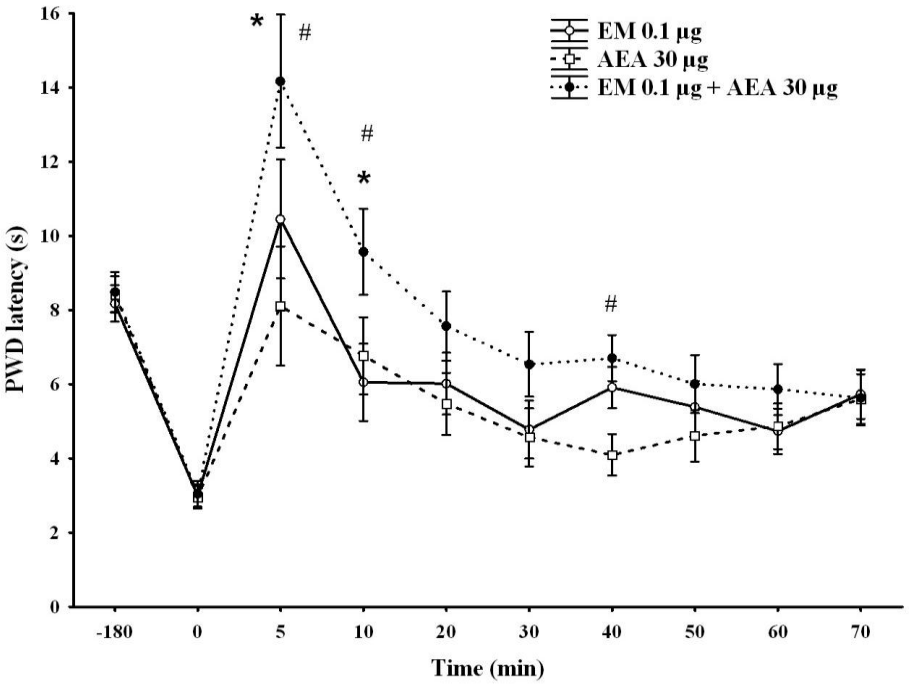


Figure 7. Time course of the antinociceptive effects of 0.1 µg EM with 30 µg AEA. Each point denotes the mean ± SEM. * indicates a significant ($p < 0.05$) difference compared to the EM-treated group. # denotes a significant difference compared to the AEA-treated treated groups.

As the ratio of the ED_{35} values of EM1/AEA was 33.82, the doses of the combinations were calculated in this proportion (Tallarida et al. 1989). The dose–response curves revealed that the slopes of the dose–response curves for EM1 and cocktail did not differ significantly (Fig. 8.). Similarly, the ED_{35} and ED_{60} values of the combination [1.35 (CI: 0.4–2.3) vs. 7.6 (CI: 6.1–9.1) µg] also did not differ markedly from the EM1-treated groups suggesting additive interaction between these ligands.

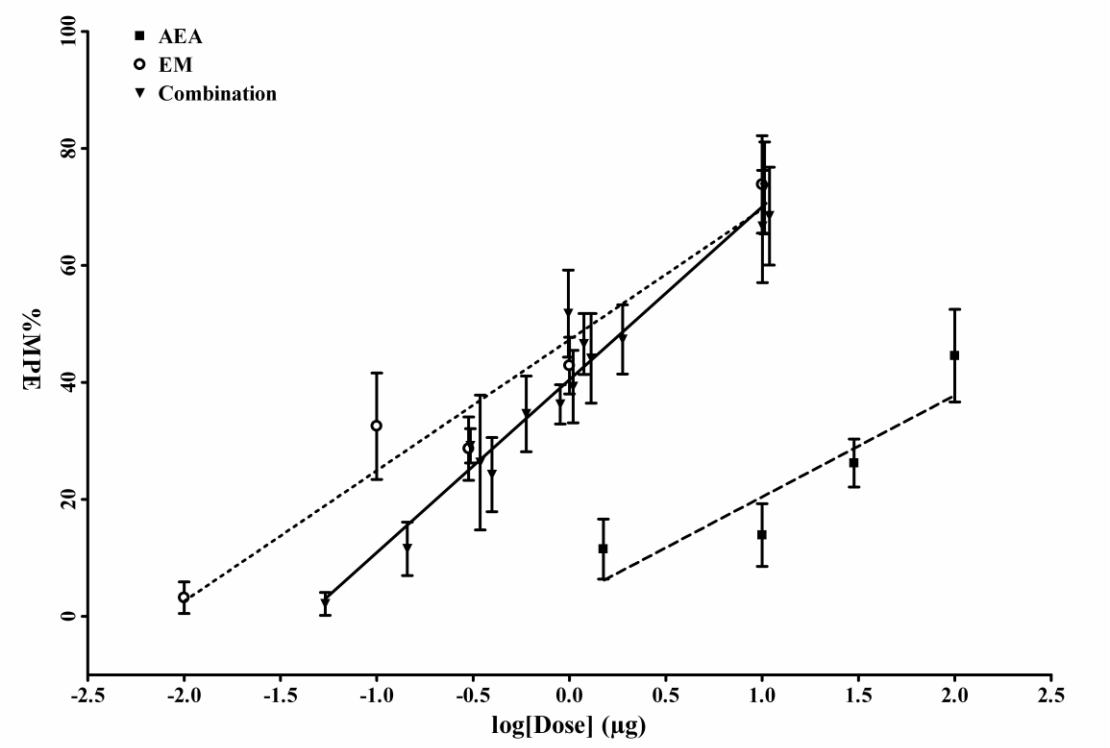
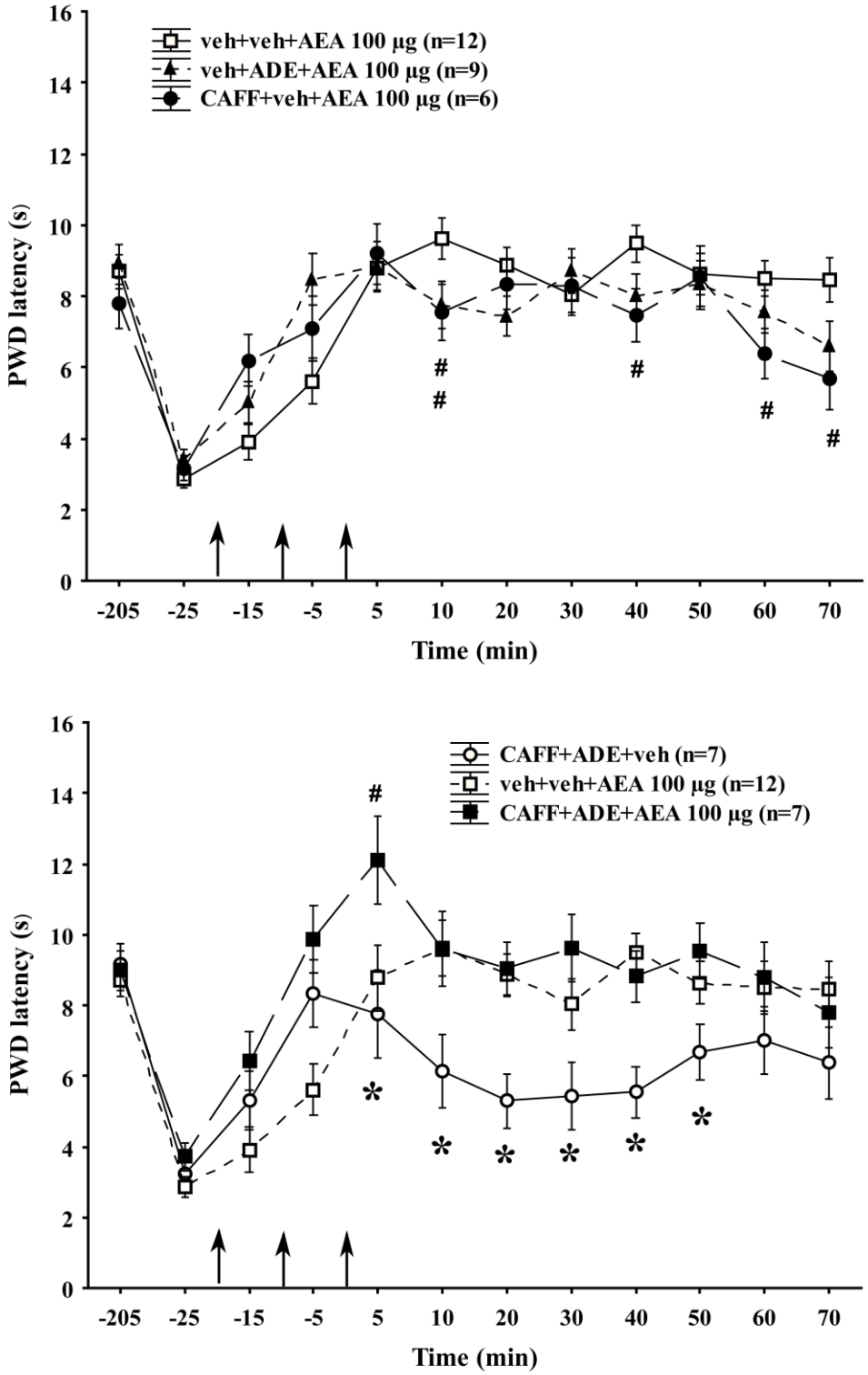


Figure 8. The magnitude of the dose-dependent effects of EM and AEA alone and their combinations.

Interaction of Anandamide with Drugs Acting on Adenosine Receptors

The CAFF and ADE cotreatment caused a significant increase in the paw withdrawal latency relative to the control group, and the post-hoc analysis revealed significant differences at -5 and 5 min, suggesting a short-lasting effect of this combination (Fig. 4). Pretreatment with ADE (100 µg) or CAFF (400 µg) did not change the effect of AEA in lower doses (1 and 33 µg) (data not shown). However, the antihyperalgesic potential of 100 µg AEA was decreased both by ADE and by CAFF (Figs. 9. and 10.). The time-response curve demonstrated that the triple combination of 100 µg AEA + ADE + CAFF was more effective than the combination of CAFF + ADE + Veh between 5 and 50 min, but comparison with the AEA-treated group showed a significant difference only 5 min after the last injection (Fig. 9.).



Figures 9., 10. Time course of the effects of double and triple combinations containing AEA. The 1st, 2nd and 3rd arrows show the injection of CAFF/vehicle, ADE/vehicle and AEA/vehicle, respectively. The symbol * denotes a significant ($p<0.05$) difference from the correspondence group without AEA. #: significant difference as compared with the AEA treatment group by itself.

Social Isolation and Ketamine Treatment

Tail-flick test

As regards the tail-flick latencies at 48 °C, three-way ANOVA revealed a significant effect of housing ($F_{1,38}$ = 17.39, p< 0.001) and time ($F_{2,74}$= 25.01; p<0.001) conditions on pain sensitivity, but ketamine treatment did not influence it. Juvenile isolation resulted in lengthened tail-flick latency when compared with nonisolated rats throughout the investigation period (Fig. 11). However, the difference between the groups decreased on the 35th day; therefore, there were no significant differences between the four groups. In contrast, tail-flick latencies in both isolated groups differed significantly from niso-sal group four weeks later, but not from the niso-ket animals, because the latency in this group moderately increased. In addition, tail-flick latency was significantly longer on the 42nd day when compared with 21st and 35th days in all groups, but there were no significant differences between the values observed on the 21st and 35th days.

The tail-flick latency at 52 °C was significantly shorter when compared with 48 °C at each time point. Three-way ANOVA revealed a significant effect of time ($F_{2,74}$=104.50, p<0.0001) and there was a trend toward significance in the effect of housing conditions (p=0.058) (Fig. 11). In all groups, the tail-flick latency was significantly longer on the 35th and 42nd day when compared with that on the 21st day, but there was no significant difference between data registered on the 35th and 42nd days. Thus the pattern of changes in tail-flick latencies differed at 48 °C and 52 °C (Figs. 11, 12).

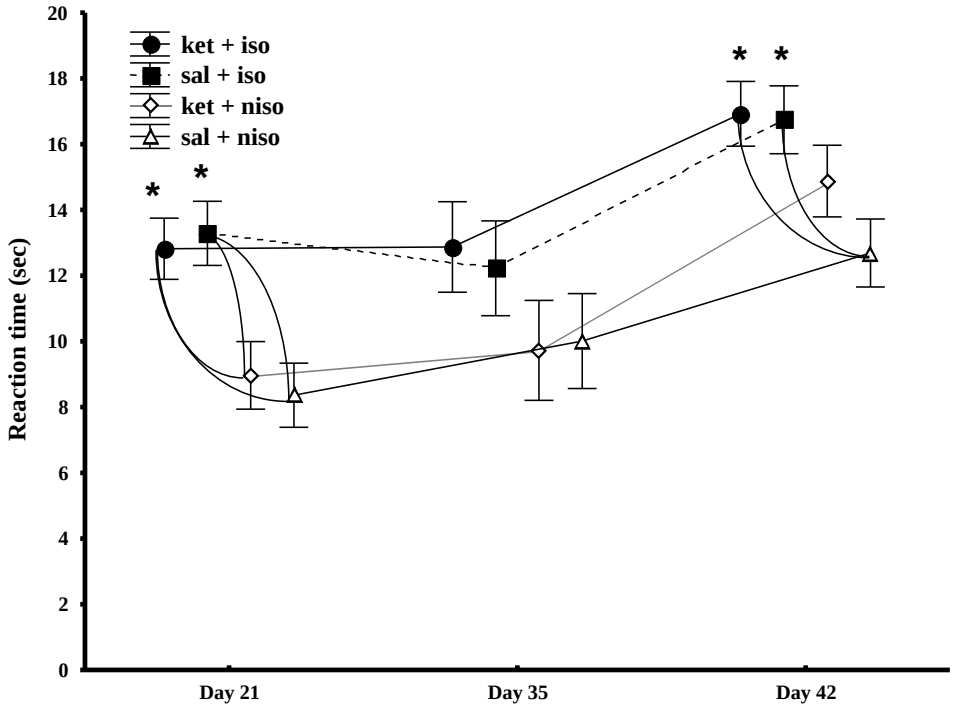


Figure 11. The tail-flick latencies at 48 °C immediately, 2 and 4 weeks after cessation of social isolation and/or ketamine treatment. Each point denotes the mean ± SEM of the results on 9–11 animals. Symbol ***** indicates a significant (p<0.05) difference between groups. Symbol # indicates a significant difference from the tail-flick latency determined on the 21st day.

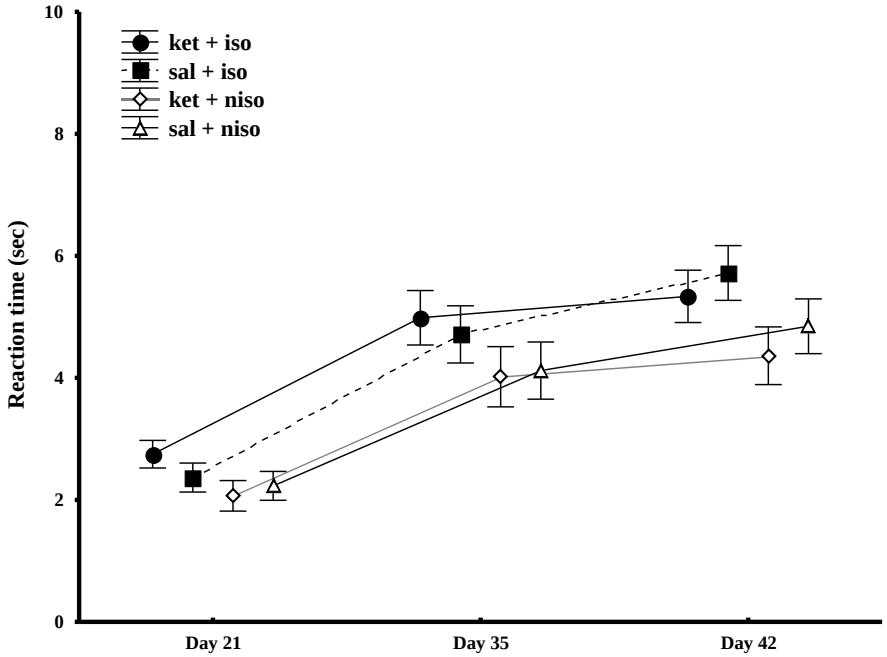


Figure 12. The tail-flick latencies at 52 °C immediately, 2 and 4 weeks after cessation of social isolation and/or ketamine treatment. Each point denotes the mean ± SEM of the results on 9–11 animals.

PWD test

The pre-carrageenan baseline latencies did not indicate significant difference between the groups, but there was a trend toward significance in the effect of housing conditions after the administration of carrageenan on the inflamed side (p=0.14). On either side, 1 mg/kg morphine was ineffective in the groups, while both 2 and 3 mg/kg morphine caused significant increases in PWD latency at several time points (Figs. 13 and 14).

2 mg/kg morphine produced antihyperalgesic effect in all groups except the niso-sal treated animals. As regards the effects of this dose on the non-inflamed side, it caused significant antinociception in each group. The highest dose of morphine produced antinociception and antihyperalgesia in all groups, and there were no significant differences between the groups.

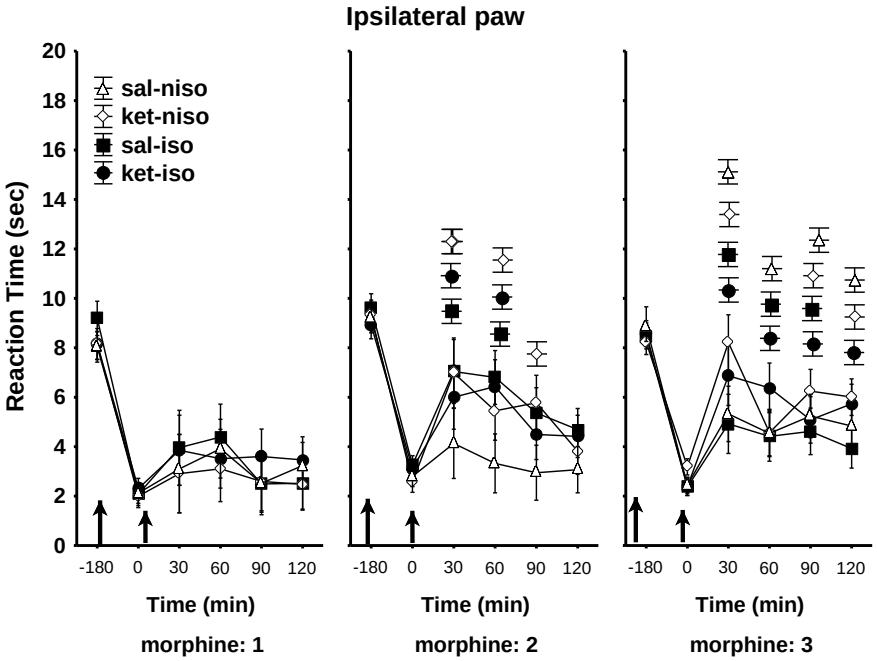


Figure 13. Time-course of the antinociceptive effects of morphine (1, 2 and 3 mg/kg) at inflamed sides on paw-withdrawal test. The first arrow shows the injection of carrageenan, the second one the administration of morphine. Each point denotes the mean ± SEM of the results in 6–8 animals. The group-symbols indicate a significant (p<0.05) difference compared to the post-carrageenan baseline value.

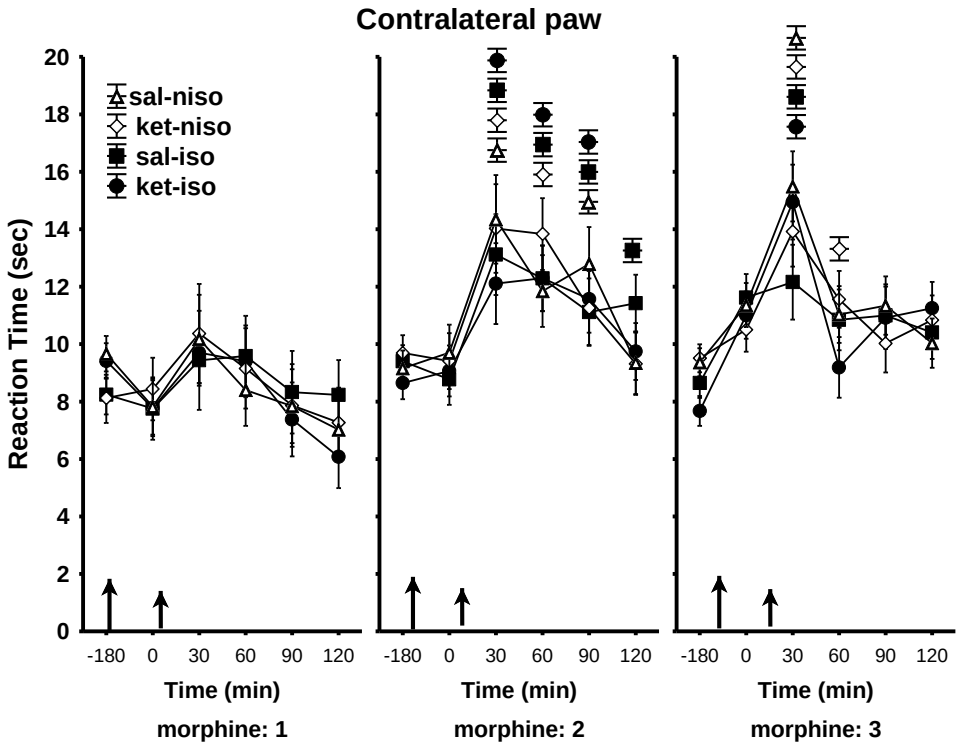


Figure 14. Time-course of the antinociceptive effects of morphine (1, 2 and 3 mg/kg) at non-inflamed sides on paw-withdrawal test. The first arrow shows the injection of carrageenan, the second one the administration of morphine. Each point denotes the mean ± SEM of the results in 6–8 animals. The group-symbols indicate a significant (p<0.05) difference compared to the post-carrageenan baseline value.

Discussion

Antinociceptive potency of drugs at spinal level

The results of the first part of my Thesis show that spinal AEA and EM1 dose-dependently decreased inflammatory thermal pain sensitivity by themselves, and EM1 has much higher potency. ADE and CAFF alone did not induce antinociception. The coadministration of AEA and EM1 in different combinations revealed that only one cocktail showed potentiated antihyperalgesia. The effects of AEA were moderately influenced by ADE, CAFF and their combinations.

As regards the action mechanism of AEA, it may produce antinociception through the activation of CB1 and CB2 receptors (Ahluwalia et al. 2000, Hohmann et al. 1999, Yaksh et al. 2006). Some data have shown that the CB1 antagonists have blocked the antinociceptive effects of AEA, while others indicated only partial antagonism, or even no inhibition at all (Di Marzo et al. 2000, Harris et al. 2001, Yaksh et al. 2006, Welch et al. 1998). Since microglial activation is also associated with pain and CB2 receptors can depress immune cell activation at spinal level**,** it cannot be excluded that the administration of AEA also has anti-inflammatory potency, and this may contribute to its antinociceptive effects (Pertwee 2005, Romero-Sandoval and Eisenach 2007). Di Marzo et al. suggested for the first time that AEA at the spinal level does not produce analgesia only through CB receptor activation (Di Marzo et al. 2000). As AEA activates TRPV1 receptors too, its role in the effects of AEA should be considered (Zygmunt et al. 1999). Thus, it was observed that AEA at the highest dose caused temporary painful behavior, also suggesting activation of the TRPV1 receptors, which integrate multiple pain stimuli (Hayes and Tyers 1980, Jancso and Jancso-Gabor 1980). The activation of TRPV1 receptors by capsaicin not only induces the excitation of nociceptors and the release of pain-inducing transmitters, but also causes the release of endogenous antinociceptive ligands, such as beta-endorphin or somatostatin (Bach and Yaksh 1995b, Jancsó et al. 1985, Jancsó and Lawson 1990, Szolcsányi et al. 1998a). Some data suggest that TRPV1, but not CB1 receptors, are involved in AEA induced responses in the dorsal root primary neurons in vitro, and it has

been suggested that the analgesic properties of AEA are likely to be mediated, at least to some extent, by TRPV1 activation in vivo (Ahluwalia et al. 2003, Horvath et al. 2008, Jerman et al. 2002). It has been shown that low dose of capsaicin (0.25 μg intrathecally) induced painful behavior during the injection (similarly to AEA), but it caused short-lasting analgesia (5-15 min), which was decreased by CAPZ (Horvath et al. 2008). Furthermore, Del Carmen Garcia et al. have reported that CAPZ blocked the hypotensive effect of AEA (17–35 μg), demonstrating the in vivo role of TRPV1 activation in the effect of AEA at spinal level (del Carmen Garcia et al. 2003). It may be hypothesized that through the activation of CB1 receptors, AEA at low concentration decreased the transmitter release, while in higher doses it increased the transmitter release via the TRPV1 receptors (Ahluwalia et al. 2003). We presume that the acute activation of primary sensory neurons by high dose of AEA (100 μg) might have caused the short-lasting painful behavior, while the antinociceptive potential of TRPV1 receptor activation might be due to the release of endogenous antinociceptive ligands at spinal level (Bach and Yaksh 1995a, Szolcsányi et al. 1998a, Szolcsányi et al. 1998b). An additional problem is that AEA acts as a noncompetitive inhibitor of serotonine-3 and nicotinic acetycholine receptors, directly inhibits the voltage-sensitive Na^+ channels and influences the glycine channels (Hejazi et al. 2006, Kim et al. 2005, Lozovaya et al. 2005, Oz et al. 2002, Oz 2006). Moreover, it is likely that other GPCRs are also involved in some of the actions of AEA observed in CB-receptor knockout mice (Hajos et al. 2001, Oz 2006). In summary, several systems may be influenced by AEA, and their net effect may be observed under these circumstances.

Similarly to earlier results, the administration of EM1 elicited dose-dependent antinociception with high potency (Csullog et al. 2001, Horvath et al. 1999, Horvath 2000, Tseng et al. 2000, Yu et al. 2004, Zadina et al. 1997). Some data suggested that EMs displayed lower potencies in the mechanical (paw pressure) test than in the heat-pain (TF) test in rats after intrathecal administration, but they exerted high analgesic potency in different inflammatory pain models as well (Csullog et al. 2001, Hao et al. 2000, Horvath et al. 1999, Horvath et al. 2007b, Labuz et al. 2003, Przewlocka et al. 1999, Wang et al. 1999). Since neuropathic pain has been assumed to be resistant to treatment with opioids, it is of particular interest that the EMs have high potency in decreasing neuropathic pain (Przewlocka et al. 1999). EM1, but not EM2, dose-relatedly reduced the Aβ-fiber evoked

responses, therefore, spinal EM2 exerts selective effects on noxious responses, whereas EM1 is non-selective (Chapman et al. 1997). These ligands by activation of μ-opioid receptors on primary sensory, interneurons and projecting neurons in the spinal cord inhibit the transmission of pain stimuli after its intrathecal administration.

The low potency of ADE is consistent with earlier results which indicated that ADE was almost ineffective in different pain models, while data on the antinociceptive potential of CAFF are controversial and the overall evidence from clinical studies is weak (Camann et al. 1990, Diener et al. 2005, Chiari et al. 1999, Kekesi et al. 2004b, Kekesi et al. 2004a, Lavand'homme and Eisenach 1999). Animal studies have suggested that CAFF induces antinociception, but could inhibit the antinociceptive potential of ADE analogs (Sawynok and Reid 1996, Sosnowski and Yaksh 1989). Surprisingly, the coadministration of ADE and CAFF led to a short-lasting antihyperalgesic effect, suggesting some kind of potentiation between them. At first sight this is controversial, however, their interaction might have been complicated by the fact that they influence all types of ADE receptors with different affinities to the receptor subtypes, and studies have demonstrated opposing roles for the receptor subtypes (Patel et al. 2001, Quarta et al. 2004). Furthermore, ADE receptor activation decreases not only the excitatory, but also the inhibitory transmitter release at spinal level, which could mask its antinociceptive potential (Yang et al. 2004). The A_1 receptor has been proposed to exist as a part of a μ opioid and α_2–adrenergic multireceptor complex on the basis of a demonstrated cross antagonism, cross tolerance and cross withdrawal between these systems (Aley et al. 1995). Stimulation of ADE receptors also inhibits the inflammation, therefore, this may contribute to its antinociceptive effect (Cronstein, 1994). Additionally, the mechanism of action of ADE may be complicated by its interaction with the TRPV1 receptors (Puntambekar et al. 2004). It has been shown that ADE and ADE analogs directly inhibit capsaicin-mediated TRPV1 activation, supporting a role of this nucleoside as an endogenous modulator of TRPV1. In contrast, the activation of TRPV1 in the spinal cord and the periphery promotes the increased release of ADE, possibly through increased intracellular Ca^{2+} entry through the TRPV1 (Cahill et al. 1993). CAFF also has several effects on other (nonadenosine receptor-related) systems which might be connected with pain mechanisms. Thus, it inhibits phosphodiesterases, leading to elevated levels of cAMP and cGMP, and it can also mobilize intracellular Ca^{2+} stores by

activation of the ryanodine receptors (Mandel 2002, Sawynok 1998). All of these effects could influence the pain sensitivity even in opposite ways (Yoon et al. 2006). Accordingly, although we expected antagonism between ADE and CAFF, it may be speculated that the potentiation observed here might be due to the concurrent influence of the above-mentioned receptors/systems. The mechanism of the studied interactions should be very complex, as these ligands may affect multiple receptors pre- and/or postsynaptically in the spinal cord (Table 3.) (Hohmann et al. 1999, Schulte et al. 2003, Schulte and Fredhohn 2003, Szallasi et al. 1995).

Ligand	**AEA**	**ADE**	**CAFF**
G-protein related			
CB_1/CB_2	↑		
Other GPCRs	↑		
A_1		↑	↓
A_{2a}		↑	↓
A_{2b}		↑	↓
A_3		↑	↓
Ion-channel			
Glycine-R	↓↑		
Ryanodine-R			↑
NAch-R	↓		
$VGNa^+$	↓		
5-HT3-R	↓		
TRPV1-R	↑	↓	
Enzyme			
phosphodiesterase			↓

Table 3. Action mechanisms of the ligands. ↑,↓: activation or inhibition by the ligand, respectively. CB1/CB2: cannabinoid receptor 1, 2 respectively, A: adenosine, **5-HT3-R**: serotonin-3 receptor; Nach-R: nicotinic achetylcholine receptor, $VGNa^+$: voltage-gated Na^+ channel

It is well known that exogenous opioids and cannabinoids produce synergistic antinociceptive interaction, although in the case of the endogenous ligands only one combination was more effective than the endogenous ligands by themselves. The possible cause of this difference might be the fact that EM1 exerts effects by μ-opioid receptors, while AEA has much more complex effect, as discussed above. We have found that neither ADE nor CAFF potentiated the antinociceptive effect of AEA at spinal level in these pain models, and even some kind of antagonism could be found. Further, the coadministration of ADE and CAFF moderately modifies the antinociceptive potential of AEA. A1 receptor is known to be localized on the same terminals as the CB1 receptors and utilizes the same signal transduction cascade as the CB1 receptors (Ahluwalia et al. 2000, Coggeshall and Carlton 1997). Since AEA and ADE exert opposite effects on the TRPV1 receptors in vitro, we initially expected that the action of AEA on TRPV1 receptors would be inhibited by ADE. We presumed that after blockade of the ADE receptors (by CAFF), ADE would act mainly as a TRPV1 antagonist, and the triple combination of these drugs would therefore antagonize the effect of AEA on the TRPV1 receptors. Our earlier result that CAPZ decreased the antinociceptive potential of AEA led us to expect similar results (Horvath et al. 2007a). However, the triple combination did not change significantly the effectivity of AEA, which might be due to their multifaceted interactions. As μ-opioid, cannabinoid CB1, adenosine and TRPV1 receptors are expressed in the dorsal horn of the spinal cord, and because they are coexpressed, at least partially on primary sensory neurons, EM1, ADE and AEA could all regulate the release of transmitters from the sensory neurons by acting at these receptors (Hohmann 2002, Szallasi and Blumberg 1999). The interactions at the level of the signal transduction pathway on the same synapses or at different synapses are both plausible explanations. The results suggest that TRPV1 receptor activation by AEA might complicate the interaction of CB1 and μ-opioid or adenosine receptors under these circumstances. These effects may change the release of both excitatory and inhibitory transmitters presynaptically from primary sensory neurons and/or postsynaptically from the interneurons, and they can modify activation of the projecting neurons as well.

An important attribute of the present potentiation is the lack of side effects (except in the case of AEA applied in the highest dose), suggesting that combined drug delivery

can, in principle, serve to enhance the therapeutic ratio of the treatment. Furthermore, the coadministration of endogenous ligands might simulate the physiological behavior of the organism. In conclusion, EM1 and AEA cotreatment may be a beneficial combination for pain therapy, however, ADE and AEA cotreatment will presumably not be an ideal combination for inflammatory pain, but further studies are required in other pain models (e.g. neuropathy) to explore their interactions in pain which is induced by different mechanisms.

Pain sensitivity changes in schizophrenia models

This part of the thesis suggests that social isolation for three weeks after weaning causes a long-lasting decrease in acute heat pain sensitivity, that is, single housing condition produced a significant increase in the pain threshold in tail-flick test at 48 °C, and there was also a trend toward significance at 52 °C, suggesting a more pronounced effect on the C-fiber-mediated nociception. These results suggest that the equilibrium of the nociceptive/antinociceptive systems might have changed for a long period of time in our models.

It is well-known that housing conditions are an important factor contributing to modifications in pain perception in animals, and our result is in concordance with earlier data showing that juvenile isolation caused significant changes in pain sensitivity, which might be due to changes mainly in the number and activity of μ-opioid receptors (DeFeudis et al. 1976, Puglisi-Allegra and Oliverio 1983, Szikszay and Benedek 1989, Van den Berg et al. 1999b). However, Becker et al. have found that isolation caused hyperalgesia in hot-plate test, while the threshold in tail root stimulation test did not change (Becker et al. 2006). The differences may be explained by the diversity in the organization of the tests, that is, hot-plate test is mainly structured supraspinally, while tail-flick test is structured largely at the spinal level. Furthermore, Becker et al. used adult Sprague-Dawley animals, and the duration of isolation was shorter (2 vs. 3 weeks), while in the present experiment, juvenile Wistar rats were isolated or socially housed during weeks 4–6 of age, a period with high levels of social play (Pellis et al. 1997, Vanderschuren et al. 1997, Weiss and Feldon 2001). In addition, Becker et al. used social isolation after ketamine or saline treatment; therefore, injections and social isolation were performed consecutively instead of parallel as in this work. Our work focused on the responses to heat stimuli therefore, discussion of functional significance is confined to this sensory modality. It is well-known that C- and Aδ-fibers convey different modalities of pain, that is, myelinated heat nociceptors have higher threshold than unmyelinated fibers (Leem et al. 1993, Treede et al. 1998). Furthermore, they have different chemical phenotypes, different pharmacological

sensitivities, and play different roles in animal models of pain (Lu et al. 2004, Lumb 2002, Yeomans et al. 1996). In order to directly test the hypothesis that ketamine and/or social isolation may exert differential control of activity evoked by different classes of nociceptors, we applied tail-flick tests at both low and high temperatures. The current work demonstrates that social isolation differentially modulates reflexes evoked by myelinated vs. unmyelinated heat nociceptors. Thus, juvenile isolation not only attenuated responses significantly evoked by unmyelinated nociceptors, but it also slightly influenced myelinated nociceptor-induced responses. In this model, preferential inhibition of activity evoked by unmyelinated heat nociceptors would increase nociceptive threshold but maintain transmission of high-resolution input. The nociceptive responses to both high and low rates of skin heating are mediated by neuronal circuits in the spinal cord, but descending control of spinal nociception is a major determinant of pain sensitivity (Yeomans et al. 1996). Activation of neurons in the PAG significantly increases response thresholds to C-fibers but not those to Aδ-fibers; furthermore, PAG lesion decreased the analgesic effect of social isolation suggesting that PAG mediates stress-induced analgesia (McMullan and Lumb 2006, Wiedenmayer et al. 2000). Thus, a possible explanation for our data is that juvenile isolation developmentally alters the maturation of this descending inhibitory system resulting in C-fiber-mediated hypoalgesia. Similar to pain pathways, parallel magnocellular and parvocellular visual pathways exist, and recent results have shown that early-stage perceptual dysfunctions, which may reflect the abnormality of precortical magnocellular pathways, are related to schizophrenia in humans (Cimmer et al. 2006, Keri et al. 2005).

In contrast to the isolation, subchronic ketamine treatment in young animals neither influenced the pain sensitivity nor enhanced the effect of social isolation. At a first glance, this result appears surprising with regard to the established role of NMDA receptors in pain mechanism; however, earlier studies also have shown that ketamine does not influence acute pain sensitivity (Joo et al. 2000, Klimscha et al. 1998). These observations are in agreement with a recent study showing that ketamine by itself does not influence heat pain sensitivity after subchronic administration (Becker et al. 2006). Thus, it seems that subchronic ketamine treatment could not reproduce the hypoalgesia characterized in schizophrenia, while social isolation can replicate it for a long time in tail-flick test, suggesting that this dose and/or duration of ketamine injections do not simulate the

schizophrenic hypoalgesia, thus NMDA function does not play a significant role in acute heat pain sensitivity changes in schizophrenia. Further experiments are needed to elucidate whether longer treatment and/or higher dose of ketamine could lead to more pronounced changes in the isolation-induced hypoalgesia.

During the paw-withdrawal test, we did not find significant differences between the groups as regards the basal values; however, there was a trend toward significance in the effect of housing conditions, on the inflammatory pain sensation. Furthermore, we found an increased anti-hyperalgesic effect of 2 mg/kg morphine 5 weeks after both social isolation and ketamine treatment. The highly test-dependent nature of pain sensitivity changes supports the view that the mechanisms that modulate thermal nociceptive responses evoked from the tail and hindpaw are not uniform (Ackley et al. 2001). Furthermore, inflammation significantly increases the activity of both unmyelinated and myelinated fibers, thus we suppose that neither social isolation nor ketamine treatment could influence significantly these processes for this long period (5 weeks) during carrageenan-induced inflammation (Coggeshall et al. 2004). Becker et al. observed increased antinociception after morphine administration in isolated and ketamine treated animals, but not in the other groups. However, they measured the effect of morphine in acute pain tests (HP, tail-root stimulation) immediately after the isolation, which might lead to the observed differences. It has been shown that juvenile isolation caused region-specific increases in the number of μ-opioid receptor binding sites and a general upregulation of κ-receptors (Van den Berg et al. 1999a). Similarly, Becker et al. have found that the number of opioid receptor binding sites and the relative efficacy of μ-opioid receptors were increased in isolated and ketamine pretreated rats and isolation increased their activity, which might have contributed to the enhanced analgesic potency of morphine (Becker et al. 2006). The involvement of opioids in schizophrenia is a subject of controversial discussion. Whilst some investigators have found different concentrations and alterations in the number of binding sites and genetic polymorphisms, others have reported similar levels in schizophrenics and respective control populations (Danos et al. 2002, Gulya 1990, Zhang et al. 2004). Thus, it has been suggested that hypoalgesia might be due to changes in opioid functions in both schizophrenia and in isolated rat models. However, since neurotransmitters and their receptors (e.g., dopamine,

acetylcholine), which contribute to schizophrenia, also influence pain sensitivity, we should also be concerned about these systems (Altier and Stewart 1999, Sommer 2004).

We observed a progressive increase in tail-flick latency in these young animals during our investigated period at both temperatures. Several studies have shown that juvenile organisms are hyperresponsive to cutaneous stimuli (Al Amin et al. 2004, Falcon et al. 1996). The tail-flick latency showed a progressive increase with age, where it increased after puberty and was maintained throughout adulthood (Al Amin et al. 2004). Changes in responsiveness to suprathreshold noxious stimuli involve maturation of both spinal and descending supraspinal structures. This work was not designed primarily to assess the effects of aging as the animals were tested over a short period only, while most of the studies reviewed by Gagliese and Melzack (2000) have their testing done over one or two years. However, we observed a difference in the pattern of increase of the tail-flick latencies between the two temperatures, that is, the Aδ-fiber-mediated pain threshold increased faster, compared with the C-fiber-mediated pain sensation. The mechanisms underlying this difference are not completely understood, but are probably related to the different maturation of the central and spinal nociceptive mechanisms (Fitzgerald and Jennings 1999).

In conclusion, these results show that juvenile isolation for three weeks produces a long-lasting decrease mainly in the C-fiber-mediated pain sensitivity, suggesting a selective disturbance in the different parallel sensory pathways. In addition, ketamine treatment did not produce effects on acute heat pain sensitivity, but potentiated the antihyperalgesic effect of morphine. Since both social isolation and NMDA treatment are well-known animal models of schizophrenia, our results showed that juvenile isolation but not ketamine administration could simulate hypoalgesia associated with this disease. However, ketamine treatment also influenced the potency of morphine, suggesting that this model also shows some effects on the nociceptive mechanisms.

General Conclusions

1. We have found that the combination of the endogenous ligands EM1 and AEA produced additive antinociceptive interaction, thus their combination may provide a new and beneficial combination for pain therapy with potentially fewer side effects at spinal level.

2. Neither ADE nor CAFF by themselves potentiated the antinociceptive effect of AEA at spinal level in our pain models, and even some kind of antagonism could be found. Further, the coadministration of ADE and CAFF moderately modified the antinociceptive potential of AEA. Thus, ADE and AEA cotreatment will presumably not be a beneficial combination for inflammatory pain, but further studies are required in other pain models (e.g. neuropathy) to explore their interactions in pain which is induced by different mechanisms.

3. We wish to draw the attention to the rapidly evolving recognition that the endogenous ligands may exert effects on several receptors and/or systems, therefore we consider that their in vivo interaction must be very complex and the net outcome after their coadministration could not been predicted from the in vitro results.

4. We firstly demonstrated that juvenile isolation for three weeks (but not ketamine treatment) produces a long-lasting decrease mainly in the C-fiber-mediated acute heat pain sensitivity, suggesting a selective disturbance in the different parallel sensory pathways. In addition, both treatments and their combination potentiated the antihyperalgesic effect of morphine. Since both social isolation and NMDA treatment are well-known animal models of schizophrenia, our results showed that these paradigms can disturb the balance between the endogenous pro- and antinociceptive mechanisms.

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