From the Department of Clinical Neuroscience,
Karolinska Institutet, Stockholm, Sweden

Neurobiological Effects of Early Life Cannabis Exposure in Relation to the Gateway Hypothesis

Maria Ellgren

Karolinska
Institutet

To my dear Fredrik

ABSTRACT

The use of *Cannabis sativa* preparations, such as hashish and marijuana, is wide-spread among young people, including pregnant women. Despite this concern, the consequences of cannabis exposure on the brain during periods of active brain development, such as the prenatal phase and adolescence, is not well known. Several epidemiological studies support the cannabis gateway hypothesis, where early cannabis use is suggested to increase the risk of initiating use of other illicit drugs, e.g., amphetamine or heroin. However, the nature of such direct links are unclear. Therefore, the aim of this thesis was to test experimentally the cannabis gateway hypothesis, i.e., to determine whether cannabis exposure during periods of active brain development alters reward-related behavior and neurobiology for psychostimulant and opioid drugs by the use of animal models.

In the first study, we examined the effects of early adolescent exposure (postnatal day; PND; 28-32, one injection per day) with the synthetic cannabinoid CB_1 receptor agonist WIN55,212-2 and the main psychoactive substance in *C. sativa*, $\Delta 9$-tetrahydrocannabinol (THC) on amphetamine-induced motor behavior and dopamine release in the nucleus accumbens during adolescence. No alterations were evident in the cannabinoid exposed rats, results which did not support the cannabis gateway hypothesis in relation to subsequent psychostimulant abuse.

Next, we investigated the effects of adolescent exposure on subsequent opioid reward-related behavior and the neurobiology of opioid and cannabinoid systems during adulthood. We studied THC exposure across the full adolescent period (PND 28-49), and administered the drug once every third day in order to better mimic the pattern of intermittent use seen in teenagers. The results revealed discrete opioid-related alterations within brain regions highly implicated in reward and hedonic processing (e.g., increased proenkephalin gene expression in the nucleus accumbens and increased mu opioid receptors in the ventral tegmental area). This was coupled to increased heroin intake in a self-administration paradigm and increased morphine conditioned place preference, indicating altered sensitivity to the reinforcing properties of opioids.

Furthermore, in evaluating the adolescent ontogeny of the opioid and cannabinoid systems within limbic-related brain areas, we found that active endocannabinoid- and opioid- related neurodevelopment takes place to a very high extent during this period. Most pronounced were the alterations in endocannabinoid levels in cognitive brain areas, even though alterations were also apparent in reward-related regions.

Finally, we investigated the effects of prenatal cannabis exposure (gestational day 5- PND 2) on subsequent opioid reward-related behavior and neurobiology of the opioid and cannabinoid systems in adulthood. Similar to adolescent cannabis exposure, prenatal exposure induced discrete opioid-related alterations within brain regions highly implicated in reward and hedonic processing. Moreover, elevated heroin-seeking observed during extinction and after food deprivation was evident in the THC exposed rats, suggesting an increased motivation for drug use under conditions of stress.

Taken together, this thesis presents neurobiological support for the cannabis gateway hypothesis in terms of adult opiate, but not amphetamine, abuse, with underlying long-term disturbances of discrete opioid-related systems within limbic brain regions.

LIST OF PUBLICATIONS

I. Ellgren M., Hurd Y.L., Franck J. Amphetamine effects on dopamine levels and behavior following cannabinoid exposure during adolescence. *European Journal of Pharmacology 2004, 497: 205– 213*

II. Ellgren M., Spano M.S., Hurd Y.L. Adolescent cannabis exposure alters opiate intake and opioid limbic neuronal populations in adult rats. *Neuropsychopharmacology 2006, published online July 5*

III. Ellgren M., Artmann A., Gupta A., Tkalych O., Hansen H.S., Hansen S.H., Devi L.A., Hurd Y.L. Time course of limbic-related alterations within the endogenous cannabinoid and opioid systems in a rat model of adolescent cannabis use. *Manuscript*

IV. Spano M.S., Ellgren M., Wang X., Hurd Y.L. Prenatal cannabis exposure increases heroin seeking with allostatic changes in limbic enkephalin systems in adulthood. *Biological Psychiatry 2006, published online July 27*

CONTENTS

LIST OF ABBREVIATIONS

μOR	Mu opioid receptor
2-AG	2-Arachidonoylglycerol
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid CB_1 receptor
CeA	Central amygdala
CPP	Conditioned place preference
DAMGO	[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin
DOPAC	3,4-dihydroxyphenylacetic acid
DPM	Disintegration per minute
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV
FR	Fixed ratio
GABA	Gamma-aminobutyric acid
GD	Gestational day
G-protein	Guanine nucleotide binding protein
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
i.p.	Intraperitoneal
i.v.	Intravenous
ICD-10	International Classification of Diseases 10
MeA	Medial amygdala
Met-enkephalin	Methionine-enkephalin
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
PDYN	Prodynorphin
PENK	Proenkephalin
PFC	Prefrontal cortex
PND	Postnatal day
POMC	Proopiomelanocortin
PR	Progressive ratio
s.c.	Subcutaneous
SEM	Standard error of mean
SN	Substantia nigra
THC	Delta-9-tetrahydrocannabinol
VTA	Ventral tegmental area

1 INTRODUCTION

1.1 CLINICAL BACKGROUND

Cannabis is the most commonly used illegal drug around the world. The use often starts at an early age and surveys have shown that as much as 21% of 16 year olds in Europe report having tried cannabis (Hibell et al 2004). In Sweden, 7% of 16 year olds have tried cannabis and 21% report having had the possibility to try the drug. Cannabis use is also rather common during pregnancy. Statistics from health surveys in the US show that, depending on age and ethnicity, 7-17% of women used cannabis during pregnancy (SAMHSA 2002). Despite the widespread use among these groups, the neurobiological effects of exposure to cannabis during periods of active brain development, like the prenatal phase and adolescence, is poorly understood.

1.2 CANNABIS

Cannabis is produced from different parts of the hemp-plant (*Cannabis sativa*; figure 2). The most common preparations are marijuana, a combination of dried leaves and flower buds, and hashish, dried resin. It is generally smoked in hand-rolled cigarettes or pipes, but also orally ingested by eating e.g. hash brownies.

Figure 1. The hemp plant, *Cannabis sativa*. Detail of drawing from Franz Eugen Köhler's Medizinal-Pflantzen (Gera-Untermhaus 1887).

Hashish and Marijuana contain around 60 active substances of which the main psychoactive substance is Δ-9-tetrahydrolcannabinol (THC), that was isolated in the early 1960s (Gaoni and Mechoulam 1964). The acute effects of smoking cannabis

include a sense of euphoria and well-being, distorted perception of colors, sounds and other sensations, impaired cognitive functions (e.g. short-term memory, learning and problem solving), distorted sense of time, anxiety and paranoid thoughts, impaired motor coordination, increased appetite, increased heart rate and reddened eyes. Repeated intake may lead to addiction, impaired cognitive functions that continue even after acute intoxication, damage on the respiratory system, reduced fertility, and enhanced risk of developing schizophrenia and depression.

1.3   THE CANNABIS GATEWAY HYPOTHESIS

The fact that cannabis use most often precedes the use of other drugs has given rise to the cannabis gateway hypothesis, where it is proposed that early cannabis use increases the risk of initiating use of other illicit drugs, such as amphetamine or heroin. Many population surveys in western societies show that those who started using cannabis at age 14 or younger had a higher percentage of present illicit drug abuse or dependence than those starting at an older age (SAMHSA 2004). Further, several epidemiological studies have supported a link between cannabis use and the use of other illicit drugs (Agrawal et al 2004; Fergusson and Horwood 2000; Lessem et al 2006; Lynskey et al 2003; Stenbacka et al 1993; Yamaguchi and Kandel 1984). Whether there is or not a direct causal relationship between cannabis use and the progression to the use of other illicit drugs has been heavily debated over the last decades. Apparent links could be non-causal given that the same characteristics may cause vulnerability to use cannabis as other illicit drugs (e.g. (Hall and Lynskey 2005; MacCoun 1998; Morral et al 2002). The model of common liabilities explain the observed associations by e.g. genetic predisposition, peer-pressure, drug availability, risk-taking behavior and underlying possible gateway-effects of nicotine or alcohol. Indeed, support have been found for shared genetic risk factors contributing to the association between use of cannabis and other drugs in twin studies (Agrawal et al 2004; Lessem et al 2006; Lynskey et al 2003), however, they could not rule out the effects of other factors.

Possible direct causal links between early cannabis use and subsequent abuse of other illicit drugs could reflect neurobiological disturbances to early cannabis exposure that makes individuals more vulnerable to the reinforcing effects of other drugs. One strategy to evaluate the relationship of prior cannabis experience with specific drugs, independent of e.g. cultural, social and moral factors is the use of experimental animal models.

1.4   DRUG ADDICTION

Sweden is estimated to have 26 000 heavy drug addicts (individuals that have had daily drug use during the last four months or intravenous intake at least once during the last 12 months). Polysubstance abuse is very common and cannabis is reported to be the main drug for 8% of the heavy drug addicts. In most cases though, amphetamine or heroin is the main drug.

However, not all individuals that try addictive substances do become dependent, and the drug taking behavior is therefore clinically divided into different stages; use, abuse and dependence. Substance use is defined as recreational intake of drug for non-medical purposes (e.g. social alcohol drinking). Diagnostic criteria of substance abuse and dependence are given in the Diagnostic and Statistic Manual of Mental Disorders (DSM-IV) by the American Psychiatric Association (APA 1994). Substance abuse is

2

defined as continued drug use despite harmful effects on a social and/or personal level (e.g. failure to fulfill obligations at work, school or home or physical hazards). The hallmark for substance dependence is a loss of control over the drug use, leading to compulsive intake. It is a chronic relapsing disorder often associated with tolerance and withdrawal. Diagnostic criteria for substance dependence are also given in the International Classification of Diseases and Health Problems (ICD-10) from the World Health Organization (WHO 1992), that are essentially the same as DSM-IV. They both use the term "substance dependence", but "drug addiction" is a term that also is used to a wide extent. The latter emphasizes a behavioral disorder and is less likely to be confused with physical dependence, and is therefore preferred in this thesis. The Swedish language has a word for dependence ("beroende"), but lack a specific word for addiction.

Addictive drugs can motivate repeated drug use by acting as positive reinforcers (producing euphoria) or as negative reinforcers (alleviating withdrawal symptoms or other negative states). The positive reinforcing properties of a drug is usually most important in the initiation of drug use, but is decreased with repeated use.

Different theories have evolved in attempts to understand the mechanism behind the transition from controlled intake of drug to drug addiction. Koob and LeMoal have proposed a theory of hedonic allostasis, where addiction is presented as a cycle of spiraling dysregulation of reward systems (Koob et al 1997; Koob and Le Moal 2001). Physiological systems are kept stable by homeostasis, a self-regulating process for maintaining body parameters around a set point critical for survival. However, during certain conditions, counteradaptive processes fail to return within the normal homeostatic range. Koob and LeMoal suggest that repeated drug intake leads to dysregulation of reward circuits, creating an allostatic state, i.e. a chronic change in hedonic set point. Activation of brain and hormonal stress responses is also involved in this process. The allostatic state drives further intake, ultimately compulsive intake, and in turn exaggerates the allostasis.

The incentive-sensitization theory of addiction proposed by Robinson and Berridge focus on how drug cues trigger excessive incentive motivation for drugs, leading to compulsive drug seeking, drug taking and relapse (Robinson and Berridge 1993; Robinson and Berridge 2001). Addictive drugs are hypothesized to alter mesolimbic brain systems that mediate a basic incentive-motivational function, the attribution of incentive salience, so they become hypersensitive or "sensitized". Dissociation is made between the incentive value of the drug ("wanting") and its pleasurable hedonic effects ("liking"), and the sensitization of neural systems leads to pathological "wanting" to take drugs. The psychomotor-activating effects of many drugs of abuse can also be sensitized and these are mediated by brain systems that overlap with those involved in reward (Wise and Bozarth 1987).

1.4.1 Animal models in drug addiction research

Animal models are very important tools in the work towards understanding the addiction process and in the search for medical intervention. Passive administration of drugs by the experimenter, giving a precise dose and timing, is a good method to use when the general acute or chronic pharmacological effects are studied. However, more

refined methods are available for the evaluation of the reinforcing properties of a drug and for modeling different features of human addiction behavior.

1.4.1.1 Self-administration

In this model, the animal controls its own drug intake and normally develops a behavioral pattern with many similarities to human addictive behavior. The animal is trained to perform an operant (press a lever or do nose poke) to receive the drug, most typically by the means of an intravenous infusion, but it could e.g. also be an intracranial infusion or presentation of alcohol for oral intake. The drug delivery is accompanied by a presentation of stimuli, e.g. a light or the sound of the activated pump, which becomes conditioned cues to the drug effects. If the self-administered substance has positive reinforcing effects, lever pressing will be continued and the animal will acquire a stable self-administration behavior. The basic paradigm is the fixed ratio 1 (FR-1) schedule, where one lever press results in one drug delivery and the animal can then easily regulate the drug intake. However, the level of intake on an FR-1 schedule does not dissociate between "liking" and "wanting". One way of measuring the "wanting" is to use a progressive ratio (PR) paradigm, where the number of lever presses required for a drug delivery progressively increases. By finding out how many times the animal is willing to press the lever before giving up provides a measure of the motivation to get the drug. The "wanting" could also be studied in terms of drug-seeking in a reinstatement paradigm. Extinction of the self-administration behavior is achieved after repeated sessions where no drug is delivered upon lever-pressing, only the conditioned cues are presented. Once the self-administration behavior is extinguished, different events such as presentation of drug-associated cues, stress and drug exposure could reinstate drug-seeking, similar to what is seen to induce relapse in the clinic.

1.4.1.2 Conditioned place preference

Reinforcing properties of substances can also be tested in a conditioned place preference paradigm, where the drug exposure is paired with specific environmental stimuli. Typically, two distinct compartments are used, where one is repeatedly paired (conditioned) with the drug and the other with saline. Subsequently, the animal gets free access to both compartments and if the drug constitutes positive reinforcing effects, the animal will spend more time in the drug-paired environment. Also this paradigm can be used in studies of relapse behavior, after extinction of the conditioned behavior by repeated exposure to the drug-paired compartment in the absence of drug-administration.

1.4.1.3 Intracranial self-stimulation

Another way of evaluating the reinforcing effects of substances is by the use of intracranial self-stimulation (ICSS). In this model, an animal receives an electric pulse within a reward-related brain site and the animal must subsequently perform an operant, e.g. press a lever, in order to receive an additional pulse. The lowest dose to maintain responding can be estimated if the current of the electrical pulse is varied, and gives a measure of the threshold current that induces reward. The administration of several drugs of abuse including cocaine, amphetamine, morphine, ethanol and nicotine

4

reduce the ICSS reward threshold (Kornetsky and Bain 1992; Olds and Fobes 1981; Wise 1996) and in contrast, an elevation of the threshold has been observed in drug-dependent animals during withdrawal (e.g. Markou and Koob 1990).

1.5 NEUROCHEMICHAL AND NEUROANATOMICAL SUBSTRATES IN DRUG ADDICTION

1.5.1 Dopamine

1.5.1.1 Dopamine synthesis and metabolism

In the late 1950s, Arvid Carlsson and co-workers discovered that dopamine was a neurotransmitter on its own, and not only a precursor for other catecholamines like noradrenaline and adrenaline (Carlsson 1959; Carlsson et al 1957). Dopamine is synthesized from the amino acid tyrosine, which is converted into L-hydroxyphenylalanin (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase. L-DOPA is quickly converted to dopamine, which is stored in vesicles and released upon nerve stimulation in a calcium-dependent manner. Dopamine transmission is regulated by the reuptake of released dopamine back into the cell by the dopamine transporter, and is there metabolized by monoamine oxidase (MAO) into 3,4-dihydroxyphenylacetic acid (DOPAC). DOPAC diffuses out of the cell and is converted to homovanillic acid (HVA) by cathecol-O-methyltransferase (COMT). Extracellular dopamine can also be metabolized by COMT to 3-methoxytyramine, which in turn is converted by extracellular MAO into HVA.

1.5.1.2 Dopamine receptors

Dopamine receptors are divided into two classes, D_1-like (including D_1 and D_5 subtypes) and D_2-like (including D_2, D_3 and D_4 subtypes) dopamine receptors (Sibley et al 1993). All dopamine receptors belong to the seven-transmembrane G-protein coupled receptor family; the D_1-like receptors are coupled to stimulatory G_s proteins and thereby stimulate the formation of cyclic adenosine 3' 5' monophosphate (cAMP), whereas the D_2-like receptors are coupled to inhibitory $G_{i/0}$ proteins, decreasing the levels of cAMP. Both types of receptors are found post-synaptically, but only the D_2-like receptors are expressed pre-synaptically and thereby function as autoreceptors.

1.5.1.3 Dopamine projections

There are two major dopaminergic projection systems, the mesocorticolimbic and the nigrostriatal. The mesocorticolimbic dopamine neurons originate in the ventral tegmental area (VTA) and project to cortical and subcortical forebrain areas, including the prefrontal cortex, cingulate cortex, nucleus accumbens, amygdaloid complex, bed nucleus of stria terminalis, hippocampus, septum and olfactory tubercle (Dahlström and Fuxe 1964; Ungerstedt 1971) and is implicated in limbic functions, e.g. reward (see section 1.4.3). Dopaminergic cells are also found in substantia nigra compacta (Dahlström and Fuxe 1964), innervating the dorsal part of the striatum, the caudate putamen, and forming the nigrostriatal dopamine system. This core component of the basal ganglia is involved in regulating motor behavior.

1.5.2 Opioid peptides

The opioid receptors were discovered in the early 1970s, when three laboratories independently discovered endogenous binding sites for opioids (Pert and Snyder 1973; Simon et al 1973; Terenius 1973). There are three main classes of opioid receptors, μ, κ and δ, that are all inhibitory seven-transmembrane G-protein coupled receptors, negatively coupled to adenylate cyclase. The μ- and δ opioid receptor ligands mediate euphoria and the κ opioid receptor mediate dysphoria, as e.g. shown in a place preference experiment where μ- and δ opioid receptor ligands induced place preference while the κ opioid receptor ligand induced place aversion (Mucha and Herz 1985).

The classical endogenous opioid peptides derive from three different genes that each code for a precursor protein: proopiomelanocortin (POMC), proenkephalin and prodynorphin. POMC was the first one to be cloned (Nakanishi et al 1979) and is the precursor of β-endorphin and non-opioid peptides like α, β and γ melanocyte stimulating hormone (MSH) and adrenocorticotropin releasing hormone (ACTH). Proenkephalin gives rise to four Met-enkephalin, one Leu-enkephalin and two longer opioid peptides, Met-enkephalin-Arg-Phe and Met-enkephalin-Arg-Gly-Leu. Dynorphins like dynorphin A, dynorphin B, α- and β-neoendorphin, dynorphin B-29 and dynorphin 32 are generated from prodynorphin. Dynorphin A, dynorphin B and β-neoendorphin can be enzymatically converted to Leu-enkephalin (Nyberg and Silberring 1990). β-endorphin has high affinity for both μ- and δ opioid receptors, enkephalin has the highest affinity for δ opioid receptors but high affinity for μ opioid receptors as well, while dynorphins have the highest affinity for κ opioid receptors. Endomorphin is another group of opioid peptides, discovered as late as in the 1990s, that binds selectively to the μ opioid receptor (Zadina et al 1997). Also, a dynorphin-like peptide was discovered, nociceptin/orphaninFQ (Meunier et al 1995). It does not bind to any of the opioid receptors however, but to the opioid receptor-like 1 (ORL-1) receptor.

1.5.2.1 Enkephalin

Enkephalins are widely distributed in the brain, with high densities in e.g. the nucleus accumbens, central amygdala, bed nucleus of stria terminalis, medial prefrontal cortex and the ventral pallidum (Watson et al 1982; Williams and Goldman-Rakic 1993), brain areas highly implicated in reward and stress. Opioid neurotransmission in the nucleus accumbens shell is thought to participate in generating hedonic impact for drug rewards and for natural sensory pleasures such as sweetness (Kelley et al 2002; Pecina and Berridge 2005). Specifically enkephalin has been shown to mediate the basal affective state (Skoubis et al 2005), since the non-selective opioid receptor antagonist naloxone induced conditioned place aversion in β-endorphin k-o mice (as in wild-types), but failed to do so in enkephalin knock-out mice.

1.5.3 The reward system

Brain regions involved in reward behavior, which constitutes the "reward system", was first identified in the 1950s by Olds and Milner (Olds and Milner 1954). The investigators were studying mechanisms of learning by the use of intracranial electrical stimulation and noticed that rats seemed to like electrode placements in specific brain areas. The rats liked it so much, that if they were given the opportunity to regulate the

stimulation themselves, they often did so to such extent that other needs were neglected. It was later revealed that what Olds and Milner had stimulated was the median forebrain bundle, which e.g. contains the mesocorticolimbic dopamine neurons that originate in the VTA and project to the nucleus accumbens. The natural function of the reward system is to motivate actions necessary for survival of the individual or the species, e.g., intake of food and water as well as sexual behavior. However, substances with addictive potential have been shown to also activate this system and to have a common feature of enhancing dopamine release in the nucleus accumbens (e.g. Di Chiara and Imperato 1988).
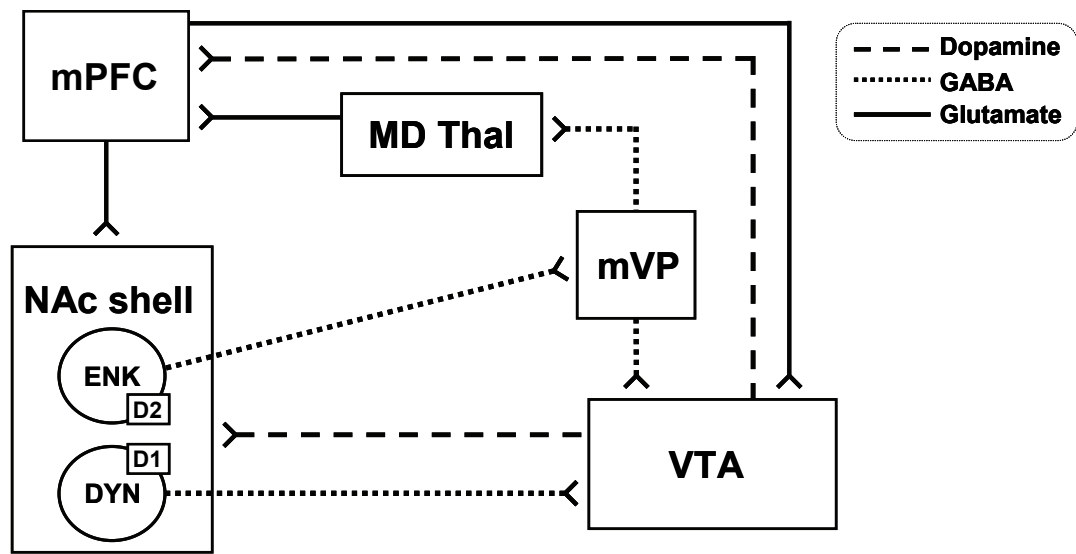

Figure 2. Schematic overview of brain areas of the mesocorticolimbic dopamine system most associated with reward, focused on the VTA projections to the nucleus accumbens shell with selected important related projections. Other brain areas are also included within the mesocorticolimbic dopamine system, like the nucleus accumbens core, basolateral amygdala, extended amygdala and hippocampus which are also highly implicated in different aspects of addiction behavior. There are two types of GABAergic medium spiny neurons projecting from nucleus accumbens; Enkephalin-containing neurons (ENK) express inhibitory dopamine D_2 receptors and project to the medial ventral pallidum and dynorphin-containing neurons (DYN) express dopamine D_1 receptors and project back to the VTA. mPFC, medial prefrontal cortex; MD Thal, mediodorsal thalamus; mVP, medial ventral pallidum; NAc shell, nucleus accumbens shell; VTA, ventral tegmental area.

The nucleus accumbens contain GABAergic medium spiny neurons, which co-express different neuropeptides. Sub-populations that express the dopamine D_1 receptor primarily contain the opioid peptide dynorphin and substance P, while those that express the dopamine D_2 receptor predominantly contain the opioid peptide enkephalin. The nucleus accumbens is divided into two anatomically and functionally distinct compartments, shell and core (Heimer et al 1991). The shell is the more limbic related component where D_1/dynorphin expressing GABAergic neurons project to the VTA and D_2/enkephalin GABAergic neurons project to the medial ventral pallidum that in turn projects to the VTA and mediodorsal thalamus. The shell compartment is also tightly interconnected with the extended amygdala (de Olmos and Heimer 1999), with

projections to the lateral hypothalamus and the brainstem. The nucleus accumbens core on the other hand is more motor-related, innervating the VTA, but also substantia nigra compacta/reticulata.

1.6   THE CANNABINOID SYSTEM

1.6.1   Cannabinoid receptors

Evidence for a high affinity binding site for cannabinoids in brain membranes was found in the end of the 1980s (Devane et al 1988) and a few years later the cannabinoid CB_1 receptor was cloned from brain tissue (Matsuda et al 1990). Subsequently, another cannabinoid receptor was identified, the CB_2 subtype, which has only 44% homology with the CB_1 receptor (Munro et al 1993). The cannabinoid CB_1 receptor is widely expressed in the brain (Wang et al 2003) with high densities in the cerebral cortex, hippocampus, amygdala, basal ganglia (striatum, globus pallidus and substantia nigra), and cerebellum (Herkenham et al 1990; Tsou et al 1998). The anatomical distribution of the CB_1 is consistent with the behavioral effects of THC such as impairment of cognition, learning, memory, motor function and distorted sense of time. The cannabinoid CB_1 receptors emerge early in the brain during fetal development (Berrendero et al 1998; Romero et al 1997) and the gene is predominantly expressed in limbic brain areas (Wang et al 2003). CB_1 receptors during early development have a higher density in white matter areas than during adulthood, which together with other data, implies that the cannabinoid system is involved in neurodevelopmental events such as synaptogenesis, proliferation and migration of neuronal cells (see e.g. review by Fernandez-Ruiz et al 2000).

Cannabinoid CB_2 receptors are found in peripheral tissues, mainly within the immune system. The CB_1 and CB_2 receptors are seven-transmembrane G-protein coupled receptors, coupled to inhibitory $G_{i/0}$ proteins, causing inhibition of adenylate cyclase and stimulation of the mitogen-activated protein (MAP) kinase pathway. Activation of the CB_1 subtype also leads to inhibition of voltage-activated calcium channels and activation of potassium channels (for review see e.g. Howlett et al 2002). Several pharmacological studies indicate that there are additional cannabinoid receptor subtypes (Breivogel et al 2001; Hajos et al 2001; Kofalvi et al 2003; Ma et al 1987). However, only CB_1 and CB_2 have been cloned thus far. A number of synthetic cannabinoid receptor agonists and antagonist have been developed, of which the most frequently used are the agonists WIN 55,212-2, CP 55,940 and HU-210, and the antagonist SR141716A (rimonabant).

1.6.2   Endocannabinoids

The search for an endogenous ligand began shortly after the cannabinoid receptors were discovered. A lipid cannabinoid-like component was isolated, which was identified as the ethanolamide of arachidonic acid, arachidonoylethanolamine (Devane et al 1992). It was named "anandamide" after the sanskrit word "ananda" that means inner bliss. Later, another endocannabinoid was identified, 2-arachidonoylglycerol (2-AG; Mechoulam et al 1995; Sugiura et al 1995), which is approximately 200 times higher in concentration in the brain than anandamide (Sugiura et al 1995) and acts as a full agonist at the CB_1 receptor compared to anandamide that is a partial agonist. 2-AG is involved in multiple

routes of lipid metabolism, which can explain the high concentration in the brain. The endocannabinoids are released from cells upon demand by stimulus-dependent cleavage of phospholipid precursors (*N*-arachidonoyl-phosphatidylethanolamine and phospatidylinositol for anandamide and 2-AG respectively) situated in the plasma membrane (Di Marzo et al 1994). The synthesis and release of anandamide and 2-AG are calcium dependent and they function through retrograde signaling; they are released from the postsynaptic neuron upon stimulation and bind to the cannabinoid CB_1 receptor that is predominantly situated on the presynaptic neuron. After release, anandamide and 2-AG may be eliminated by a two-step mechanism consisting of carrier-mediated transport into cells (Beltramo and Piomelli 2000; Beltramo et al 1997; Hillard et al 1997) and once inside the cells, anandamide and 2-arachidonoyl glycerol are immediately degraded to arachidonic acid and ethanolamine or glycerol, respectively, through the enzymatic hydrolysis by fatty acid amid hydrolas (FAAH) (Di Marzo et al 1998; Di Marzo et al 1994). Even though the transport carrier has not been identified, the reuptake can be pharmacologically blocked by the transport inhibitor AM404 (Beltramo et al 1997; Calignano et al 1997).

1.6.3 Interactions with the reward system

Smoking cannabis induces subjective feelings of well being and euphoria in humans (Chait and Zacny 1992), effects that can be blocked by the cannabinoid CB_1 receptor antagonist SR141716A (Huestis et al 2001), indicating that these effects are mediated through the cannabinoid receptor. Additionally, similar to other addictive drugs, both THC and synthetic cannabinoid receptor agonists are self-administered by animals (Fattore et al 2001; Justinova et al 2003; Tanda et al 2000), induce conditioned place preference (Lepore et al 1995; Valjent and Maldonado 2000) and lower the threshold dose for intracranial self stimulation (Gardner et al 1988; Gardner and Vorel 1998), indicating activation of reward neuronal pathways. Indeed, cannabinoids have been shown to activate the mesocorticolimbic dopamine neurons in terms of increased dopamine cell firing in the VTA (French et al 1997) as well as increased dopamine release in the nucleus accumbens (Chen et al 1990b; Tanda et al 1997) and prefrontal cortex (Chen et al 1990a). How this effect is achieved is however not clear. Cannabinoid CB_1 receptors are not present directly on the mesolimbic dopamine neurons (Herkenham et al 1991; see figure 3), nevertheless, cannabinoid agonists increase the activity of dopamine neurons in the VTA in brain slices (Cheer et al 2000). The fact that prior application of a $GABA_A$ receptor antagonist, bicuculline, blocks this effect implies that cannabinoids work through activation of CB_1 receptors on GABA interneurons within the VTA, disinhibiting the dopamine neurons in a similar manner as opioids (Johnson and North 1992). Supporting this theory, THC have been shown to be self-administered locally into the VTA (Zangen et al 2006), however, direct infusion of THC into the VTA did not elevate dopamine release in the nucleus accumbens in another study (Chen et al 1993). Further support for the capacity of THC to activate the dopaminergic activity was found recently, in a study where chronic treatment with a rather low dose of THC was shown to enhance the number of dendritic branches in the nucleus accumbens and medial prefrontal cortex, output regions for the mesocorticolimbic dopamine neurons, but not in the striatum, hippocampus and some other cortical regions (Kolb et al 2006). The fact that cannabinoid-induced dopamine release in the nucleus accumbens is attenuated by systemic and intra-VTA

administration of naloxone (Chen et al 1990b; Tanda et al 1997) has given rise to the suggestion that the dopamine-enhancing mechanism is mediated through elevation of endogenous opioids in the VTA. Indeed, THC has been shown to increase the level of beta-endorphin within the VTA (Solinas et al 2004b).
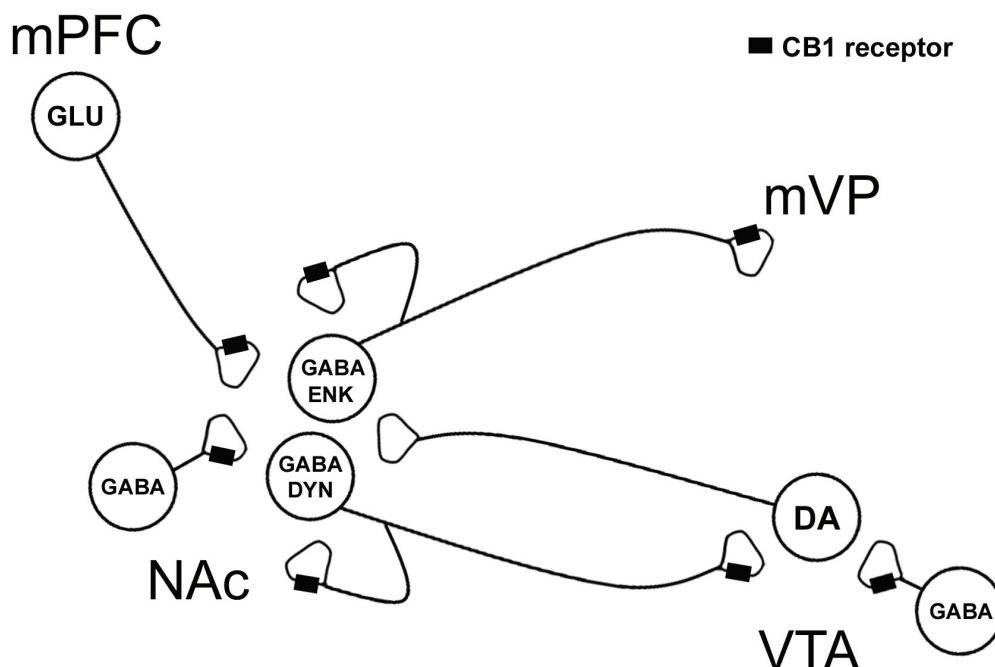

Figure 3. Putative locations of the cannabinoid CB_1 receptor within the reward system, including the mesolimbic dopamine projection (DA), enkephalin and dynorphin containing medium spiny neurons (GABA/ENK and GABA/DYN), glutamatergic prefrontal cortex projections (GLU) and GABAergic interneurons in the nucleus accumbens and the VTA (GABA). For functional implications, see the text. mPFC, medial prefrontal cortex; mVP, medial ventral pallidum; NAc, nucleus accumbens shell; VTA, ventral tegmental area.

Direct actions within the nucleus accumbens also seem to play a role in the rewarding effects of cannabinoids, since the study by Zangen et al (2006) showed that THC was self-administered directly into the nucleus accumbens. Further, local administration was shown to increase extracellular levels of dopamine within the same area (Chen et al 1993). WIN 55,212-2 and CP55,940 have been shown to decrease GABA release onto medium spiny neurons in the nucleus accumbens (Hoffman and Lupica 2001; Manzoni and Bockaert 2001) which matches the localization of CB_1 receptors on GABAergic neurons in the nucleus accumbens (Pickel et al 2004). CB_1 receptors are also located presynaptically on glutamatergic neurons in the nucleus accumbens (Pickel et al 2004), and activation of these receptors could explain the inhibition of glutamate release in the nucleus accumbens observed after cannabinoid receptor agonist administration (Robbe et al 2001). Furthermore, increased enkephalin levels have been detected in the nucleus accumbens after acute cannabinoid exposure (Valverde et al 2001). Clearly, more research is needed to evaluate the importance of these events in the rewarding properties of cannabinoids.

1.7 CONSEQUENCES OF CANNABIS EXPOSURE DURING DEVELOPMENT

As previously mentioned, cannabis smoking is very common among young people, including pregnant women. During periods of brain formation and development, such as the prenatal stage and adolescence, the brain is likely to be more sensitive to external and internal variables, such as drug exposure, stress and gonadal hormones.

1.7.1 Prenatal exposure

Approximately one third of the plasma THC levels of a cannabis-smoking mother cross the placenta (Behnke and Eyler 1993) and it is also transferred to maternal milk during lactation, resulting in that the offspring becomes exposed to THC. Two longitudinal birth cohorts have followed cannabis-exposed children and evaluated different cognitive and behavioral parameters from birth to adolescence — the Ottawa Prenatal Prospective Study (Fried 1995) and the Maternal Health Practices and Child Development Project in Pittsburg (Day and Richardson 1991). These studies found impairment in memory, verbal function and reasoning in children of cannabis-smoking mothers at 3 years of age (Day et al 1994; Fried and Watkinson 1990) as well as deficits in visuospatial reasoning and memory and increased impulsivity, inattention and hyperactivity around 10 years of age (Fried et al 1998; Goldschmidt et al 2000; Richardson et al 2002). Interestingly, nicotine and cannabis use in 16-21 year olds was increased in offspring exposed to cannabis, but not those exposed only to nicotine (Porath and Fried 2005). This effect was more pronounced in males.

Studies on human fetuses, at mid-gestation development, in our lab have shown a reduction in dopamine D_2 receptor mRNA expression in the basolateral amygdala primarily in male subjects (Wang et al 2004) and in the nucleus accumbens (Wang et al., unpublished results). No differences were seen in dopamine D_1 receptor or cannabinoid CB_1 receptor mRNA expression. Selective changes were also detected within the opioid system, showing increased µ opioid receptor mRNA expression in the amygdala, decreased κ opioid receptor mRNA in the mediodorsal thalamus and decreased proenkephalin mRNA in the caudal putamen (Wang et al 2006).

Animal studies on the effects of prenatal cannabinoid exposure have revealed disturbances of both the opioid and dopamine systems. Sexually dimorphic disturbances was found in PENK, POMC and PDYN mRNA expression in THC exposed offspring at the end of the prenatal stage (Perez-Rosado et al 2000). However, most other investigations have used an extended period of cannabis exposure that also covers the lactation phase, ending at PND 24. These studies have found disturbances in µ opioid receptor levels (Vela et al 1998) and ontogeny of dopamine receptors (Rodriguez de Fonseca et al 1991; Rodriguez de Fonseca et al 1992). On a behavioral level, perinatal THC exposure alters basal motor behavior and novelty reactions (Navarro et al 1994). Moreover, elevated morphine conditioned place preference was found in perinatally THC exposed offsprings (Rubio et al 1998). THC exposed females, but not males, had a higher rate of acquiring morphine self-administration behavior (Vela et al 1998), but no differences were seen between pre-exposure groups in a progressive ratio paradigm (Gonzalez et al 2003). An altered function of dopamine autoreceptors has also been suggested, since THC exposed rats showed elevated

sensitivity to dopamine D$_2$ receptor agonists in behavioral tests (Moreno et al 2003). However, in all these studies of prenatal and perinatal THC exposure an oral maternal THC administration route was used. This might not be optimal since it results in slower onset and enhanced metabolism of the substance, compared to the pharmacokinetics seen in marijuana-smoking women (Grotenhermen 2003).

1.7.2 Adolescent exposure

Active neural changes, e.g. synapse formation and elimination, take place during adolescence (Charmandari et al 2003; Rice and Barone 2000). Consequently, cannabis exposure during the adolescent period may increase the vulnerability to neuropsychiatric disorders and clinical evidence has confirmed such links. Early-onset cannabis use increases the risk of developing schizophrenia (Arseneault et al 2002; Fergusson et al 2003; van Os et al 2002; Zammit et al 2002) as well as a worsen course of the disorder (Green et al 2004; Veen et al 2004). It has also been shown that subclinical positive and negative symptoms were more strongly associated with first time use before age 16 than after, and was independent of lifetime frequency of use (Stefanis et al 2004). Adolescent cannabis exposure have been shown to also affect cognition, in terms of impaired attentional functions (Ehrenreich et al 1999) and lower verbal IQ (Pope et al 2003). In agreement, animal studies demonstrate that chronic cannabinoid exposure in adolescent but not adult rats produce long-lasting memory impairment and increased anxiety (O'Shea et al 2004), impaired spatial and non-spatial learning (Cha et al 2006) as well as impaired prepulse inhibition (Schneider and Koch 2003). All together, these studies further implicate the influence of adolescent cannabis exposure on neuropsychiatric functioning.

Furthermore, adolescent cannabis use is suggested to influence the subsequent abuse of other drugs. As previously mentioned, several epidemiological studies report that early regular use of cannabis increases the risk of initiation of use of other illicit drugs (Agrawal et al 2004; Fergusson and Horwood 2000; Lynskey et al 2003; Yamaguchi and Kandel 1984), supporting the cannabis gateway hypothesis of cannabis as a steppingstone towards abuse of other drugs. Cross-interactions between cannabis and other illicit drugs have been evaluated to some extent in animal models (see section 1.8). However, these studies have been carried out on adult rats, whereas in humans most individuals start smoking cannabis in adolescence. Prior to this thesis, no information was available about the consequences of adolescent cannabis exposure on the reinforcing properties of other illicit drugs.

1.8 CANNABIS INTERACTIONS WITH OTHER DRUGS OF ABUSE

1.8.1 Psychostimulants

Amphetamine and cocaine are the most common psychostimulant drugs and they are both highly addictive. Cocaine is derived from the coca plant (*Erythroxylum coca*) and increases the dopaminergic tone in the brain by blocking the dopamine transporter that normally is responsible for the reuptake of dopamine from the synaptic cleft into neurons. Amphetamine is a synthetic drug that also increases the dopamine tone, but to an even higher extent than cocaine, by reversing the dopamine transporter resulting in release of dopamine, and at high doses, also blockade of dopamine reuptake. Amphetamine also affects the vesicular dopamine transporter that normally packs

12

dopamine from the cytosol into vesicles. Both cocaine and amphetamine are well documented to induce a marked increase in extracellular dopamine in reward-related brain areas, like the nucleus accumbens, to increase locomotor activity and to induce stereotypic behavior (Di Chiara and Imperato 1988; Hurd and Ungerstedt 1989; Hurd et al 1989; Kuczenski and Segal 1989; Zetterström et al 1983). Repeated administration of psychostimulants leads to sensitization of dopaminergic and behavioral responses which is frequently interpreted to reflect drug dependence vulnerability (Robinson et al 1988; Roy et al 1978).

1.8.1.1 *Cross-interactions between cannabinoids and psychostimulants*

Investigations of cross-interactions between cannabinoids and psychostimulants, e.g., amphetamine and cocaine, have shown diverse results. Chronic THC-exposure sensitized the amphetamine-induced locomotor activity response (Gorriti et al 1999), while chronic WIN 55,212-2 exposure did not (Muschamp and Siviy 2002). Although, stereotypic behavior was enhanced in both studies. Amphetamine-induced locomotor activity was also found to be increased after chronic THC exposure in rats defined as high responders to a novel environment (Lamarque et al 2001). However, no locomotor cross-sensitization was seen between the CB_1 receptor agonists CP 55,940 or HU-210 and cocaine (Arnold et al 1998; Ferrari et al 1999).

Investigations of the importance of a functioning cannabinoid CB_1 receptor for the rewarding effects of psychostimulants have also been carried out. CB_1 receptor knock-out mice did not differ from wild-type in cocaine-induced conditioned place preference or acute cocaine self-administration behavior (a single 30 min session; Cossu et al 2001; Martin et al 2000). The same lack of effect on acute cocaine self-administration and cocaine induced place preference was seen with acute blockade of the CB_1 receptor with the antagonist SR 141716A (Chaperon et al 1998; Lesscher et al 2005). However, in a resent study, CB_1 knock-out mice did show altered cocaine self-administration behavior in terms of decreased acquisition, maintenance responding and break point in a progressive ratio paradigm (Soria et al 2005). Further evidence for cannabinoid involvement in cocaine reinforcement is that the cannabinoid CB_1 receptor agonist promotes reinstatement of cocaine seeking after extinction of self-administration behavior, while the antagonist SR 141716A blocked cocaine priming-induced reinstatement (De Vries et al 2001).

1.8.2 Opiates

Opiates are derived from the opium poppy (*Papaver somniferum*). Opium is obtained from the fruit capsules of the poppy and contains several alkaloids, e.g. morphine. Heroin is synthesized from morphine, and the difference between the substances is that heroin passes the blood-brain-barrier faster and thereby gives a more intense initial effect. Heroin is however quickly metabolized to morphine, exerting its effects by binding to μ and δ opioid receptors in the brain. Both heroin and morphine are highly addictive substances, shown to, e.g., increase mesolimbic dopamine signaling.

1.8.2.1 *Cross-interactions between cannabinoids and opiates*

Converging lines of evidence indicate strong links between the cannabinoid and opioid systems. Cannabinoid CB_1 receptors and μ opioid receptors are often co-localized, even

in the same subcellular compartments, in many brain regions including the striatum and the VTA (Pickel et al 2004; Rodriguez et al 2001). Recent evidence have led to the suggestion that CB_1 receptors heterodimerize with e.g. μ and δ opioid receptors (Mackie 2005; Rios et al 2006), and such cooperation might lead to altered functioning.

Behavioral studies using cannabinoid CB_1 and μ opioid receptor knock-out mice have shown that the cannabinoid system is needed for opiate reward-related behavior and *vice versa*, since CB_1 knock-outs failed to self-administer heroin or to show conditioned place preference to morphine and μ opioid receptor knock-outs did not develop place preference to THC or CP 55,940 (Cossu et al 2001; Ghozland et al 2002; Ledent et al 1999; Martin et al 2000). Further more, acute block of the opioid receptors with the antagonist naltrexone attenuates CP 55,940 conditioned place preference as well as THC self-administration (Braida et al 2001; Justinova et al 2004). Similarly, acute block of the cannabinoid CB_1 receptor with SR141716A attenuates opioid-induced conditioned place preference and self-administration behavior (Caille and Parsons 2003; De Vries et al 2003; Navarro et al 2004; Navarro et al 2001) preferentially in dependent animals and in progressive ratio paradigms (De Vries et al 2003; Solinas et al 2003). In addition to blocking opioid self-administration, systemic SR141617A also attenuated heroin- and morphine-induced reduction in GABA levels in the ventral pallidum, but not the dopamine increase in the nucleus accumbens (Caille and Parsons 2003; Caille and Parsons 2006). Local administration of the cannabinoid receptor antagonist into the nucleus accumbens, but not the ventral pallidum, attenuated heroin self-administration and together these experiments indicate that the attenuating effect of SR141617A on opioid reinforcement is mediated through CB_1 receptors within the nucleus accumbens and regulation of GABA levels in the ventral pallidum.

As mentioned previously, chronic cannabis exposure during the perinatal phase induced opioid-related neurobiological and behavioral alterations (see paragraph 1.5.3.1). However, opioid-related alterations are also seen after chronic cannabinoid exposure in adult rats. Chronic CP 55,940 exposure was shown to enhance morphine behavioral sensitization and alter morphine self-administration behavior (Norwood et al 2003). Chronic THC exposure also enhanced subsequent heroin-intake, but not the motivation and/or reinforcing efficacy of the drug as assessed by the progressive ratio self-administration paradigm (Solinas et al 2004a). On a neurobiological level, chronic CP 55,940 exposure induced transient increases in μ opioid receptor levels in the caudate putamen, nucleus accumbens shell and core, medial amygdala, substantia nigra and the VTA that normalized to vehicle levels after 7-14 days of treatment (Corchero et al 2004). Also, chronic administration of CP-55,940 increased PENK mRNA levels in the caudate putamen, nucleus accumbens and hypothalamus (Manzanares et al 1998).

2 AIMS OF THE STUDY

The general aim of this thesis was to test experimentally the cannabis gateway hypothesis, whether cannabis exposure during periods of active brain development alters reward-related behavior and neurobiology for psychostimulant- and opioid-drugs.

More specifically, by the use of rat models to:

- Examine the effects of early adolescent cannabinoid exposure on amphetamine-induced motor behavior and dopamine release in the nucleus accumbens during adolescence.

- Evaluate the effects of prenatal and adolescent cannabis exposure on opiate reward related behavior during adulthood.

- Characterize alterations in the opioid and cannabinoid systems within limbic-related brain areas induced by prenatal and adolescent cannabis exposure.

- Study the ontogeny of the opioid and cannabinoid systems within limbic-related brain areas during the adolescent period.

3 MATERIALS AND METHODS

3.1 SUBJECTS

Sprague-Dawley (B&K Universal, Sollentuna, Sweden; paper I) and Long-Evan (M&B Taconic, N.Y., USA; paper II and III) male rats, 28 days old at the start of the experiments, were used in the adolescent studies. Male and female Long-Evan rats (approximately 6-7 weeks old) were obtained from Charles River, Germany for the prenatal study in paper IV. They were housed in a temperature-controlled environment with ad libitum access to food and water. The rats were kept on a 12-h light/dark cycle with lights turned off at 7 p.m. (paper I) or 11 a.m. (paper II-IV; reversed day cycle). Hence, all experiments were performed during the light phase in study I and during the dark phase in studies II-IV. The rats were allowed to acclimate in their new environment and were handled daily for one week before the experiments were initiated. All animal experiments were performed in accordance with the guidelines of The Swedish National Board for Laboratory Animals under protocols approved by the Ethical Committee of Northern Stockholm, Sweden.

3.2 DRUGS

THC (10 mg/ml in ethanol solution; Sigma-Aldrich, Sweden) was evaporated under nitrogen gas, dissolved in 0.9% NaCl with 1% Tween 80 and administered intra-peritoneally (i.p.) in a volume of 1.25 ml/kg in paper I. In paper II-IV the evaporated THC was dissolved in 0.9% NaCl with 0.3% Tween 80 and administered i.p. at a volume of 2 ml/kg and 0.5 ml/kg intravenously (i.v.). WIN 55,212-2 mesylate (WIN; Sigma-Aldrich, Sweden) was dissolved in 0.9% NaCl with 1% Tween 80 and administered i.p. at a volume of 1.25 ml/kg. Rimonabant (also known as SR 141716A; Sanofi-Aventis) was dissolved in 0.9% NaCl and administered at a volume of 2 ml/kg i.p. Amphetamine sulphate (Apoteket AB, Sweden) was dissolved in 0.9% NaCl and administered i.p. Heroin HCl (Apoteket AB, Sweden) was dissolved in 0.9% NaCl and administered i.v. at a volume of 85 µl/infusion. Morphine sulphate was dissolved in 0.9% NaCl and administered sub cutaneously (s.c.) at a volume of 1 ml/kg.

3.3 CANNABIS EXPOSURE

3.3.1 Adolescent cannabis exposure

In paper I, WIN (0.625, 1.25 or 2.5 mg/kg), THC (0.75, 1.5 or 3.0 mg/kg) or vehicle was given once a day between PND 28-32. After a one week drug-free period, *in vivo* microdialysis and behavioral experiments were performed on PND 40; behavioral testing was performed also on PND 68. The pre-exposure design was chosen in order to that both cannabinoid pre-exposure and testing could be carried out within the adolescent period (days 28-42; (Spear 2000).
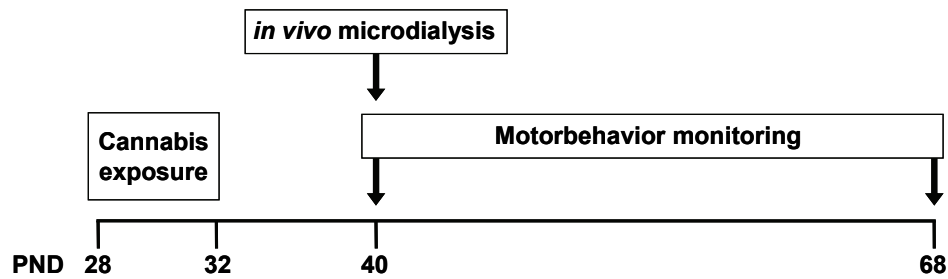
Figure 4. The experimental design used in paper I.

In papers II and III, the rats were exposed to THC (1.5 mg/kg i.p.) or vehicle (0.9% NaCl with 0.3% Tween 80) during postnatal days 28 to 49 to extend beyond the prototypic adolescent period. In order to mimic the intermittent use seen in teenagers, the drug was given once every third day; this resulted in a total of eight injections. There was a one week drug-free period between the cannabis exposure and the behavioral and post mortem experiments starting on PND 57 in paper II. In paper III, rats were sacrificed for neurochemical studies 24 h after the first, fourth and eighth injection, i.e. on PND 29 (early adolescence), 38 (mid adolescence) and 50 (late adolescence) respectively.
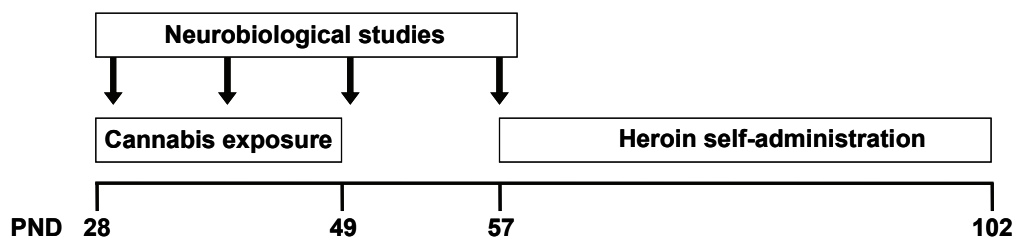
Figure 5. Experimental design in papers II (molecular studies on PND 57 and heroin self-administration) and III (molecular studies on PND 29, 38 and 50); the same cannabis pre-exposure design was used for these two studies.

3.3.2 Prenatal cannabis exposure

Pregnant females received daily intravenous injections of THC (0.15 mg/kg) or vehicle from gestational day 5 to PND 2 (which corresponds to the mid-gestation ($\approx$ week 20) development stage in humans (Bayer et al 1993), the time period examined in our human fetal studies). An intravenous route of administration was used since it more closely mimics the pharmacokinetics of cannabis smoking, the normal route used in pregnant women (Grotenhermen 2003). The dose of THC is an extrapolation from current estimates of low cannabis cigarettes (about 16 mg of THC), correcting for differences in route of administration and body weight (Grotenhermen 2003). During the course of the drug treatment, gestational parameters were recorded, such as maternal weight gain, gestational length and fetal weights. On PND 2, pups from both groups were cross-fostered and culled 8 to 10 from vehicle mothers; brains were taken

from other sets of animals at this time period. On PND 21, male offspring were weaned from their mothers and housed 4/cage. Brains were taken from one group of rats for post-mortem studies at PND 62 when the behavioral studies were initiated in the other animals.
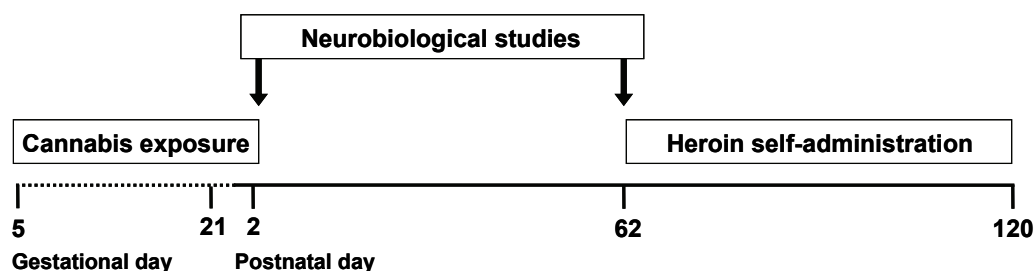

Figure 6. The experimental design in paper IV.

3.4 BEHAVIORAL AND *IN VIVO* NEUROCHEMICAL STUDIES

3.4.1 *In vivo* microdialysis

3.4.1.1 *Stereotaxic surgery*

Two days prior to the microdialysis testing, animals were anesthetized with isoflurane (Forene, Apoteket, Sweden) and a guide cannula (CMA Microdialysis, Sweden) was implanted above the nucleus accumbens using stereotaxic surgery. The coordinates for the position of the guide cannula were AP +1.5 mm, ML +1.0 mm and DV _2.5 mm from bregma or the dura, according to the atlas of Paxinos and Watson (1997) and to empirical tests in 150-g rats. The guide cannula was secured to the scull with stainless steel screws and dental cement (AgnTho's, Sweden). After surgery, the rats were kept in single cages.

3.4.1.2 *In vivo microdialysis*

On the evening prior to the test day, a microdialysis probe (CMA 12, CMA Microdialysis, Sweden) was lowered into the nucleus accumbens (DV _7.5) via the guide cannula. In the morning, the probe was connected to an infusion pump (Univentor syringe pump 801, AgnTho's, Sweden) and artificial cerebrospinal fluid (148 mM NaCl, 2.7 mM KCl, 0.85 mM MgCl2, 1.2 mM CaCl2, pH 7.1; Apoteket, Sweden) was pumped through the probe at a constant rate of 1 Al/min. After approximately 30 min of equilibration, dialysate samples were collected every 20 min (Univentor microsampler 810, AgnTho's, Sweden) in vials containing 2.2 Al of 1 M perchloric acid (to give a final concentration of 0.1 M perchloric acid) to minimize catecholamine degradation. The microdialysis experiment was performed in the rat's homecage, and immediately after the end of the experiment, the rats were anaesthetized with CO2, followed by decapitation. The brain was removed, frozen in ice-cold isopentane and sliced in a cryostat. The sections were validated for correct probe localization with the guidance of the brain atlas of Paxinos and Watson (1997).

3.4.1.3 HPLC analysis

The dialysate samples were injected into a high-performance liquid chromatograph (HPLC) equipped with a reverse phase column (Reprosil, 150_4 mm, 3 Am particle size) for separation and a coulometric detector (Coulochem II, ESA) to quantitate the dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels. The oxidation electrode was set to 400 mV, and the reduction electrode to _200 mV. The mobile phase consisted of 55 mM sodium acetate, 0.5 mM octanesulfonic acid, 0.01 mM Na_2EDTA and 10% methanol (pH was adjusted to 4.1 with acetic acid) and was perfused at a constant flow rate of 0.7 ml/min.

3.4.2 Motor behavior monitoring

3.4.2.1 Locomotor activity

Locomotor activity was measured in two activity chambers (45x45 cm; ActiMot, TSE Systems, Germany) with transparent walls. Behavioral activity was monitored by three sets of 32 infrared emitters and receivers with 14 mm distance between beams, two sets in the x–y axis to register horizontal activity and one set 8–15 cm above the floor to monitor rearing behavior. Novelty was measured during the first 30 min of the session, and thereafter, a challenge amphetamine injection was given. Locomotor activity was measured for an additional 90 or 120 min. All tests were carried out between 8:00 AM and 12:30 PM.

3.4.2.2 Stereotyped behavior

The rats were videotaped during the locomotor activity tests and stereotyped behavior was scored manually according to a scale developed by Kalivas et al. (1988). The scale ranged from 1–10 as follows: 1. asleep or still; 2. inactive, grooming or mild licking; 3. locomotion, rearing or sniffing; 4. any combination of locomotion, rearing or sniffing; 5. continuous sniffing without locomotion or rearing; 6. continuous sniffing with locomotion or rearing; 7. patterned sniffing for 5 s; 8. patterned sniffing for 10 s; 9. continuous gnawing; 10. bizarre diskinetic movements or seizures. The behavior was analyzed for 10 s every 10 min for 90 min, giving a maximum score of 90.

3.4.3 Intravenous heroin self-administration

3.4.3.1 Surgery and post-operative care

Intravenous catheters (Brian Fromant, Cambridge, UK) were implanted into the right jugular vein under isoflurane (Isofluran Baxter, Apoteket AB, Sweden) anesthesia. During the first three days of recovery, the catheters were flushed with 0.1 ml of a saline solution containing 10U heparine (Heparin LEO, Apoteket AB, Sweden) and ampicillin (50 mg/kg; Doctacillin, Apoteket AB, Sweden). During this period, the rats were also given the analgesic carprofen (0.5 mg/kg s.c.; Rimadyl vet, Apoteket AB, Sweden). From the fourth day, the catheters were flushed with 0.1 ml of saline containing 30U heparin. This was also done before and after all self-administration sessions.

3.4.3.2 Apparatus

Heroin self-administration studies were carried out in operant chambers (29.5 x 32.5 x 23.5 cm) equipped with two retractable levers and infrared locomotor sensors (Med Associates Inc., Vermont, U.S.); each enclosed in sound-attenuating chambers. Depression of one lever (defined as active) resulted in an intravenous drug injection, while depression on the other lever (defined as inactive) had no programmed consequence but was always recorded. A single active lever press resulted in a 5s drug infusion (85 μl); concurrently a white light cue above this lever was turned on for 5s. A 10s timeout period was then introduced, during which the white cue light was turned off and both levers were retracted. Each self-administration session started with the extension of the two levers. A red house light was illuminated throughout the sessions. Assessment of the self-administration schedule and data collection were controlled by Med Associates PC software.
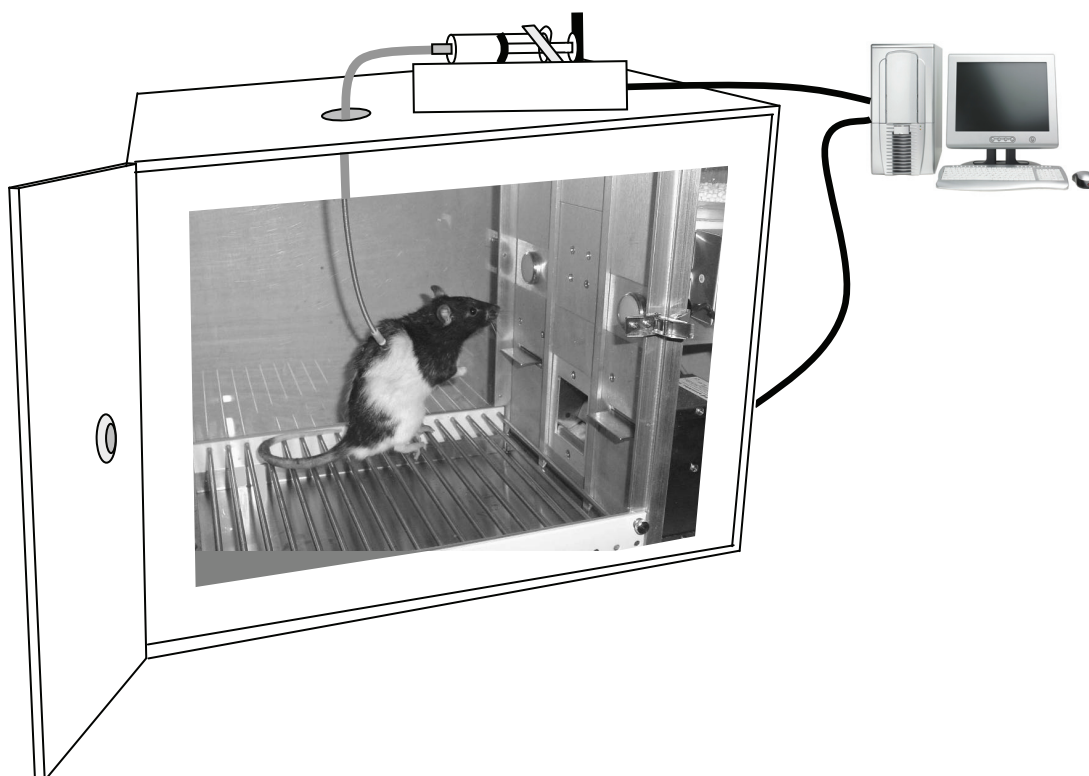
Figure 7. Illustration showing the set up of the operant chambers used in the self-administration experiments in papers II and IV.

3.4.3.3 Self-administration training

Animals were allowed to self-administer heroin (15μg/kg/inf) under a fixed-ratio 1 schedule in 3 h daily sessions. The self-administration sessions were conducted daily during the dark cycle (11:00–18:00). To ensure patency, catheters were flushed daily before and after each IVSA session with a sterile saline solution containing heparin (30U). After 6 days, the dose of heroin was increased to 30μg/kg/inf and the rats received one priming infusion at the start of the following 1-4 sessions, until they

started to press on their own. The rats were food-restricted (20 g food pellets/day) during the acquisition phase that was carried out until stable baseline responding was reached. Drug self-administration was continuous with no drug-free periods during the acquisition and maintenance phases. THC and vehicle-pretreated rats were processed simultaneously throughout all phases of the self-administration procedure. The rats were kept on a maintenance heroin dose of 30 or 60 µg/kg FR-1 schedule. Stable heroin intake behavior was established for at least 3 days prior to all behavioral or pharmacological manipulations.

3.4.3.4 Dose response test

A between session dose response test was performed (30, 7.5, 100, 15 and 60 µg/kg/infusion heroin). In paper II, each dose was tested for one session while in paper IV, each dose was tested for three consecutive sessions.

3.4.3.5 Drug seeking

Heroin seeking behavior was studied using a paradigm where the number of responses on the active lever were counted but had no programmed consequences; no heroin was delivered or cue light presented during this session. The effect of acute pretreatment with the CB_1 antagonist Rimonabant (0.3, 1.0 and 3.0 mg/kg i.p.) was evaluated.

3.4.3.6 Mild stress test

24 hours food deprivation was used to examine the consequence of exposure to a mild stress on base-line heroin responding.

3.4.3.7 Extinction and reinstatement test

An extinction phase started following the procedures described above. Responding for heroin was extinguished in daily sessions by replacing the heroin solution in the syringe with physiological saline. Since not all catheters were patent for the entire extinction phase, saline infusion was only given during the first week of extinction. Subsequently, animals were connected to the liquid swivel and an empty syringe was connected to the infusion tubing to seal the system, but was not placed in the syringe pump. All others parameters were left unchanged. Drug-reinforced behavior was considered extinguished when responding on the active lever had decreased by at least 85% for 3 consecutive days. After extinction criteria were reached, each rat was given one of the following priming injections to test its effect on reinstating heroin-seeking behavior: saline, heroin (0.25 mg/kg, s.c.; 10 min before the session) and CB_1 antagonist, Rimonabant (3mg/kg, i.p.; 30 min before the session and 20 before heroin priming) + heroin combination.

3.4.4 Morphine conditioned place preference

An open field activity chamber with place preference inserts (45x45 cm; MED Associates Inc., Vermont, U.S.) creating two distinct compartments was used. One compartment had white walls and metal rod floor and the other had black walls and metal grid floor. A removable door separated the two compartments. The experiment started 8 days after the last THC or vehicle injection, on postnatal day 57, and was carried out under dim light during the first six hours of the dark phase of the light/dark

cycle. A biased conditioned place preference paradigm was used such that on experiment day 1, the rats were allowed to explore both compartments of the conditioned place preference box during a 15-minute habituation session. The pre-conditioning test was conducted on day 2 where the rats, similar to the habituation session, were allowed to explore both compartments of the conditioned place preference box during 15 minutes to measure the baseline preference. On days 3, 5 and 7 the rats were given an injection of low dose morphine (4 mg/kg s.c.) and after 15 minutes in the home cage placed in the least preferred compartment, as revealed in the pre-conditioning test, for 30 minutes. On days 4, 6 and 8 the rats were given an injection of saline and after 15 minutes placed in the other compartment for 30 minutes. On day 9, a post-conditioning test was performed identical to the pre-conditioning test, where the rats had free access to both compartment for 15 minutes. Preference was calculated as the difference in time spent in the drug-paired compartment between the pre-conditioning and post-conditioning tests.

3.5 *IN SITU* BRAIN STUDIES

3.5.1 Brain section preparation

Twenty-four hours after the last heroin self-administration session, rats were killed as described below and brains were processed for post-mortem studies. Rats were anaesthetized in a CO_2 chamber and decapitated. Brains were quickly removed, frozen in isopentane ($\approx$ -30°C) for 1 min, and stored at -80°C until cutting. Coronal sections (20-μm thick) of the striatum and midbrain were cut in a refrigerated cryostat (-15°C; Frigocut 2800E, Leica Instruments, Nobloch, Germany) according to Paxinos and Watson rat brain atlas (1997) and mounted onto Superfrost Plus slides (Brain Research Laboratories, Newton, Massachusetts). The sections were stored at -30°C until processed as described below.

3.5.2 *In situ* mRNA hybridization

PENK and PDYN mRNA expression levels were studied by ISHH. The PENK riboprobe was complementary to a 333 kb fragment of the rat PENK cDNA (bp291-624 GenBank accession number Y07503). The PDYN riboprobe was complementary to a 534 kb fragment of the rat PDYN cDNA (bp349-883; accession number NM_019374). The RNA probes were transcribed in the presence of [^{35}S]uridine5'-[α-thio]triphosphate (specific activity 1000-1500 Ci/mmol; New England Nuclear, Boston, MA, USA). The ISHH procedure was similar to published protocols (Hurd et al 2001). Briefly, the labeled probe was applied to the brain sections in a concentration of 2×10^3 cpm/mm^2 of the coverslip area. Two adjacent sections from each subject were studied. Hybridization was carried out overnight at 55°C in a humidified chamber. After hybridization, the slides were apposed to β-max Hyperfilm (Amersham, Bucks, UK) along with ^{14}C-standards (American Radiolabelled Chemicals, St Louis, MO, USA): 1-4 days for PENK; 6 days for PDYN.

3.5.3 GTPγS stimulation

WIN 55,212-2- and DAMGO-induced [^{35}S]GTPγS stimulation was measured in order to asses whether THC pretreatment altered CB_1 and μOR receptor function, respectively. Brain sections were rinsed in Binding Buffer (50 mM Tris HCl, 3mM

MgCl$_2$, 0.2mM EGTA, 100 mM NaCl, pH 7.4) at 25°C for 10 min, followed by a 15 min preincubation in Binding Buffer containing 2mM Guanosine D phosphate (GDP) at 25°C. Sections were then incubated in Binding Buffer with 2mM GDP, 100 μM DTT, 0.04nM [^{35}S]GTPγS (specific activity 1099 Ci/mmol; Amersham Biosciences, England, UK) and appropriate agonists at 25°C for 2 hr. Agonists used were: 3μM DAMGO (Sigma-Aldrich MO, USA) and 10μM WIN 55,212-2 (Sigma-Aldrich MO, USA). Incubation solutions with WIN 55,212-2 contained 0.5% (wt/vol) BSA (fatty acid-free). Non-specific binding was determined in the absence of the agonist and in the presence of 10μM unlabeled GTPγS. After incubation, the slides were rinsed twice (2 min), in cold Tris Buffer (50mM Tris-HCl, pH=7.4) and subsequently in cold distilled water. The slides were dried overnight and exposed to film for 24 hr along with ^{14}C-microscales (Amersham Bucks, UK).

3.5.4 Receptor autoradiography

3.5.4.1 [^{3}H]-WIN 55,212-2 (CB$_1$) autoradiography

[^{3}H]-WIN 55,212-2 binding was measured in order to determine potential changes of cannabinoid receptor density in association with the THC pretreatment. Sections were preincubated in 20mM HEPES buffer with 0.5% (wt/vol) bovine serum albumin (BSA; fatty acid-free), pH 7.0 for 20 min at 30°C and then incubated in 1nM [^{3}H]-WIN 55,212-2 [R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazinyl)(1-naphthalenyl)methanonemesylate] (PerkinElmer, Life Science Inc., Boston MA) for 80 min at 30°C. Non-specific binding was assessed in the presence of 10μM non-labeled WIN 55,212-2. Slides were rinsed four times for 10 min each in the preincubation buffer at 25°C and then twice in cold distilled water. Slides were dried overnight and exposed to β-max Hyperfilm for 14 days.

3.5.4.2 [^{3}H]-DAMGO (μOR) autoradiography

[^{3}H]-DAMGO binding was carried out in order to examine whether THC pretreatment altered μOR density. Slide-mounted brain sections were preincubated at 25°C for 15 min in 50mM Tris-HCl (pH 7.4), 100mM NaCl, 1% BSA and briefly washed three times in 50 mM Tris pH 7.4. μOR binding sites were labeled by incubating sections for 1h at 25°C in the presence of 2nM [^{3}H][D-Ala^2,N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO; PerkinElmer, Life Science Inc.). Non-specific binding was defined in the presence of 1 μM naloxone. After incubation, slides were rinsed three times for 5 min each in the ice-cold incubation buffer, dipped in ice cold distilled water, dried overnight and placed in a β–Imager (Biospace Mesures, France) which allows direct on-line beta emission quantification.

3.5.5 Data analyses

Films images were digitalized into the 256 levels of optical density with a Microtek scanner (SM III, Microtek Europe, Rotterdam, The Netherlands) and quantification of film autoradiograms was carried out using computer-assisted optical densitometry (NIH Image, version 1.58, Wayne Rasband, NIMH). Densitometric readings were taken of the nucleus accumbens, dorsal striatum (caudate putamen), the VTA and substantia nigra in accordance with the Paxinos and Watson Rat Atlas (1997); identification of different brain nuclei was determined from adjacent sections which were stained with

cresyl-violet. Values obtained from duplicate brain sections for each subject were averaged. For in situ hybridization experiments, the values were expressed as dpm/mg of tissue by reference to the co-exposed ^{14}C standard. For receptor autoradiography, specific binding was calculated by subtracting non-specific binding from total binding in the specific area of interest. Optical densities measured from film receptor autoradiograms were transformed into μCi/g tissue using a standard curve generated with the ^{3}H-standards. The percent agonist-induced stimulation from the [^{35}S]GTPγS autoradiography experiments was calculated as [stimulated-basal]/basal x 100. Images from the β-Imager were analyzed directly as cpm/mm^{2}using Betavision Analysis (Biospace Mesures).

3.6 BRAIN TISSUE STUDIES

3.6.1 Fluorescent immunosorbent assay

In the fluorescent immunosorbent assay (FLISA) we used both a traditional antibody aimed towards both naïve and activated μOR and a conformation specific antibody recognizing an activated N terminus conformation state of the receptor (Gupta et al 2006). To prepare cell membranes, the dissected tissue was homogenized in 50mM Tris-HCl (pH 7.4) + 10 % sucrose and 1X protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). Following a 10 min. centrifugation (15900 rpm at +4°C), the pellet was resuspended in 50mM Tris-HCl (pH 7.4) with protease and phosphatase inhibitors. After 30 min. incubation on ice, the samples were centrifuged (15900 rpm at +4°C for 20 min.) and resuspended in 50mM Tris-HCl (pH 7.4) with protease and phosphatase inhibitors. 3 μg membrane proteins were plated on a high binding 96 well ELISA plates (Fisher scientific research, NJ, USA) and was left to dry over night at room temperature. The following day, the membranes were washed with phosphate buffered saline (PBS) and blocked with 3 % natural goat serum in PBS for 1 hour at room temperature. Primary antibody solutions (1:500 dilution) were added to the wells followed by incubation at 4°C over night. The plates were then washed three times with PBS before incubation at 37°C for 1 hour with secondary antibody solution (fluorescence-linked goat raised anti-rabbit IgG or goat raised anti-mouse IgG, depending on the origin of the primary antibody; IRDye, Li-cor biosciences, Nebraska, USA), diluted 1:2000. The plates were once again washed three times with PBS before the fluorescence, corrected integrated intensity (I.I.), at 700 or 800 nm emission wavelength was determined for each well using the Odyssey Infrared Imaging System (Li-cor biosciences, Nebraska, USA).

3.6.2 Radioimmunoassay

Met-enkephalin was analyzed in the supernatant collected from the membrane purification (see above). Approximately 10-30 μl of sample supernatant was analyzed for ir-Met-enkephalin using the Met-enkephalin radioimmunoassay (RIA). For this, the samples were incubated with a 1:2000 dilution of Met-enkephalin antiserum (Bachem Bioscience Inc, Philadelphia, USA) in a RIA buffer (10 mM Tris-Cl buffer pH 7.5 containing 0.1% gelatin; Bio-Rad, California, USA; 0.1% bovine serum albumin ;protease-free, Sigma; 0.1% Triton X-100 and 0.02% sodium azide). On the following day, ^{125}I-Met-enkephalin (~10,000 cpm/tube; Bachem Bioscience Inc.) was added and the tubes were incubated overnight at 4°C. To terminate the reaction, 100 μl of goat

anti-rabbit globulin and 100 µl of normal rabbit serum were added in RIA buffer (without gelatin). The antigen-antibody complex was separated from the unbound radioligand by centrifugation and the radioactivity in the pellet was measured in a gamma counter (Perkin Elmer Life Sciences, Shelton, CT, USA). A standard curve consisting of 0-1000nM Met-enkephalin was used to calculate the amount of ir-Met-enkephalin in each sample.

3.6.3 Liquid chromatography- mass spectrometry

Synthetic standards of anandamide and 2-AG, $^2H_8$-AEA and $^2H_8$-2-AG were bought from Cayman Chemical (Ann Arbor, MI, USA). Anandamide and 2-AG were extracted from the brain tissues according to Kingsley and Marnett (Kingsley and Marnett 2003). In brief, the brain tissue was homogenized in ethylacetate:hexane (9:1; 40 ml per gram of tissue) containing 500 pmol of each internal standard. Lipid extracts were purified via solid phase extraction (SPE) and the eluate were analyzed by LC-MS as described by (Giuffrida et al 2000) using a Hewlett Packard 1100 Series HPLC/MS system equipped with a Phenomenex HyperClone ODS column. Quantitative analysis were performed by positive electrospray detecting diagnostic ions (protonated molecular ions $[M + H]^+$ and sodium adducts of molecular ions $[M + Na]^+$) in selected ion monitoring (SIM) mode. Data analysis was performed using HP Chemstation software.

3.7 STATISTICAL ANALYSIS

Data were analyzed with one- or two-way analysis of variance (ANOVA) with repeated measures where appropriate. Significant ANOVA results were followed by Planned Comparisons and Bonferroni correction for multiple comparisons or Tukey post hoc analysis. The stereotypic ratings in Paper I was analyzed with Mann-Whitney non-parametrical rank test. Statistical significance was set as p<0.05 and trends considered for p<0.10.

4 RESULTS AND DISCUSSION

4.1 ADOLESCENT CANNABIS EXPOSURE

4.1.1 Effects of early adolescent cannabinoid exposure on dopamine and motor behavior responses to amphetamine (paper I)

Since nucleus accumbens shell dopamine release and locomotor sensitization are features highly implicated in reward, these parameters were studied in the evaluation of cross-interactions between cannabis and amphetamine in view of the gateway hypothesis. We found that sub-chronic early adolescent exposure (at PND 28-32) with the synthetic cannabinoid agonist WIN 55,212-2 (1.25 mg/kg) failed to cross-sensitize with amphetamine (0.5 mg/kg on PND 40) on nucleus accumbens shell dopamine release (figure 8A). The amphetamine challenge per se did induce a marked increase in dopamine release (almost 10 times higher than the base-line level; figure 8A) resembling the response normally observed in adult rats (Di Chiara and Imperato 1988; Hurd and Ungerstedt 1989; Kuczenski and Segal 1989; Zetterström et al 1983), while the WIN 55,212-2 challenge only induced a trend to significant increase (figure 8B). One other study evaluated the effects of chronic adolescent cannabis exposure on amphetamine-induced dopamine related effects (Pistis et al 2004). They noted a development of tolerance in rats treated with cannabis during adolescence (slightly older rats than in our study, 5-6 weeks old), but not adulthood, to amphetamine induced inhibition of mesolimbic dopamine neuron firing. This could however be interpreted as either sensitized or desensitized dopamine release.
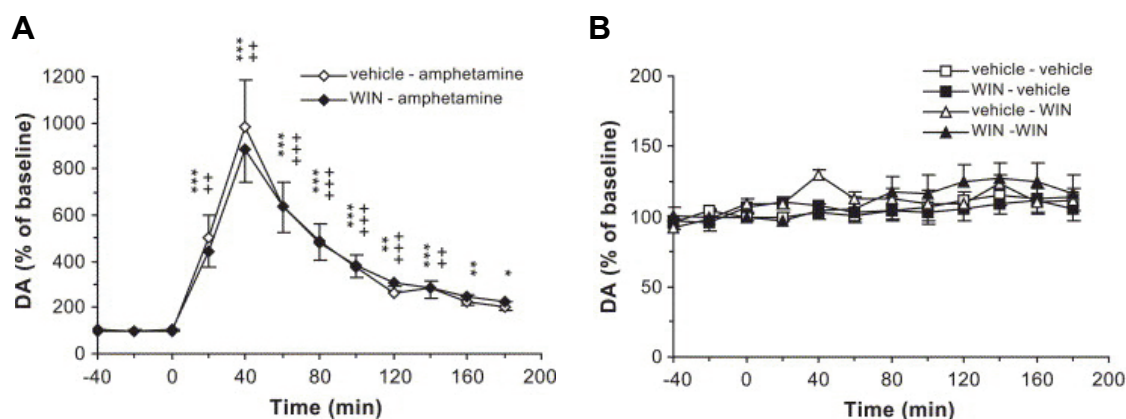
Figure 8. Effects of (A) amphetamine (0.5 mg/kg) and (B) WIN 55,212-2 or vehicle administration on extracellular dopamine levels in the nucleus accumbens shell in adolescent rats, 7 days after subchronic WIN 55,212-2 or vehicle exposure. The challenge injection was given at time 0. Data are expressed as mean ±SEM percent of baseline level. Symbols indicate significant challenge effect; * $P<0.05$, ** $P<0.01$, ** $P<0.001$ WIN-amphetamine vs. WIN-vehicle; [++] $P<0.01$, [+++] $P<0.001$ vehicle-amphetamine vs. vehicle-vehicle.

Furthermore, exposure with different doses of either WIN 55,212-2 (0.625-2.5 mg/kg) or THC (0.75-3.0 mg/kg) during the same age span did not alter amphetamine-induced locomotor activity or stereotyped behavior (figure 9). We also tested the effect of early adolescent THC exposure on varying amphetamine doses (0.5 or 2.0 mg/kg),

administered to adolescent or adult rats (PND 40 or 68; see figure 5 in paper I), but the amphetamine response was again not altered.
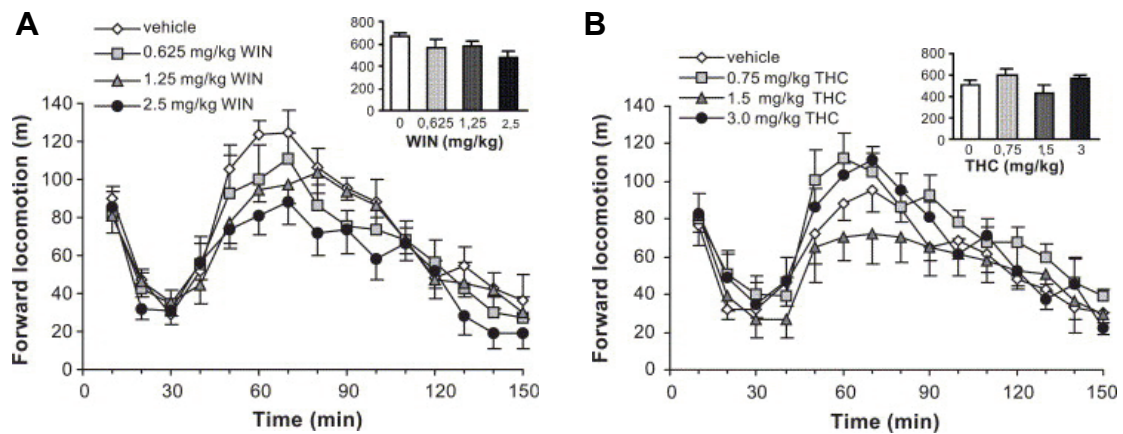
Figure 9. Amphetamine (0.5 mg/kg)-induced locomotor activity in adolescent rats, 7 days after subchronic (5 days) pretreatment with (A) WIN 55,212-2 and (B) THC. The amphetamine injection was given after 30 min. Data are presented as mean forward locomotion ±SEM. The insert shows total forward locomotion ±SEM during the period 20–80 min after amphetamine administration. n=5–6.

Earlier investigations of behavioral cross-sensitization between chronic cannabinoid pre-treatment and amphetamine in adult rats have shown somewhat diverse results. A sensitized locomotor activity response to amphetamine was evident after chronic pretreatment with THC (Gorriti et al 1999) but not WIN 55, 212-2 (Muschamp and Siviy 2002), although stereotypic behavior was sensitized in both studies. The locomotor response to amphetamine was also sensitized after chronic THC pretreatment in rats defined as high responders to a novel environment (Lamarque et al 2001).

In summary, these results suggest a lack of cross-sensitization between cannabinoids and amphetamine on dopamine release in nucleus accumbens shell or motor behavior and thereby did not support the cannabis gateway hypothesis in relation to psychostimulant sensitivity.

4.1.2 Effects of chronic intermittent adolescent cannabis exposure on opioid reward-related behavior (paper II)

4.1.2.1 Heroin self-administration

In light of the vast support for cannabinoid-opioid interactions in behavioral studies, we decided to evaluate the effect of adolescent cannabis exposure on subsequent opioid vulnerability in the next series of studies. We extended the pre-exposure period to cover the whole adolescent period reaching the border of young adulthood (see figure 5 in the methods section). A heroin self-administration model was used, which most reliably mimics addiction behavior in humans. We found that adult rats with a history of adolescent cannabis exposure had altered heroin self-administration behavior. We did not detect any differences in acquisition of heroin self-administration behavior (figure 10), but the THC exposed rats continued to shift their heroin intake upward over the

course of the acquisition phase stabilizing eventually at approximately 25 total responses per session as compared to 15 in the vehicle group. The THC exposed group
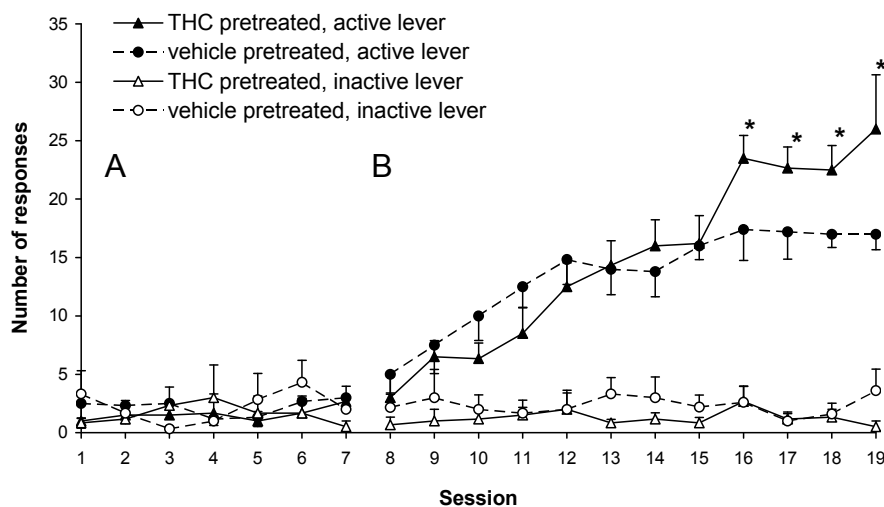
Figure 10. Heroin acquisition behavior on the active and inactive levers during (A) 15 µg/kg/infusion and (B) 30 µg/kg/infusion on an FR-1 schedule of reinforcement in adult rats (beginning postnatal day 57) following adolescent (postnatal days 28-49) THC (n=6) or vehicle (n=5) exposure. Data are presented as mean number of responses per session ±SEM. * P <0.05 vs. vehicle controls for each session.

also responded more for heroin at moderate to low doses (30 and 60 mg/kg/infusion; figure 11) in the dose-response test and had a higher intake during maintenance throughout the course of the experiment (figure 12). The switch from controlled to uncontrolled heroin intake that characterizes addiction is usually potentiated by increased heroin intake over time (Ahmed et al 2000) and thereby the behavior demonstrated by the THC exposed group would predict an enhanced risk of subsequent drug addiction.
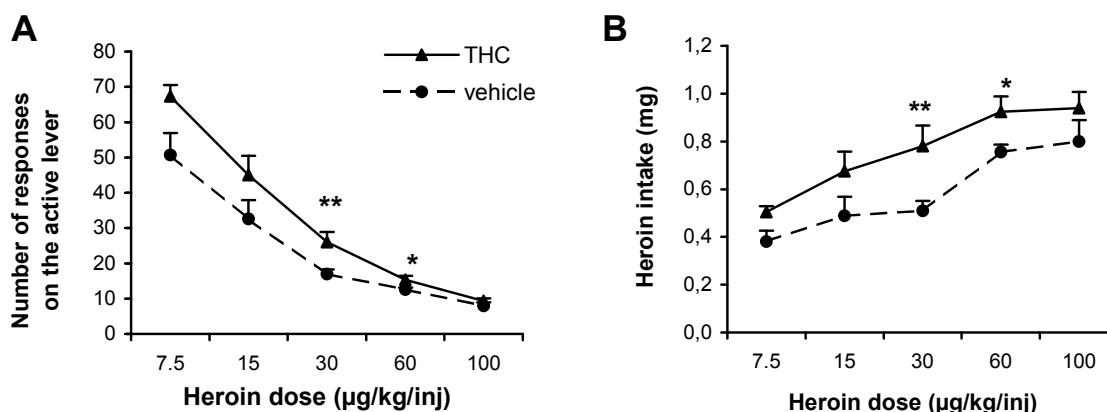
Figure 11. Between-session heroin self-administration behavior dose-response curve (7.5, 15, 30, 60, 100 µg/kg/infusion; randomized order) on a FR-1 reinforcement schedule in rats pretreated with THC (n=6) or vehicle (n=5) during adolescence. (A) Responding for heroin (number of active lever presses) and (B) heroin intake (mg) are shown as mean ± SEM. *, P < 0.05; **, P< 0.01 as compared to respective vehicle control.

28
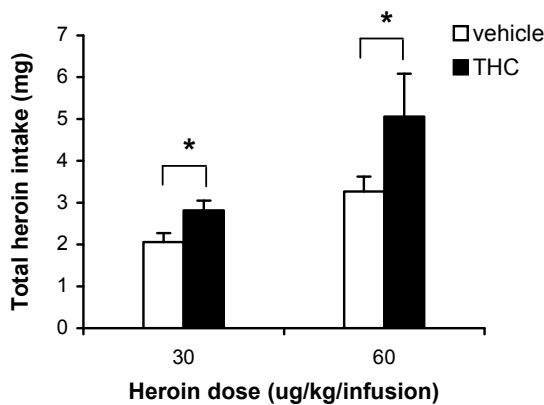
Figure 12. Total heroin intake (± SEM) over 4 consecutive sessions of stable response at 30 and 60 µg/kg/infusion in adult rats exposed to THC (n=6) or vehicle (n=5) during adolescence. * P < 0.05 vs. vehicle control.
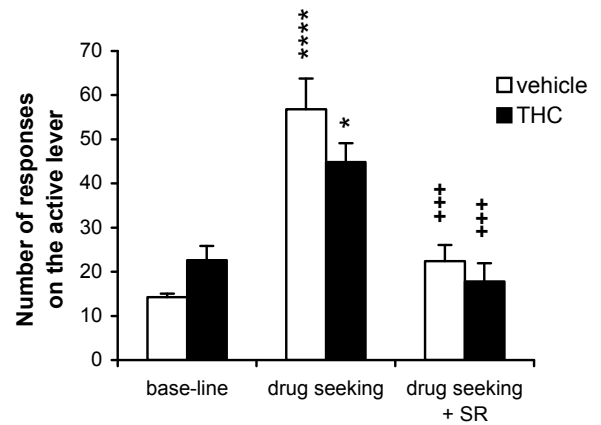
Figure 13. Heroin-seeking behavior (responding on the active lever in the absence of expected drug delivery; no cue light presentation) in THC (n=5)- or vehicle (n=5)-exposed rats and the effect of acute CB1 antagonist pretreatment. * P < 0.05; **** P< 0.0001 as compared to base-line. +++ P< 0.001 vs. without CB1 antagonist pretreatment.

Increased intake on an FR-1 reinforcement schedule cannot provide information whether the rats have increased drug liking/wanting or blunted reinforcement that could lead to a compensating higher drug intake. However, increased intake on the descending part of the dose-response curve as we noted here has been hypothesized to reflect a decrease in reward function (Ahmed and Koob 2005) in view of the opponent process hypothesis of addiction (Koob et al 1997; Koob and Le Moal 2001). This would suggest that the adolescent THC exposure resulted in a hedonic deficit that is alleviated by increased heroin intake. It could also be associated with increased motivation to take the drug. However, in the absence of heroin and associated cues, both THC and vehicle exposed rats showed similar increases in drug seeking (figure 13) and there was no difference in the number of active responses between the groups. Vehicle-exposed rats had a higher percent increase in the number of responses on the active lever, but the behavior was non-specific since they also had a higher responding on the inactive lever. Using a progressive ratio paradigm which best assesses motivational behavior, Solinas et al (2004a) showed that adult exposure to THC does not alter the motivation to take heroin even though the intake was elevated. There may of course be differences in the incentive salience of heroin in association with adolescent compared to adult THC exposure, so whether adolescent cannabis exposure alters the motivation to respond for heroin in a progressive ratio paradigm or not needs to be addressed in the future.

4.1.2.2 Morphine conditioned place preference

Morphine-induced conditioned place preference in a separate group of animals treated similarly with THC or vehicle during the adolescent phase is shown in figure 14. THC and vehicle treated animals did not differ in locomotor activity when exposed to the novel environment of the conditioned place preference apparatus during the habituation session (5160 ± 896 counts in the vehicle group compared to 6623 ± 687 counts in the

THC group), indicating that adolescent THC exposure does not induce long-lasting alterations in novelty behavior or base-line locomotor activity. Since a biased conditioned place preference model was used, all rats but two (one in each pre-exposure group) showed an initial preference for the black compartment during habituation and these two rats were excluded from the study. There were no differences between the groups in time spent in the drug-paired compartment during the pre-conditioning test. In the post-conditioning test however, rats exposed to THC during adolescence had a significant preference to the environment associated with the low dose morphine which vehicle controls failed to show; THC exposed rats had a significantly higher increase in time spent in the drug-paired compartment [$F(1,10) = 10.21$, $P<0.01$]. The fact that rats showed enhanced conditioned place preference to a low dose morphine would suggest that THC-pretreated animals had heightened reward response to opiates.

A limitation of the study is that several investigations have shown preference to 4 mg/kg morphine. There are a number of differences in the experimental designs that could explain this discrepancy, e.g. ours is the only study performed during the dark phase. It is also important to emphasize that both THC and vehicle animals were simultaneously processed in the morphine conditioned place preference paradigm. Additional dose response studies will help to confirm these results. Nevertheless, this study clearly showed that THC exposed animals were more sensitive to the rewarding effects of morphine.
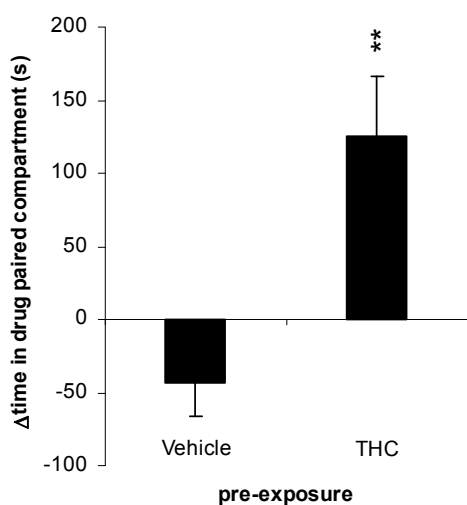
Figure 14. Morphine conditioned place preference showed as Δtime spent in the drug paired compartment (postconditioning test-preconditioning test) in rats exposed to vehicle or THC during adolescence. ** P< 0.01 as compared to the vehicle group. n=5-7.

Taken together, chronic intermittent cannabis exposure during the adolescent period alters opioid reward-related behavior in adult animals, both in terms of increased intake of heroin in a self-administration experiment and elevated sensitivity to morphine conditioned place preference. Conditioned place preference and the self-administration model measures different aspects of drug reward/reinforcement, thus different components of drug reward-related mechanisms may be differentially affected by the early THC exposure. Nevertheless, the findings that THC-exposed rats had both increased heroin self-administration and enhanced sensitivity to morphine conditioned place preference would suggest dysfunction in hedonic processing leading to an enhanced risk for subsequent drug dependence.

4.1.3 Neurobiological alterations within the opioid and cannabinoid systems after adolescent cannabis exposure (paper II and III)

4.1.3.1 PENK mRNA expression and Met-enkephalin ir levels

The endogenous opioid neuropeptide most associated with regulating hedonic state is enkephalin (Skoubis et al 2005), and the nucleus accumbens (in particular the shell division) is the most limbic-related striatal subregion tightly coupled with reward behavior (Everitt and Wolf 2002; Koob 1992). We found that PENK mRNA expression was elevated specifically in the nucleus accumbens shell one week after the adolescent THC exposure (on PND57, when the heroin self-administration was initiated in another group of animals; figure 15). The selective enhancement of PENK mRNA expression in the nucleus accumbens shell could therefore underlie the altered heroin intake behavior evident in THC-exposed animals. The enhancement of nucleus accumbens shell PENK gene expression in THC animals was still evident even after 45 days of heroin self-administration, which suggests a persistent disturbance of the enkephalin reward system after adolescent THC exposure.
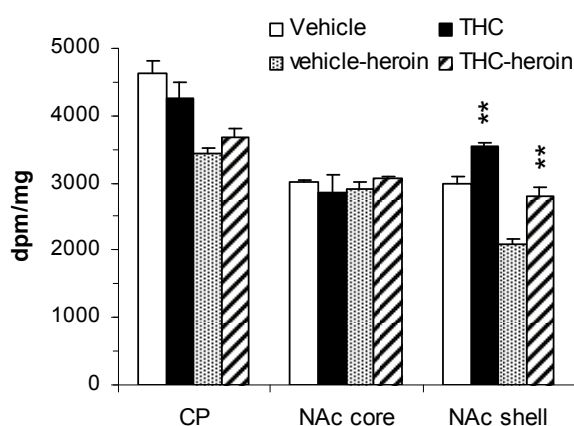
Figure 15. Proenkephalin mRNA expression levels (expressed as dpm/mg; Mean ± SEM) in the caudate putamen (CP), nucleus accumbens (NAc) core and shell of adult rats with adolescent exposure to THC or vehicle. The data represents animals at the time point (PND 57) corresponding to the start of heroin self-administration and animals that had self-administered heroin for approximately 45 days. (n=4-6) **, P < 0.01 as compared to respective vehicle control

Given the significant effect of adolescent THC exposure on PENK levels, we evaluated the time course effects of adolescent THC exposure on the enkephalin system in paper III. The enkephalin containing medium spiny neurons in the nucleus accumbens express cannabinoid CB_1 receptors as well as dopamine D_2 receptors (Lu et al 1998; Pickel et al 2004). THC exposure is likely to activate the CB_1 receptors and THC administration increases dopamine release in the nucleus accumbens which would lead to activation of the D_2 receptors. Activation of these receptors would predict decreased enkephalin expression and peptide release from axon collaterals. However, reductions in Met-enkephalin immunoreactivity following THC exposure was only seen in brain tissue homogenates from nucleus accumbens core after the full treatment paradigm in late adolescence, collected 24 hours after the last THC injection (figure 16).
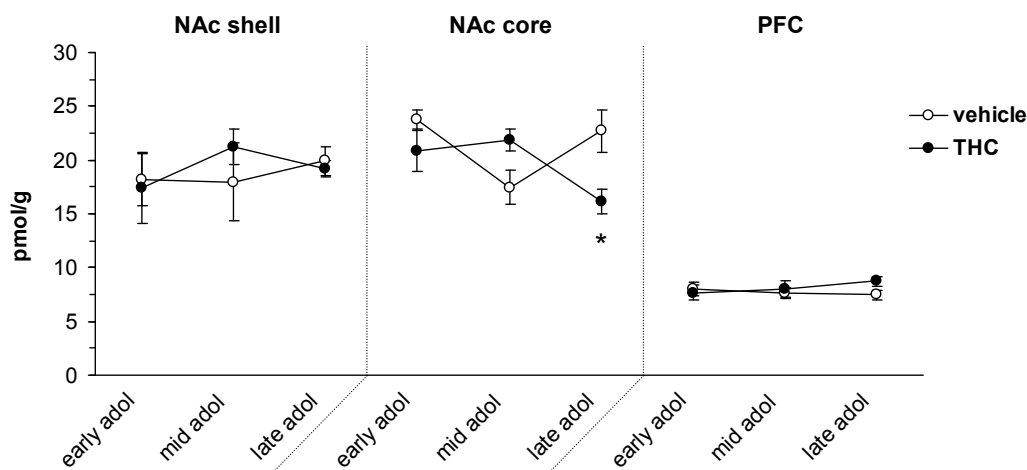
Figure 16. Levels of Met-enkephalin immunoreactivity at different time-points during adolescence and the effect of THC exposure. Early, mid and late adolescence groups had received 1, 4 or 8 injections of THC or vehicle respectively. Data is shown as mean +/- SEM. * = significant treatment effect, P<0.05, THC vs. vehicle control. NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; PFC, prefrontal cortex.

The fact that increased PENK mRNA expression was detected following the drug-free period into adulthood could reflect an allostatic response to counteract a potential down-regulation of the PENK during THC exposure. Further supporting this theory is that our model of prenatal THC exposure showed decreased PENK mRNA expression in the nucleus accumbens shell shortly after the exposure but had increased expression levels in the same area when they had reached adulthood; these rats also showed enhanced opioid-induced reward related behavior in adulthood (see paragraph 4.2 and paper IV). A previous investigation revealed increased PENK mRNA expression in the nucleus accumbens (no subcompartment specified) shortly after chronic cannabinoid exposure (Manzanares et al 1998). However, methodological differences may account for the discrepant result, such as the use of the full CB_1 receptor agonist CP55,940 and adult animals in the latter study, but it does still support a disturbance of the enkephalin system after cannabis exposure.

4.1.3.2 μ opioid receptor density and function

There was no difference in μ opioid receptor density between THC and vehicle exposed rats, in terms of [^{3}H]DAMGO binding one week after the last THC injection (when the heroin self-administration was initiated in another group of animals, PND 57). However, we found elevated μOR function in terms of DAMGO-stimulated [^{35}S]GTPγS binding in the VTA and substantia nigra in THC exposed rats at the same time point (figure 17, paper II). Increased μOR function in the brain stem would be expected to potentiate dopamine levels in forebrain areas such as the nucleus accumbens and caudate putamen considering that stimulation of these midbrain receptors would disinhibit the inhibitory GABAergic regulation on dopamine cell firing (Johnson and North 1992). Interestingly, heroin intake behavior (total number of active lever presses) exhibited on the last day of testing was significantly correlated with agonist activation of the μOR GTP coupling in the nucleus accumbens shell (Spearman's correlation r=0.756, P<0.05). These findings continue to substantiate an important role of the nucleus accumbens μOR in heroin reinforcement.
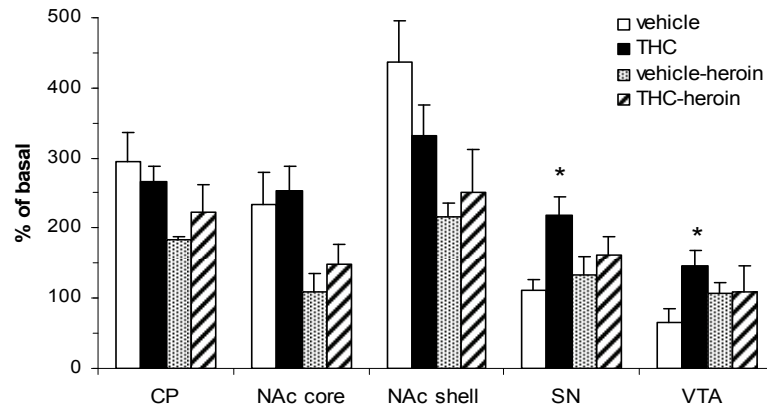
Figure 17. [^{35}S]DAMGO-stimulated GTPγS binding in various striatal and brainstem regions in animals exposed during adolescence to THC or vehicle. Animals were studied as young adults (PND 57, when heroin self-administration was initiated in the behavioral study) and after approximately 45 days of heroin self-administration. Data are expressed as mean percent stimulation ± SEM. CP, caudate putamen; NAc core, nucleus accumbens core; NAc shell, nucleus accumbens shell; SN, substantia nigra; VTA, ventral tegmental area.
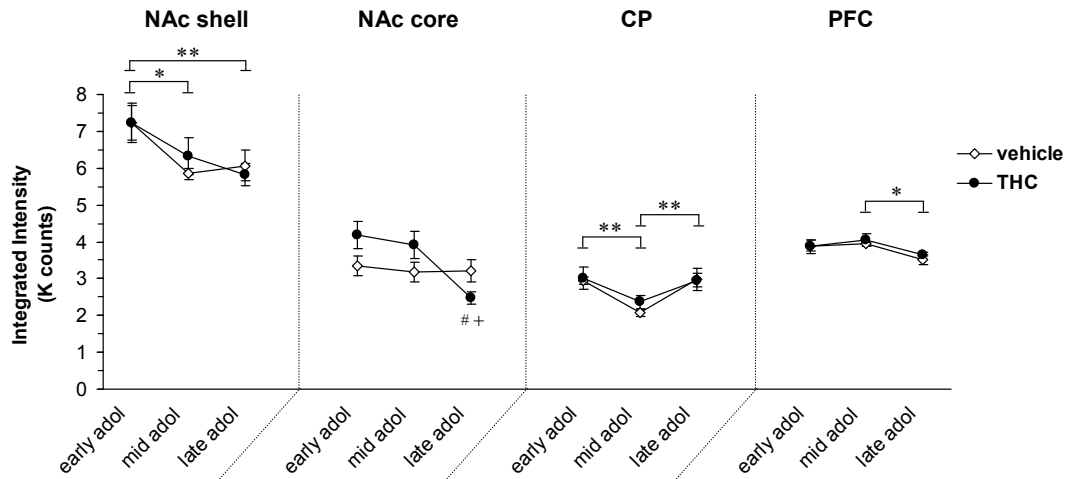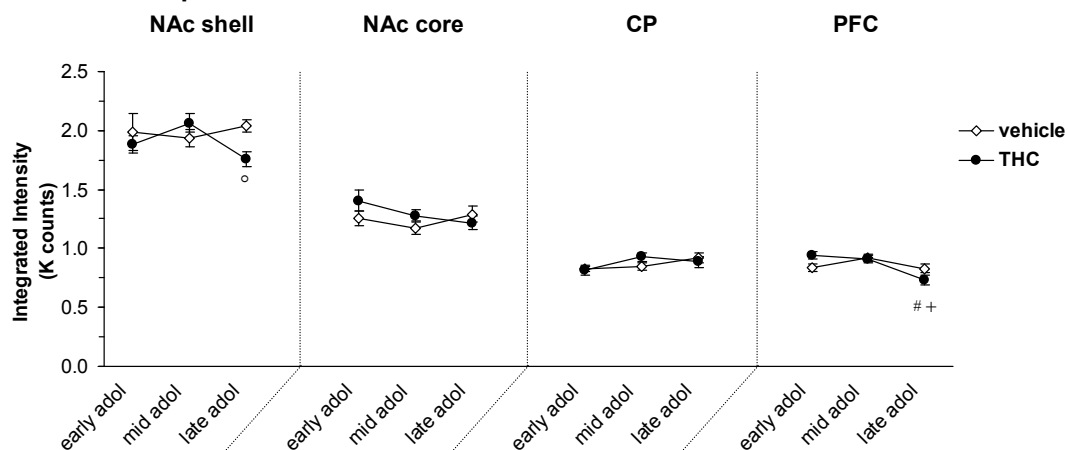
Figure 18. Density of naïve + activated μOR (A) and specifically activated μOR (B) at different time-points during adolescence and the effect of THC exposure. Early, mid and late adolescence groups had received 1, 4 or 8 injections of THC or vehicle respectively. Data is shown as mean +/- SEM. * = significant difference between age groups, P<0.05; ** = P<0.01. ° = significant treatment effect, THC vs. vehicle control P<0.05. # = significantly different from THC early adolescence, P<0.05. + = significantly different from THC mid adolescence, P<0.05. NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; CP, caudate putamen; PFC, prefrontal cortex.

33

In paper III, we studied the time course effects of the adolescent cannabis exposure on µOR density in the nucleus accumbens shell and core, caudate putamen and the prefrontal cortex. Using FLISA with an antibody that recognizes µ opioid receptors in an activated conformational state (Gupta et al 2006), we noted an decrease in the amount of activated µOR in nucleus accumbens shell 24 hours after the last THC injection (on PND 50; figure 18).

4.1.3.3 Endogenous cannabinoid levels

The time course effects of adolescent THC exposure on the levels of endogenous cannabinoids were also studied in the nucleus accumbens, caudate putamen and prefrontal cortex. We found that adolescent rats exposed to THC had increased amounts of anandamide in the nucleus accumbens, particularly after the first injection, but there was still a trend towards increased levels after the full treatment paradigm
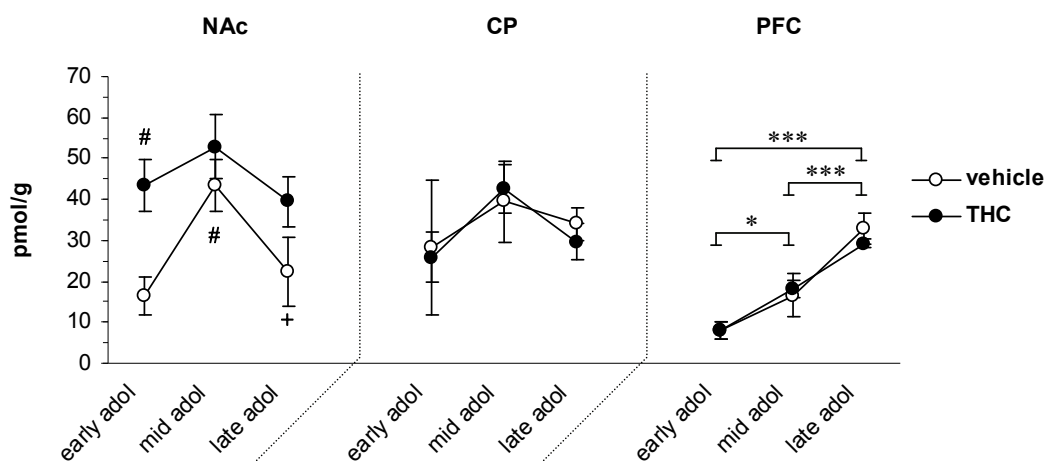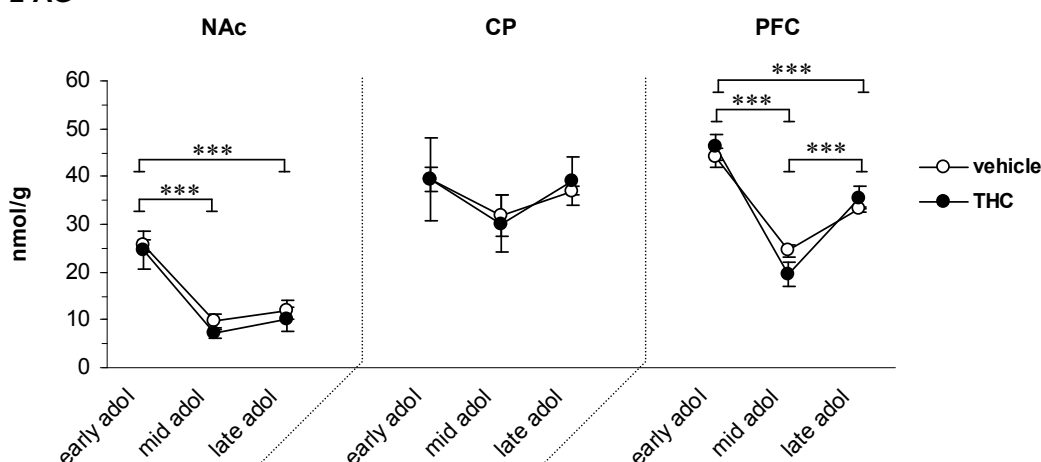
Figure 19. Levels of Anandamide (A) and 2-AG (B) at different time-points during adolescence and the effect of THC (1.5 mg/kg) exposure. Early, mid and late adolescence groups had received 1, 4 or 8 injections of THC or vehicle respectively. Data is shown as mean +/- SEM. * = significant difference between age groups, $P<0.05$; *** = $P<0.001$. # = significantly different from vehicle early adolescence, $P<0.05$. + = significantly different from vehicle mid adolescence, $P<0.05$. NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; CP, caudate putamen; PFC, prefrontal cortex.

(figure 19). A previous study examined the effects of THC exposure on endogenous cannabinoid anandamide and similar to the current findings observed increased anandamide in limbic forebrain areas that contained the nucleus accumbens (Di Marzo et al 2000). The anandamide level in the nucleus accumbens is stimulated by cortical glutamate afferent signaling and dopamine D_2 receptor activation (Patel et al 2003). Thus our finding of enhanced anandamide levels could indicate enhanced glutamate and/or dopamine signaling in the nucleus accumbens as a consequence of THC exposure. Glutamate release in the nucleus accumbens is inhibited by acute THC administration (Robbe et al 2001), while dopamine release is increased (Chen et al 1990b; Tanda et al 1997). This might also be the case after chronic THC exposure in our adolescent animal model, which is further supported by the apparent decreased Met-enkephalin levels in NAc core. Since the enkephalin containing medium spiny neurons in the NAc also express dopamine D_2 receptors (Lu et al 1998; Pickel et al 2004), increased dopamine activation would lead to decreased enkephalin expression.

4.1.3.4 CB₁ density and function

We did not detect any alterations in [^{3}H]WIN55,212,2 binding or [^{35}S]WIN55,212,2-stimulated GTPγS coupling after adolescent THC exposure, studied one week after the last injection. However, there was a significant positive correlation between the CB_1 binding in the substantia nigra and heroin self-administration behavior (Spearman's correlation r=0.731, P<0.05). Increased CB_1 binding in this brainstem region would disinhibit dopaminergic neurons leading to an enhanced dopamine tone in the striatum.

In summary, THC exposure during adolescence strongly alters enkephalin and µOR limbic systems, while the cannabinoid alterations were specific to the nucleus accumbens where anandamide levels were increased. This suggests an altered reward processing and/or hedonic state as a consequence of adolescent cannabis exposure.

4.1.4 Ontogeny of the cannabinoid and opioid systems in limbic-related brain areas during adolescence (paper III)

Until now, the ontogeny of endogenous cannabinoids and opioids specifically in limbic-related areas during adolescence has not been examined. In our study of adolescent rats, developmental changes in endogenous cannabinoid levels were evident in both the nucleus accumbens and the prefrontal cortex (figure 19). Most profound was the continuous increase in the prefrontal cortex anandamide levels throughout the adolescent period; the levels were almost three times higher in late adolescence than early adolescence. The levels of 2-AG on the other hand was markedly lower in the later part of adolescence than in the beginning in both the nucleus accumbens and the prefrontal cortex. This indicates that active neurodevelopment takes place in brain areas involved in reward and, to an even bigger extent, cognition. Blocking endocannabinoid function by administering the CB_1 antagonist SR141617A (rimonabant) to adult rats have been shown to improve working memory (Lichtman 2000; Terranova et al 1996) supporting a role for endocannabinoids in cognitive functions. Studies also indicate that endocannabinoids are involved in neurogenesis and neuroprotection, e.g. impaired adult neurogenesis was evident in CB_1 knock-out mice (Jin et al 2004). The fluctuations in endocannabinoid levels we detected may therefore reflect the neurodevelopmental remodeling in these brain areas during the adolescent period.

Met-enkephalin levels were stable throughout the adolescent period. Even so, developmental changes were seen within the opioid system; the µOR density in the nucleus accumbens shell and the prefrontal cortex was decreased during the course of adolescence, whereas the density in caudate putamen was temporarily down-regulated during mid adolescence (figure 18).

A potential limitation of the time course experiment in control animals could be that the increasing number of vehicle injections and probable stress could account for the neurobiological differences between the age groups. However, it is unlikely that it would induce such marked alterations in endocannabinoid levels, in opposite directions, as we note here. It would be good though to validate our findings in naïve animals in the same age groups.

Taken together, the results show robust alterations in endocannabinoid levels during the adolescent period specific to brain areas involved in cognitive function and motivation. There were also opioid-related alterations in the same brain areas, but to a smaller extent.

4.2 PRENATAL CANNABIS EXPOSURE

4.2.1 Effects of prenatal cannabis exposure on heroin self-administration behavior (paper IV)

In addition to adolescence, the prenatal period is another critical time point of brain development when THC exposure could occur. To evaluate the consequences of prenatal cannabis exposure, we studied behavioral and neurobiological paradigms in a rat model of prenatal cannabis exposure (see figure 6 in the methods section). We found that prenatal cannabis exposure did not alter the acquisition of heroin self-administration behavior (see figure 1 in paper IV) or maintenance intake in adult rats. Even so, a number of specific behavioral findings point to enhanced opiate sensitivity as a consequence of prenatal cannabis exposure. THC exposed rats exhibited a shorter latency to the first active lever press during the stable phase of the acquisition period, less than 1 minute compared to almost 2 minutes in the vehicle group (see figure 2 in paper IV). They also responded more to lower doses of heroin in the dose response curve (figure 20). A vertical upward shift of the dose-response curve is usually hypothesized to reflect both a decrease in the rewarding effects of heroin (leading to an
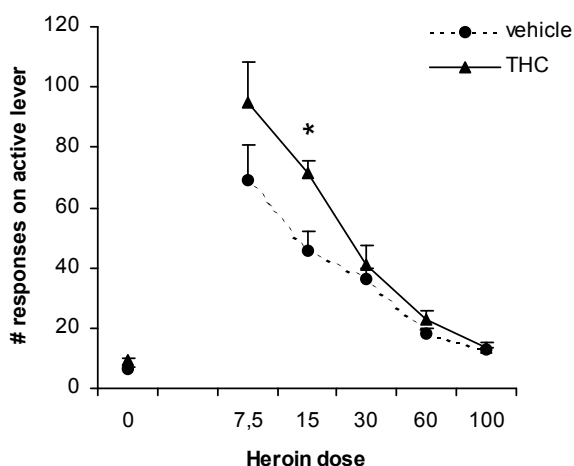
Figure 20. Heroin self-administration behavior (active lever presses) dose-response curve in adult rats with prenatal THC or vehicle exposure. Data refer to the last self-administration session of a given dose. Values are expressed as mean ± SEM. *, P<0.05 as compared to vehicle

increased consumption) and increased motivation for the drug (evidenced by an increased peak of the dose-response curve (Ahmed and Koob 2005). The rats prenatally exposed to THC did not, as mentioned earlier, have an increased heroin intake during maintenance. But under the influence of mild stress (24 hours of food restriction) they showed increased rates of responding for heroin (figure 21).
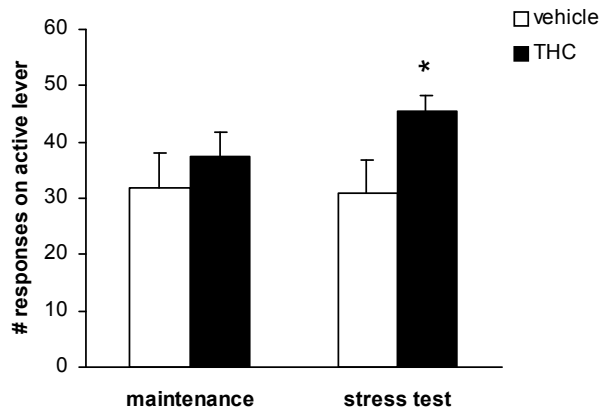

Figure 21. Effect of mild stress (1 day food deprivation) in adult rats with prenatal THC or vehicle exposure. Each bar represents the mean ± SEM of the number of active lever presses for 5 animals per group. *, $P<0.02$ as compared to vehicle control.

Further supporting a long-term vulnerability in the motivation to self-administer heroin rather than just altered sensitivity to the reinforcing properties of the drug is that THC exposed rats had higher levels of heroin-seeking during extinction compared to the vehicle group (figure 22). There was no significant differences in relapse behavior between the groups, although the THC exposed rats showed a trend for higher heroin seeking induced by a heroin priming injection after approximately 21 days of drug extinction. The drug induced relapse behavior was blocked by the CB$_1$ antagonist SR 142716A, to a similar extent in both groups, strengthening previous studies showing a cross interaction between cannabinoid and opioid systems in regulation of relapse mechanisms (De Vries et al 2003; Fattore et al 2003; Navarro et al 2001).
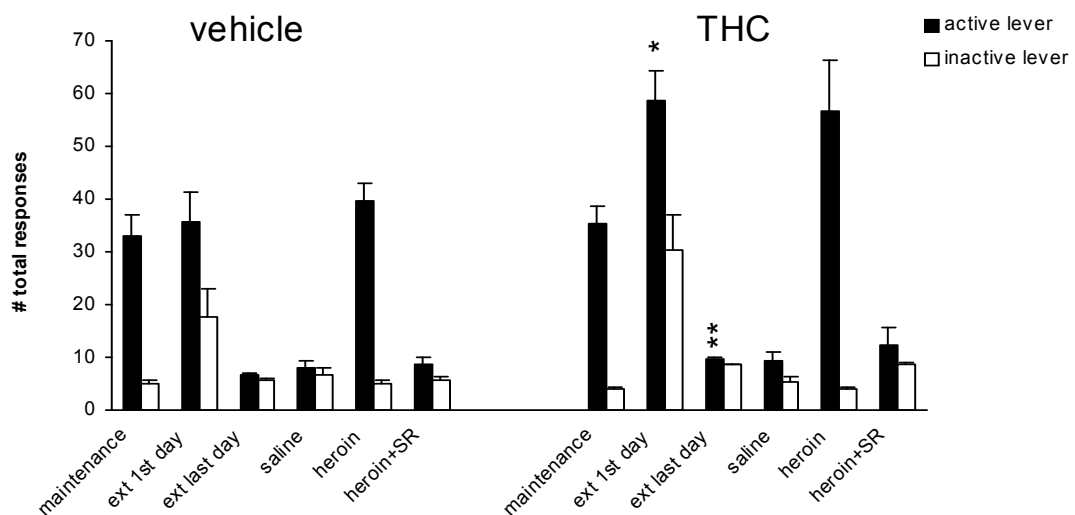
Figure 22. Effect of acute primings on the reinstatement of heroin-seeking behavior following heroin extinction in adult rats with perinatal THC or vehicle exposure. Each bar represents the mean ± SEM of active and inactive lever press over the last 3 days of heroin self administration (maintenance), during drug-free extinction (ext) and during the reinstatement sessions with heroin prime (0.25 mg/kg, s.c.) and SR 141716A (Rimonabant; 3 mg/kg, i.p.) administration. N=5 animals per group. *, $P<0.02$; **, $P<0.01$ as compared to vehicle control.

Altogether, the results suggest that adult rats prenatally exposed to THC do not show altered heroin self-administration behavior during normal conditions. However, they had increased heroin-seeking during extinction and after food deprivation, suggesting an increased motivation for drug use under the influence of stress.

4.2.2 Neurobiological alterations within the cannabinoid and opioid systems after prenatal cannabis exposure (paper IV)

4.2.2.1 *PENK mRNA expression*

Immediately following the prenatal THC exposure, on PND 2 that corresponds to the mid-gestational period in humans, there was a down-regulation of PENK mRNA expression in the nucleus accumbens (figure 23). We noted a profound disruption of the PENK mesocorticolimbic system even approximately 60 days following THC prenatal exposure, with increased levels of PENK mRNA in the nucleus accumbens shell and core as well as the central and medial amygdala, brain areas highly involved in emotional regulation, reward, goal directed behavior and motivation (Cardinal et al 2002; Kelley 2004; Koob 1999). There were no alterations in PENK levels in the caudate putamen, that is most associated with sensorimotor function (Nakano et al 2000), at any of the time points studied. A previous investigation found the opposite though, alterations in caudate putamen but not in the nucleus accumbens in male offspring on gestational day 21 (Kelley 2004; Perez-Rosado et al 2000; Skoubis et al 2005). The discrepancy may partly be explained by different routes of administration (intravenous vs. oral). However, in our current model of prenatal THC exposure, PENK gene expression is selectively altered in mesocorticolimbic neural populations, well known to modulate hedonic state (Kelley 2004; Perez-Rosado et al 2000; Skoubis et al 2005). These results fit with the observations of our model of adolescent THC exposure (see paragraph 4.1, papers II and III), which showed the
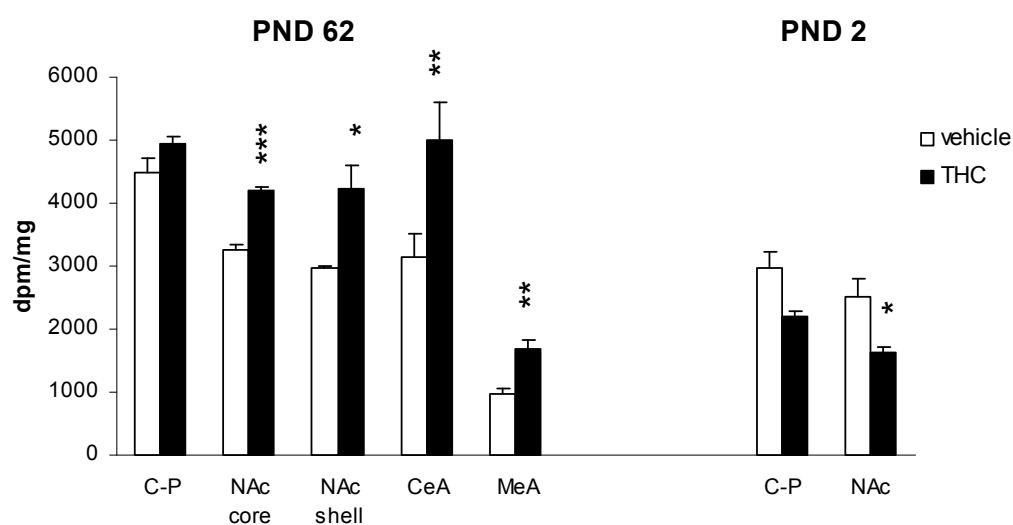
Figure 23. PENK mRNA expression levels in the various brain areas of adult postnatal day 62 (left panel) and post-natal day 2 (right panel) rats with perinatal THC or vehicle exposure. C-P, caudate-putamen; CeA, central amygdala; MeA, medial amygdala. Values are expressed as mean ± SEM (dpm/mg) for 5 animals per group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ as compared to the vehicle

control. CP, caudate putamen; NAc core, nucleus accumbens core; NAc shell, nucleus accumbens shell; CeA, central amygdala; MeA, medial amygdala.

same pattern of enkephalin regulation in the nucleus accumbens (decreased Met-enkephalin immunoreactivity immediately after THC exposure and increased PENK mRNA in young adulthood), also together with an increased opioid reward-related behavior. Enkephalin containing medium spiny neurons in the nucleus accumbens express CB_1 receptors (Lu et al 1998; Pickel et al 2004) and since they are coupled to $G_{i/o}$ G-proteins (Pertwee and Ross 2002) activation of these receptors would cause a decrease in PENK gene expression. This down-regulation of the PENK during THC exposure could be counteracted by the observed increased PENK mRNA expression in the young adult animals creating a state of allostasis.

The medial and central amygdala are components of the extended amygdala, and PENK disturbance in these areas has predominantly been associated with stress and anxiety (Drolet et al 2001; Kang et al 2000). The elevated PENK mRNA levels in central and medial amygdala are consistent with our behavioral findings that drug abstinence and mildly stressful events such as food deprivation increase heroin seeking behavior. The choice of food deprivation as a stress model could be questioned since the cannabinoid system is involved in food related behavior (Di Marzo and Matias 2005). The fact that the pre-exposure groups did not differ in weight neither at birth nor as young adults at the start of the self-administration experiment supports that the increase in heroin intake following food deprivation is not related to an alteration of feeding behavior. Drug abstinence, particularly during its early phase, is a very stressful event for drug-dependent subjects. Thus, the marked increase of heroin-seeking in THC-pretreated animals, especially during the first day of heroin extinction, could reflect a behavioral response to stress which intensifies the motivation for drug use. Several studies report cannabinoid involvement in anxiety related behavior, both in human (Hall 1998; Patton et al 2002) and rodents (Arevalo et al 2001; Onaivi et al 1990) and the hypothalamic-pituitary-adrenal axis have been shown to be stimulated during cannabinoid administration (Corchero et al 1999; Puder et al 1982) and withdrawal (Rodriguez de Fonseca et al 1997). Increased PENK though, is normally associated with a reduced anxiety response (Kang et al 2000). Nevertheless, acute and chronic elevation of corticosterone that facilitate potentiation and termination of stress response, respectively, both elevate PENK in various brain regions (Ahima et al 1992). Systematic evaluation of the glucocorticoid system is necessary with the current prenatal THC model to fully understand the role of the stress system in contributing to the PENK mesocorticolimbic mRNA levels. It also has to be determined whether the elevated PENK mRNA state reflects compensation for tonically reduced enkephalin peptide levels.

4.2.2.2   PDYN mRNA expression

In addition to enkephalin, striatal output function is also modulated by dynorphin. PDYN mRNA is known to be involved in the long-term regulation of both psychostimulants (Svensson and Hurd 1998) and opiates (Tjon et al 1997). However, no alterations in PDYN levels were seen in the THC-exposed offspring. Dynorphin is primarily expressed in the striatonigral GABAergic medium spiny neurons, so our findings of specific PENK alterations indicate that prenatal cannabis exposure selectively affects the striatopallidal medium spiny neurons, where PENK is mainly expressed.

4.2.2.3 μOR coupling

Long-term alteration of prenatal THC on the adult nucleus accumbens opioid system was also apparent for the μOR coupling (figure 24). THC-exposed offspring had significant decrease in DAMGO-stimulated GTPγS coupling in the nucleus accumbens shell, which would suggest decreased opioid reward. Possible evidence of reward deficit in the THC animals was the apparent vertical shift of the dose-response curve. There were also non-limbic opioid alterations in adult animals with prenatal THC exposure, with increased μOR coupling in the SN. This would predict elevated dopamine levels in output regions, like e.g. dorsal striatum, since stimulation of μOR receptors reduce inhibitory GABA-mediated regulation of dopamine cell firing (Johnson and North 1992). Such conclusion would not fit with our observation of increased heroin-induced locomotor activity in THC exposed animals though.
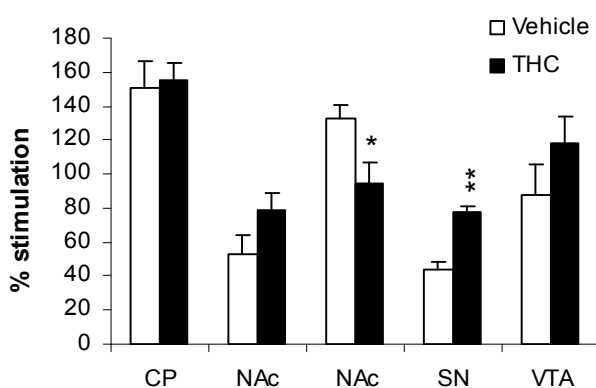
Figure 24. Effect of THC or vehicle prenatal exposure (GD 5 to PND 2) on DAMGO stimulated [^{35}S]GTPγS binding in adult rat brain (PND 62). Values are presented as mean ± SEM. *, $p<0.05$; **, $p<0.01$ as compared to the vehicle control. CP, caudate putamen; NAc core, nucleus accumbens core; NAc shell, nucleus accumbens shell; SN, substantia nigra; VTA, ventral tegmental area.

In summary, long-term alterations are evident in the limbic opioid system after prenatal THC exposure, especially in brain areas related to reward and stress, indicating altered hedonic processing as well as vulnerability to stress.

40
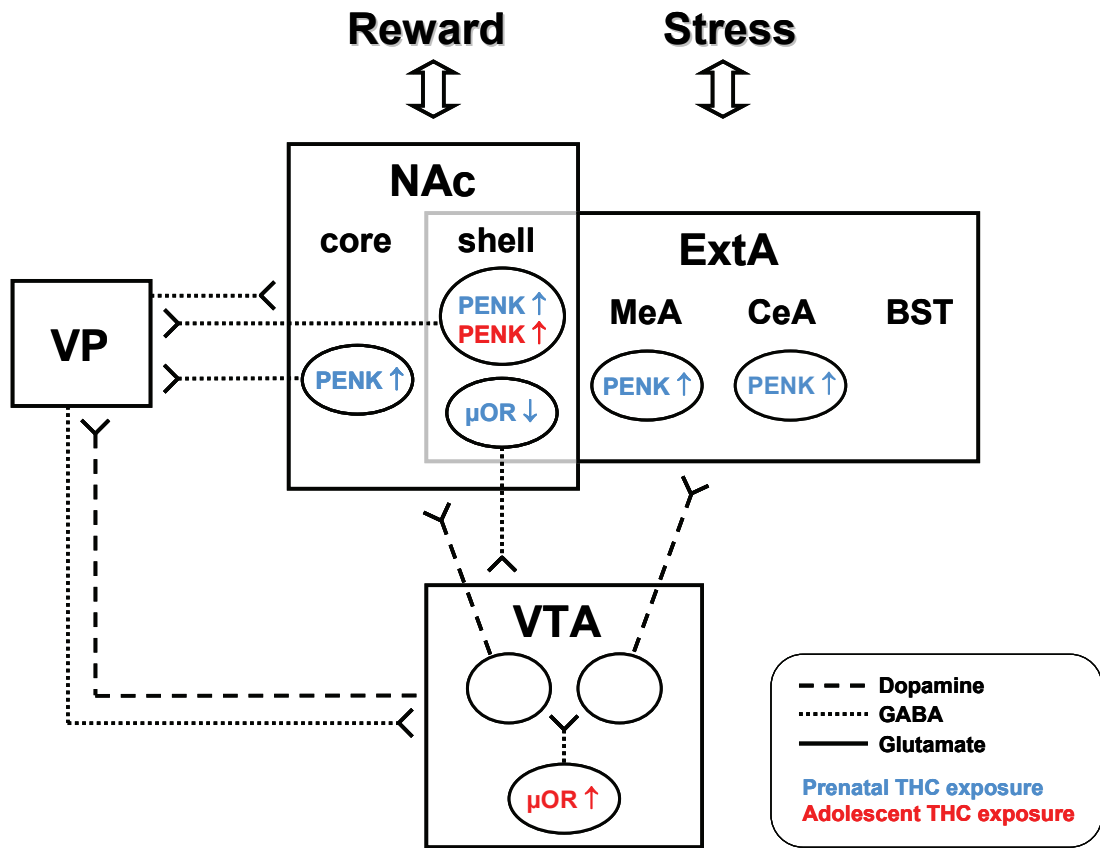
Figure 25. Summary of the opioid alterations in limbic brain areas associated with regulation of reward and stress in adult rats following prenatal or adolescent THC exposure; as compared to the dorsal striatum where no changes were seen. Limbic PENK expression is coupled to hedonic processing and the observed alterations in this system is reflected by the enhanced opioid reward-related behavior evident after both prenatal and adolescent THC exposure. The prenatally exposed animals however, did only show elevated heroin-seeking under the influence of mild stress. This behavior might be explained by the increased PENK expression seen in stress-related brain areas in these rats. After adolescent THC exposure, μOR coupling was enhanced in the VTA, which may potentially lead to elevated heroin-induced dopamine levels in the nucleus accumbens via enhanced disinhibition of dopamine neurons. (see section 4.1.3 and 4.2.2 for further discussion) VP, ventral pallidum; NAc, nucleus accumbens; VTA, VTA; ExtA, extended amygdala; MeA, medial amygdala; CeA, central amygdala; BST, bed nucleus of stria terminalis; BLA, basolateral amygdala.

5 CONCLUSIONS

This thesis presents neurobiological support for the cannabis gateway hypothesis in terms of subsequent opiate abuse. Cannabis exposure during the prenatal phase as well as during adolescence induced discrete opioid-related alterations within brain regions highly implicated in reward and hedonic state processing. These alterations were coupled to altered opioid-induced reward-related behavior, and specifically, THC exposure during development induced:

- increased heroin-intake at low to moderate doses

- increased heroin-intake after exposure to mild stress in prenatal THC exposed rats

- enhanced drug-seeking during extinction in rats exposed to THC during the prenatal period

- enhanced conditioned place preference to morphine in rats exposed to THC during adolescence

Together these behavioral findings suggest enhanced sensitivity to opioid-induced reinforcement as a consequence of early life cannabis exposure.

In contrast to opiate sensitivity, early adolescent cannabinoid exposure did not induce cross-sensitized responses to amphetamine, in terms of dopamine release in the nucleus accumbens or motor behavior. Thus, we did not find support for the cannabis gateway hypothesis in relation to subsequent psychostimulant abuse.

Furthermore, our findings shows that active endocannabinoid- and opioid-related neurodevelopment takes place to a very high extent during the adolescent period, most pronounced in the endocannabinoids levels in cognitive brain areas though also to some extent in reward-related regions.

6 ACKNOWLEDGEMENTS

7 REFERENCES

Agrawal A, Neale MC, Prescott CA, Kendler KS (2004): A twin study of early cannabis use and subsequent use and abuse/dependence of other illicit drugs. *Psychol Med* 34:1227-37.

Ahima RS, Garcia MM, Harlan RE (1992): Glucocorticoid regulation of preproenkephalin gene expression in the rat forebrain. *Brain Res Mol Brain Res* 16:119-27.

Ahmed SH, Koob GF (2005): Transition to drug addiction: a negative reinforcement model based on an allostatic decrease in reward function. *Psychopharmacology (Berl)* 180:473-90.

Ahmed SH, Walker JR, Koob GF (2000): Persistent increase in the motivation to take heroin in rats with a history of drug escalation. *Neuropsychopharmacology* 22:413-21.

Arevalo C, de Miguel R, Hernandez-Tristan R (2001): Cannabinoid effects on anxiety-related behaviours and hypothalamic neurotransmitters. *Pharmacol Biochem Behav* 70:123-31.

Arnold JC, Topple AN, Hunt GE, McGregor IS (1998): Effects of pre-exposure and co-administration of the cannabinoid receptor agonist CP 55,940 on behavioral sensitization to cocaine. *Eur J Pharmacol* 354:9-16.

Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE (2002): Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. *Bmj* 325:1212-3.

Association AP (1994): *Diagnostic and statistical manual of mental disorders*, 4th ed. ed. Washington, D.C.: APA.

Bayer SA, Altman J, Russo RJ, Zhang X (1993): Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *NeuroToxicology* 14:83-144.

Behnke M, Eyler FD (1993): The consequences of prenatal substance use for the developing fetus, newborn, and young child. *Int J Addict* 28:1341-91.

Beltramo M, Piomelli D (2000): Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylglycerol. *Neuroreport* 11:1231-5.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D (1997): Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* 277:1094-7.

Berrendero F, Garcia-Gil L, Hernandez ML, Romero J, Cebeira M, de Miguel R, Ramos JA, Fernandez-Ruiz JJ (1998): Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. *Development* 125:3179-88.

Braida D, Pozzi M, Cavallini R, Sala M (2001): Conditioned place preference induced by the cannabinoid agonist CP 55,940: interaction with the opioid system. *Neuroscience* 104:923-6.

Breivogel CS, Griffin G, Di Marzo V, Martin BR (2001): Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol* 60:155-63.

Caille S, Parsons LH (2003): SR141716A reduces the reinforcing properties of heroin but not heroin-induced increases in nucleus accumbens dopamine in rats. *Eur J Neurosci* 18:3145-9.

Caille S, Parsons LH (2006): Cannabinoid modulation of opiate reinforcement through the ventral striatopallidal pathway. *Neuropsychopharmacology* 31:804-13.

Calignano A, La Rana G, Beltramo M, Makriyannis A, Piomelli D (1997): Potentiation of anandamide hypotension by the transport inhibitor, AM404. *Eur J Pharmacol* 337:R1-2.

Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002): Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev* 26:321-52.

Carlsson A (1959): The occurrence, distribution and physiological role of catecholamines in the nervous system. *Pharmacol Rev* 11:490-3.

Carlsson A, Lindqvist M, Magnusson T (1957): 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature* 180:1200.

Cha YM, White AM, Kuhn CM, Wilson WA, Swartzwelder HS (2006): Differential effects of delta9-THC on learning in adolescent and adult rats. *Pharmacol Biochem Behav* 83:448-55.

Chait LD, Zacny JP (1992): Reinforcing and subjective effects of oral delta 9-THC and smoked marijuana in humans. *Psychopharmacology (Berl)* 107:255-62.

Chaperon F, Soubrie P, Puech AJ, Thiebot MH (1998): Involvement of central cannabinoid (CB1) receptors in the establishment of place conditioning in rats. *Psychopharmacology (Berl)* 135:324-32.

Charmandari E, Kino T, Souvatzoglou E, Chrousos GP (2003): Pediatric stress: hormonal mediators and human development. *Horm Res* 59:161-79.

Cheer JF, Marsden CA, Kendall DA, Mason R (2000): Lack of response suppression follows repeated ventral tegmental cannabinoid administration: an in vitro electrophysiological study. *Neuroscience* 99:661-7.

Chen J, Marmur R, Pulles A, Paredes W, Gardner EL (1993): Ventral tegmental microinjection of delta 9-tetrahydrocannabinol enhances ventral tegmental somatodendritic dopamine levels but not forebrain dopamine levels: evidence for local neural action by marijuana's psychoactive ingredient. *Brain Res* 621:65-70.

Chen J, Paredes W, Lowinson JH, Gardner EL (1990a): Delta 9-tetrahydrocannabinol enhances presynaptic dopamine efflux in medial prefrontal cortex. *Eur J Pharmacol* 190:259-62.

Chen JP, Paredes W, Li J, Smith D, Lowinson J, Gardner EL (1990b): Delta 9-tetrahydrocannabinol produces naloxone-blockable enhancement of presynaptic basal dopamine efflux in nucleus accumbens of conscious, freely-moving rats as measured by intracerebral microdialysis. *Psychopharmacology (Berl)* 102:156-62.

Corchero J, Fuentes JA, Manzanares J (1999): Chronic treatment with CP-55,940 regulates corticotropin releasing factor and proopiomelanocortin gene expression in the hypothalamus and pituitary gland of the rat. *Life Sci* 64:905-11.

Corchero J, Oliva JM, Garcia-Lecumberri C, Martin S, Ambrosio E, Manzanares J (2004): Repeated administration with Delta9-tetrahydrocannabinol regulates mu-opioid receptor density in the rat brain. *J Psychopharmacol* 18:54-8.

Cossu G, Ledent C, Fattore L, Imperato A, Bohme GA, Parmentier M, Fratta W (2001): Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. *Behav Brain Res* 118:61-5.

Dahlström A, Fuxe K (1964): Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta. Physiol. Scand. (Suppl)* 232:1-55.

Day NL, Richardson GA (1991): Prenatal marijuana use: epidemiology, methodologic issues, and infant outcome. *Clin Perinatol* 18:77-91.

Day NL, Richardson GA, Goldschmidt L, Robles N, Taylor PM, Stoffer DS, Cornelius MD, Geva D (1994): Effect of prenatal marijuana exposure on the cognitive development of offspring at age three. *Neurotoxicol Teratol* 16:169-75.

de Olmos JS, Heimer L (1999): The concepts of the ventral striatopallidal system and extended amygdala. *Ann N Y Acad Sci* 877:1-32.

De Vries TJ, Homberg JR, Binnekade R, Raaso H, Schoffelmeer AN (2003): Cannabinoid modulation of the reinforcing and motivational properties of heroin and heroin-associated cues in rats. *Psychopharmacology (Berl)* 168:164-9.

De Vries TJ, Shaham Y, Homberg JR, Crombag H, Schuurman K, Dieben J, Vanderschuren LJ, Schoffelmeer AN (2001): A cannabinoid mechanism in relapse to cocaine seeking. *Nat Med* 7:1151-4.

Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS, Howlett AC (1988): Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* 34:605-13.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992): Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258:1946-9.

Di Chiara G, Imperato A (1988): Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. USA* 85:5274-5278.

Di Marzo V, Berrendero F, Bisogno T, Gonzalez S, Cavaliere P, Romero J, Cebeira M, Ramos JA, Fernandez-Ruiz JJ (2000): Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of delta9-tetrahydrocannabinol-tolerant rats. *J Neurochem* 74:1627-35.

Di Marzo V, Bisogno T, Sugiura T, Melck D, De Petrocellis L (1998): The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. *Biochem J* 331 (Pt 1):15-9.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D (1994): Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* 372:686-91.

Di Marzo V, Matias I (2005): Endocannabinoid control of food intake and energy balance. *Nat Neurosci* 8:585-9.

Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier JF (2001): Role of endogenous opioid system in the regulation of the stress response. *Prog Neuropsychopharmacol Biol Psychiatry* 25:729-41.

Ehrenreich H, Rinn T, Kunert HJ, Moeller MR, Poser W, Schilling L, Gigerenzer G, Hoehe MR (1999): Specific attentional dysfunction in adults following early start of cannabis use. *Psychopharmacology (Berl)* 142:295-301.

Everitt BJ, Wolf ME (2002): Psychomotor stimulant addiction: a neural systems perspective. *J Neurosci* 22:3312-20.

Fattore L, Cossu G, Martellotta CM, Fratta W (2001): Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212-2 in rats. *Psychopharmacology (Berl)* 156:410-6.

Fattore L, Spano MS, Cossu G, Deiana S, Fratta W (2003): Cannabinoid mechanism in reinstatement of heroin-seeking after a long period of abstinence in rats. *Eur J Neurosci* 17:1723-6.

Fergusson DM, Horwood LJ (2000): Does cannabis use encourage other forms of illicit drug use? *Addiction* 95:505-520.

Fergusson DM, Horwood LJ, Swain-Campbell NR (2003): Cannabis dependence and psychotic symptoms in young people. *Psychol Med* 33:15-21.

Fernandez-Ruiz J, Berrendero F, Hernandez ML, Ramos JA (2000): The endogenous cannabinoid system and brain development. *Trends Neurosci* 23:14-20.

Ferrari F, Ottani A, Giuliani D (1999): Influence of the cannabinoid agonist HU 210 on cocaine- and CQP 201-403-induced behavioural effects in rat. *Life Sci* 65:823-31.

French ED, Dillon K, Wu X (1997): Cannabinoids excite dopamine neurons in the ventral tegmentum and substantia nigra. *Neuroreport* 8:649-52.

Fried PA (1995): The Ottawa Prenatal Prospective Study (OPPS): methodological issues and findings--it's easy to throw the baby out with the bath water. *Life Sci* 56:2159-68.

Fried PA, Watkinson B (1990): 36- and 48-month neurobehavioral follow-up of children prenatally exposed to marijuana, cigarettes, and alcohol. *J Dev Behav Pediatr* 11:49-58.

Fried PA, Watkinson B, Gray R (1998): Differential effects on cognitive functioning in 9- to 12-year olds prenatally exposed to cigarettes and marihuana. *Neurotoxicol Teratol* 20:293-306.

Gaoni Y, Mechoulam R (1964): Isolation, structure and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 86:1646.

Gardner EL, Paredes W, Smith D, Donner A, Milling C, Cohen D, Morrison D (1988): Facilitation of brain stimulation reward by delta 9-tetrahydrocannabinol. *Psychopharmacology (Berl)* 96:142-4.

Gardner EL, Vorel SR (1998): Cannabinoid transmission and reward-related events. *Neurobiol Dis* 5:502-33.

Ghozland S, Matthes HW, Simonin F, Filliol D, Kieffer BL, Maldonado R (2002): Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. *J Neurosci* 22:1146-54.

Giuffrida A, Rodriguez de Fonseca F, Piomelli D (2000): Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. *Anal Biochem* 280:87-93.

Goldschmidt L, Day NL, Richardson GA (2000): Effects of prenatal marijuana exposure on child behavior problems at age 10. *Neurotoxicol Teratol* 22:325-36.

Gonzalez B, de Miguel R, Martin S, Perez-Rosado A, Romero J, Garcia-Lecumberri C, Fernandez-Ruiz J, Ramos JA, Ambrosio E (2003): Effects of perinatal exposure to delta 9-tetrahydrocannabinol on operant morphine-reinforced behavior. *Pharmacol Biochem Behav* 75:577-84.

Gorriti MA, Rodriguez de Fonseca F, Navarro M, Palomo T (1999): Chronic (-)-delta9-tetrahydrocannabinol treatment induces sensitization to the psychomotor effects of amphetamine in rats. *Eur J Pharmacol* 365:133-42.

Green AI, Tohen MF, Hamer RM, Strakowski SM, Lieberman JA, Glick I, Clark WS (2004): First episode schizophrenia-related psychosis and substance use disorders: acute response to olanzapine and haloperidol. *Schizophr Res* 66:125-35.

Grotenhermen F (2003): Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet* 42:327-60.

Gupta A, Decaillot FM, Gomes I, Tkalych O, Heimann AS, Ferro ES, Devi LA (2006): Conformation state sensitive antibodies to G-protein coupled receptors. *J Biol Chem*.

Hajos N, Ledent C, Freund TF (2001): Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. *Neuroscience* 106:1-4.

Hall W (1998): Cannabis use and psychosis. *Drug Alcohol Rev* 17:433-44.

Hall WD, Lynskey M (2005): Is cannabis a gateway drug? Testing hypothesis about the relationship between cannabis and the use of other illicit drugs. *Drug Alcohol Rev* 24:39-48.

Heimer L, Zahm D, Churchill L, Kalivas P, Wholtmann C (1991): Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience* 41:89-125.

Herkenham M, Lynn A, Little M, Johnson M, Melvin L, De Costa B, Rice K (1990): Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. USA* 87:1932.

Herkenham M, Lynn AB, de Costa BR, Richfield EK (1991): Neuronal localization of cannabinoid receptors in the basal ganglia of the rat. *Brain Res* 547:267-74.

Hibell B, Andersson B, Bjarnason T, Ahlström S, Balakireva O, Kokkevi A, Morgan M (2004): *The ESPAD Report 2003. Alcohol and Other Drug Use Among Students in 35 European Countries.* Stockholm, Sweden: The Swedish Council for Information on Alcohol and Other Drugs (CAN) and the Pompidou Group at the Council of Europe.

Hillard CJ, Edgemond WS, Jarrahian A, Campbell WB (1997): Accumulation of N-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J Neurochem* 69:631-8.

Hoffman AF, Lupica CR (2001): Direct actions of cannabinoids on synaptic transmission in the nucleus accumbens: a comparison with opioids. *J Neurophysiol* 85:72-83.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG (2002): International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 54:161-202.

Huestis MA, Gorelick DA, Heishman SJ, Preston KL, Nelson RA, Moolchan ET, Frank RA (2001): Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. *Arch Gen Psychiatry* 58:322-8.

Hurd YL, Suzuki M, Sedvall GC (2001): D1 and D2 dopamine receptor mRNA expression in whole hemisphere sections of the human brain. *J Chem Neuroanat* 22:127-37.

Hurd YL, Ungerstedt U (1989): Calcium-dependency of amphetamine, nomifensine and Lu 19-005 effects on dopamine transmission. *Eur. J. Pharmacol* 166:261-270.

Hurd YL, Weiss F, Andén NE, Koob GF, Ungerstedt U (1989): Cocaine reinforcement and extracellular dopamine overflow: an in vivo microdialysis study. *Brain Res.* 498:199-203.

Jin K, Xie L, Kim SH, Parmentier-Batteur S, Sun Y, Mao XO, Childs J, Greenberg DA (2004): Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Mol Pharmacol* 66:204-8.

Johnson S, North R (1992): Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neuroscience* 12:483-488.

Justinova Z, Tanda G, Munzar P, Goldberg SR (2004): The opioid antagonist naltrexone reduces the reinforcing effects of Delta 9 tetrahydrocannabinol (THC) in squirrel monkeys. *Psychopharmacology (Berl)* 173:186-94.

Justinova Z, Tanda G, Redhi GH, Goldberg SR (2003): Self-administration of delta9-tetrahydrocannabinol (THC) by drug naive squirrel monkeys. *Psychopharmacology (Berl)* 169:135-40.

Kang W, Wilson SP, Wilson MA (2000): Overexpression of proenkephalin in the amygdala potentiates the anxiolytic effects of benzodiazepines. *Neuropsychopharmacology* 22:77-88.

Kelley AE (2004): Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. *Neurosci Biobehav Rev* 27:765-76.

Kelley AE, Bakshi VP, Haber SN, Steininger TL, Will MJ, Zhang M (2002): Opioid modulation of taste hedonics within the ventral striatum. *Physiol Behav* 76:365-77.

Kingsley PJ, Marnett LJ (2003): Analysis of endocannabinoids by Ag+ coordination tandem mass spectrometry. *Anal Biochem* 314:8-15.

Kofalvi A, Vizi ES, Ledent C, Sperlagh B (2003): Cannabinoids inhibit the release of [3H]glutamate from rodent hippocampal synaptosomes via a novel CB1 receptor-independent action. *Eur J Neurosci* 18:1973-8.

Kolb B, Gorny G, Limebeer CL, Parker LA (2006): Chronic treatment with Delta-9-tetrahydrocannabinol alters the structure of neurons in the nucleus accumbens shell and medial prefrontal cortex of rats. *Synapse* 60:429-36.

Koob GF (1992): Neural mechanisms of drug reinforcement. *Ann. NY Acad. Sci.* 654:171-191.

Koob GF (1999): The role of the striatopallidal and extended amygdala systems in drug addiction. *Ann N Y Acad Sci* 877:445-60.

Koob GF, Caine SB, Parsons L, Markou A, Weiss F (1997): Opponent process model and psychostimulant addiction. *Pharm Biochem Beh* 57:513-521.

Koob GF, Le Moal M (2001): Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* 24:97-129.

Kornetsky C, Bain G (1992): Brain-stimulation reward: a model for the study of the rewarding effects of abused drugs. *NIDA Res Monogr* 124:73-93.

Kuczenski R, Segal D (1989): Concomitant characterization of behavioral and striatal neurotransmitter response to amphetamine using in vivo microdialysis. *J. Neurosci.* 9:2051-2065.

Lamarque S, Taghzouti K, Simon H (2001): Chronic treatment with Delta(9)-tetrahydrocannabinol enhances the locomotor response to amphetamine and heroin. Implications for vulnerability to drug addiction. *Neuropharmacology* 41:118-29.

Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, Bohme GA, Imperato A, Pedrazzini T, Roques BP, Vassart G, Fratta W, Parmentier M (1999): Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* 283:401-4.

Lepore M, Vorel SR, Lowinson J, Gardner EL (1995): Conditioned place preference induced by delta 9-tetrahydrocannabinol: comparison with cocaine, morphine, and food reward. *Life Sci* 56:2073-80.

Lesscher HM, Hoogveld E, Burbach JP, van Ree JM, Gerrits MA (2005): Endogenous cannabinoids are not involved in cocaine reinforcement and development of cocaine-induced behavioural sensitization. *Eur Neuropsychopharmacol* 15:31-7.

Lessem JM, Hopfer CJ, Haberstick BC, Timberlake D, Ehringer MA, Smolen A, Hewitt JK (2006): Relationship between adolescent marijuana use and young adult illicit drug use. *Behav Genet* 36:498-506.

Lichtman AH (2000): SR 141716A enhances spatial memory as assessed in a radial-arm maze task in rats. *Eur J Pharmacol* 404:175-9.

Lu XY, Ghasemzadeh MB, Kalivas PW (1998): Expression of D1 receptor, D2 receptor, substance P and enkephalin messenger RNAs in the neurons projecting from the nucleus accumbens. *Neuroscience* 82:767-80.

Lynskey MT, Heath AC, Bucholz KK, Slutske WS, Madden PA, Nelson EC, Statham DJ, Martin NG (2003): Escalation of drug use in early-onset cannabis users vs co-twin controls. *J.A.M.A.* 289:427-433.

Ma W, Peschanski M, Ralston HJ (1987): The differential synaptic organization of the spinal and lemniscal projections to the ventrobasal complex of the rat thalamus. Evidence for convergence of the two systems upon single thalamic neurons *Neuroscience* 23:925-934.

MacCoun R (1998): In what sense (if any) is marijuana a gateway drug? *FAS Drug Policy Analysis Bulletin* 4.

Mackie K (2005): Cannabinoid receptor homo- and heterodimerization. *Life Sci* 77:1667-73.

Manzanares J, Corchero J, Romero J, Fernandez-Ruiz JJ, Ramos JA, Fuentes JA (1998): Chronic administration of cannabinoids regulates proenkephalin mRNA levels in selected regions of the rat brain. *Brain Res Mol Brain Res* 55:126-32.

Manzoni OJ, Bockaert J (2001): Cannabinoids inhibit GABAergic synaptic transmission in mice nucleus accumbens. *Eur J Pharmacol* 412:R3-5.

Markou A, Koob GF (1990): Postcocaine anhedonia an animal model of cocaine withdrawal. *Neuropsychopharmacology* 3.

Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2000): Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. *Eur J Neurosci* 12:4038-46.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990): Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346:561-4.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. (1995): Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 50:83-90.

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B, et al. (1995): Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377:532-5.

Moreno M, Trigo JM, Escuredo L, Rodriguez de Fonseca F, Navarro M (2003): Perinatal exposure to delta 9-tetrahydrocannabinol increases presynaptic dopamine D2 receptor sensitivity: a behavioral study in rats. *Pharmacol Biochem Behav* 75:565-75.

Morral AR, McCaffrey DF, Paddock SM (2002): Reassessing the marijuana gateway effect. *Addiction* 97:1493-504.

Mucha RF, Herz A (1985): Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology (Berl)* 86:274-80.

Munro S, Thomas KL, Abu-Shaar M (1993): Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365:61-5.

Muschamp JW, Siviy SM (2002): Behavioral sensitization to amphetamine follows chronic administration of the CB1 agonist WIN 55,212-2 in Lewis rats. *Pharmacol Biochem Behav* 73:835-42.

Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, Numa S (1979): Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 278:423-7.

Nakano K, Kayahara T, Tsutsumi T, Ushiro H (2000): Neural circuits and functional organization of the striatum. *J Neurol* 247 Suppl 5:V1-15.

Navarro M, Carrera MR, Del Arco I, Trigo JM, Koob GF, Rodriguez de Fonseca F (2004): Cannabinoid receptor antagonist reduces heroin self-administration only in dependent rats. *Eur J Pharmacol* 501:235-7.

Navarro M, Carrera MR, Fratta W, Valverde O, Cossu G, Fattore L, Chowen JA, Gomez R, del Arco I, Villanua MA, Maldonado R, Koob GF, Rodriguez de Fonseca F (2001): Functional interaction between opioid and cannabinoid receptors in drug self-administration. *J Neurosci* 21:5344-50.

Navarro M, de Fonseca FR, Hernández ML, Ramos JA, Fernández-Ruiz JJ (1994): Motor behavior and nigrostriatal dopaminergic activity in adult rats perinatally exposed to cannabinoids. *Pharmacol. Biochem. Behavior.* 47:47-58.

Norwood CS, Cornish JL, Mallet PE, McGregor IS (2003): Pre-exposure to the cannabinoid receptor agonist CP 55,940 enhances morphine behavioral sensitization and alters morphine self-administration in Lewis rats. *Eur J Pharmacol* 465:105-14.

Nyberg F, Silberring J (1990): Conversion of the dynorphins to Leu-enkephalin in human spinal cord. *Prog Clin Biol Res* 328:261-5.

Olds J, Milner P (1954): Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J Comp Physiol Psychol* 47:419-27.

Olds ME, Fobes JL (1981): The central basis of motivation: intracranial self-stimulation studies. *Annu Rev Psychol* 32:523-74.

Onaivi ES, Green MR, Martin BR (1990): Pharmacological characterization of cannabinoids in the elevated plus maze. *J Pharmacol Exp Ther* 253:1002-9.

Organisation WH (1992): *The ICD-10 classification of mental and behavioral disorders: clinical descriptions and diagnostic guidlines.* Geneva, Switzerland: WHO.

O'Shea M, Singh ME, McGregor IS, Mallet PE (2004): Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. *J Psychopharmacol* 18:502-8.

Patel S, Rademacher DJ, Hillard CJ (2003): Differential regulation of the endocannabinoids anandamide and 2-arachidonylglycerol within the limbic forebrain by dopamine receptor activity. *J Pharmacol Exp Ther* 306:880-8.

Patton GC, Coffey C, Carlin JB, Degenhardt L, Lynskey M, Hall W (2002): Cannabis use and mental health in young people: cohort study. *Bmj* 325:1195-8.

Paxinos G, Watson C (1997): *The Rat Brain in Stereotaxic Coordinates*, 3rd ed. New York: Academic Press.

Pecina S, Berridge KC (2005): Hedonic hot spot in nucleus accumbens shell: where do mu-opioids cause increased hedonic impact of sweetness? *J Neurosci* 25:11777-86.

Perez-Rosado A, Manzanares J, Fernandez-Ruiz J, Ramos JA (2000): Prenatal Delta(9)-tetrahydrocannabinol exposure modifies proenkephalin gene expression in the fetal rat brain: sex-dependent differences. *Brain Res Dev Brain Res* 120:77-81.

Pert CB, Snyder SH (1973): Opiate receptor: demonstration in nervous tissue. *Science* 179:1011-4.

Pertwee RG, Ross RA (2002): Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids* 66:101-21.

Pickel VM, Chan J, Kash TL, Rodriguez JJ, MacKie K (2004): Compartment-specific localization of cannabinoid 1 (CB1) and mu-opioid receptors in rat nucleus accumbens. *Neuroscience* 127:101-112.

Pistis M, Perra S, Pillolla G, Melis M, Muntoni AL, Gessa GL (2004): Adolescent exposure to cannabinoids induces long-lasting changes in the response to drugs of abuse of rat midbrain dopamine neurons. *Biol Psychiatry* 56:86-94.

Pope HG, Jr., Gruber AJ, Hudson JI, Cohane G, Huestis MA, Yurgelun-Todd D (2003): Early-onset cannabis use and cognitive deficits: what is the nature of the association? *Drug Alcohol Depend* 69:303-10.

Porath AJ, Fried PA (2005): Effects of prenatal cigarette and marijuana exposure on drug use among offspring. *Neurotoxicol Teratol* 27:267-77.

Puder M, Weidenfeld J, Chowers I, Nir I, Conforti N, Siegel RA (1982): Corticotrophin and corticosterone secretion following delta 1-Tetrahydrocannabinol, in intact and in hypothalamic deafferentated male rats. *Exp Brain Res* 46:85-8.

Rice D, Barone S, Jr. (2000): Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect* 108 Suppl 3:511-33.

Richardson GA, Ryan C, Willford J, Day NL, Goldschmidt L (2002): Prenatal alcohol and marijuana exposure: effects on neuropsychological outcomes at 10 years. *Neurotoxicol Teratol* 24:309-20.

Rios C, Gomes I, Devi LA (2006): mu opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. *Br J Pharmacol* 148:387-95.

Robbe D, Alonso G, Duchamp F, Bockaert J, Manzoni OJ (2001): Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. *J Neurosci* 21:109-16.

Robinson TE, Berridge KC (1993): The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Research Reviews*.

Robinson TE, Berridge KC (2001): Incentive-sensitization and addiction. *Addiction* 96:103-14.

Robinson TE, Jurson PA, Bennett JA, Bentgen KM (1988): Persistent sensitization of dopamine neurotransmission in ventral striatum (nucleus accumbens) produced by prior experience with (+)-amphetamine: a microdialysis study in freely moving rats. *Brain Res.* 462:211-222.

Rodriguez de Fonseca F, Carrera MR, Navarro M, Koob GF, Weiss F (1997): Activation of corticotropin-releasing factor in the limbic system during cannabinoid withdrawal. *Science* 276:2050-4.

Rodriguez de Fonseca F, Cebeira M, Fernandez-Ruiz JJ, Navarro M, Ramos JA (1991): Effects of pre- and perinatal exposure to hashish extracts on the ontogeny of brain dopaminergic neurons. *Neuroscience* 43:713-23.

Rodriguez de Fonseca F, Hernandez ML, de Miguel R, Fernandez-Ruiz JJ, Ramos JA (1992): Early changes in the development of dopaminergic neurotransmission after maternal exposure to cannabinoids. *Pharmacol Biochem Behav* 41:469-74.

Rodriguez JJ, Mackie K, Pickel VM (2001): Ultrastructural localization of the CB1 cannabinoid receptor in mu- opioid receptor patches of the rat Caudate putamen nucleus. *J Neurosci* 21:823-33.

Romero J, Garcia-Palomero E, Berrendero F, Garcia-Gil L, Hernandez ML, Ramos JA, Fernandez-Ruiz JJ (1997): Atypical location of cannabinoid receptors in white matter areas during rat brain development. *Synapse* 26:317-23.

Roy SN, Bhattacharyya AK, Pradhan S, Pradhan SN (1978): Behavioural and neurochemical effects of repeated administration of cocaine in rats *Neuropharmacology* 17:559-564.

Rubio P, Rodriguez de Fonseca F, Martin-Calderon JL, Del Arco I, Bartolome S, Villanua MA, Navarro M (1998): Maternal exposure to low doses of delta9-tetrahydrocannabinol facilitates morphine-induced place conditioning in adult male offspring. *Pharmacol Biochem Behav* 61:229-38.

SAMHSA (2002): *Substance Abuse and Mental Health Service Administration. Results from the 2001 National Household Survey on Drug Abuse NHSDA Series H-17*. Rockville, MD: DHHS Publication No. SMA 02-3758.

SAMHSA (2004): *Substance Abuse and Mental Health Service Administration. Results from the 2003 National Survey on Drug Use and Health: National Findings*. Rockville, MD: Office of Applied Studies, NSDUH Series H-25, DHHS.

Schneider M, Koch M (2003): Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory, and the performance in a progressive ratio task in adult rats. *Neuropsychopharmacology* 28:1760-9.

Sibley DR, Monsma FJ, Jr., Shen Y (1993): Molecular neurobiology of dopaminergic receptors. *Int Rev Neurobiol* 35:391-415.

Simon EJ, Hiller JM, Edelman I (1973): Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate. *Proc Natl Acad Sci U S A* 70:1947-9.

Skoubis PD, Lam HA, Shoblock J, Narayanan S, Maidment NT (2005): Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur J Neurosci.* 21:1379-1384.

Solinas M, Panlilio LV, Antoniou K, Pappas LA, Goldberg SR (2003): The cannabinoid CB1 antagonist N-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl) -4-

methylpyrazole-3-carboxamide (SR-141716A) differentially alters the reinforcing effects of heroin under continuous reinforcement, fixed ratio, and progressive ratio schedules of drug self-administration in rats. *J Pharmacol Exp Ther* 306:93-102.

Solinas M, Panlilio LV, Goldberg SR (2004a): Exposure to delta-9-tetrahydrocannabinol (THC) increases subsequent heroin taking but not heroin's reinforcing efficacy: a self-administration study in rats. *Neuropsychopharmacology* 29:1301-11.

Solinas M, Zangen A, Thiriet N, Goldberg SR (2004b): Beta-endorphin elevations in the ventral tegmental area regulate the discriminative effects of Delta-9-tetrahydrocannabinol. *Eur J Neurosci* 19:3183-92.

Soria G, Mendizabal V, Tourino C, Robledo P, Ledent C, Parmentier M, Maldonado R, Valverde O (2005): Lack of CB1 cannabinoid receptor impairs cocaine self-administration. *Neuropsychopharmacology* 30:1670-80.

Spear LP (2000): The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev* 24:417-63.

Stefanis NC, Delespaul P, Henquet C, Bakoula C, Stefanis CN, Van Os J (2004): Early adolescent cannabis exposure and positive and negative dimensions of psychosis. *Addiction* 99:1333-41.

Stenbacka M, Allebeck P, Romelsjo A (1993): Initiation into drug abuse: the pathway from being offered drugs to trying cannabis and progression to intravenous drug abuse. *Scand J Soc Med* 21:31-9.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995): 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 215:89-97.

Svensson PS, Hurd YL (1998): Specific reductions of striatal prodynorphin and D1 dopamine receptor messenger RNAs during cocaine abstinence. *Mol. Brain Res.* 56:162-168.

Tanda G, Munzar P, Goldberg SR (2000): Self-administration behavior is maintained by the psychoactive ingredient of marijuana in squirrel monkeys. *Nat Neurosci* 3:1073-4.

Tanda G, Pontieri FE, Di Chiara G (1997): Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. *Science* 276:2048-50.

Terenius L (1973): Characteristics of the "receptor" for narcotic analgesics in synaptic plasma membrane fraction from rat brain. *Acta Pharmacol Toxicol (Copenh)* 33:377-84.

Terranova JP, Storme JJ, Lafon N, Perio A, Rinaldi-Carmona M, Le Fur G, Soubrie P (1996): Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716. *Psychopharmacology (Berl)* 126:165-72.

Tjon GHK, Voorn P, Vanderschuren LJMJ, De Vries TJ, Michiels NHLM, Jonker AJ, Klop H, Nestby P, Mulder AH, Schoffelmeer ANM (1997): Delayed occurrence of enhanced striatal preprodynorphin gene expression in behaviorally sensitized rats: differential long-term effects of intermittent and chronic morphine administration. *Neuroscience* 76:167-176.

Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM (1998): Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83:393-411.

Ungerstedt U (1971): Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol. Scand. Suppl.* 367:69-93.

Valjent E, Maldonado R (2000): A behavioural model to reveal place preference to delta 9-tetrahydrocannabinol in mice. *Psychopharmacology (Berl)* 147:436-8.

Valverde O, Noble F, Beslot F, Dauge V, Fournie-Zaluski MC, Roques BP (2001): Delta9-tetrahydrocannabinol releases and facilitates the effects of endogenous enkephalins: reduction in morphine withdrawal syndrome without change in rewarding effect. *Eur J Neurosci* 13:1816-24.

van Os J, Bak M, Hanssen M, Bijl RV, de Graaf R, Verdoux H (2002): Cannabis use and psychosis: a longitudinal population-based study. *Am J Epidemiol* 156:319-27.

Wang X, Dow-Edwards D, Anderson V, Minkoff H, Hurd YL (2004): In utero marijuana exposure associated with abnormal amygdala dopamine D2 gene expression in the human fetus. *Biol Psychiatry* 56:909-15.

Wang X, Dow-Edwards D, Anderson V, Minkoff H, Hurd YL (2006): Discrete opioid gene expression impairment in the human fetal brain associated with maternal marijuana use. *Pharmacogenomics J* 6:255-64.

Wang X, Dow-Edwards D, Keller E, Hurd YL (2003): Preferential limbic expression of the cannabinoid receptor mRNA in the human fetal brain. *Neuroscience* 118:681-94.

Watson SJ, Khachaturian H, Akil H, Coy DH, Goldstein A (1982): Comparison of the distribution of dynorphin systems and enkephalin systems in brain. *Science* 218:1134-1136.

Veen ND, Selten JP, van der Tweel I, Feller WG, Hoek HW, Kahn RS (2004): Cannabis use and age at onset of schizophrenia. *Am J Psychiatry* 161:501-6.

Vela G, Martin S, Garcia-Gil L, Crespo JA, Ruiz-Gayo M, Javier Fernandez-Ruiz J, Garcia-Lecumberri C, Pelaprat D, Fuentes JA, Ramos JA, Ambrosio E (1998): Maternal exposure to delta9-tetrahydrocannabinol facilitates morphine self-administration behavior and changes regional binding to central mu opioid receptors in adult offspring female rats. *Brain Res* 807:101-9.

Williams SM, Goldman-Rakic PS (1993): Characterization of the dopaminergic innervation of the primate frontal cortex using a dopamine-specific antibody. *Cereb Cortex* 3:199-222.

Wise RA (1996): Addictive drugs and brain stimulation reward. *Annu Rev Neurosci* 19:319-40.

Wise RA, Bozarth MA (1987): A psychomotor stimulant theory of addiction. *Psych. Rev.* 94:469-492.

Yamaguchi K, Kandel DB (1984): Patterns of drug use from adolescence to young adulthood: III. Predictors of progression. *Am J Public Health.* 74:673-681.

Zadina JE, Hackler L, Ge LJ, Kastin AJ (1997): A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 386:499-502.

Zammit S, Allebeck P, Andreasson S, Lundberg I, Lewis G (2002): Self reported cannabis use as a risk factor for schizophrenia in Swedish conscripts of 1969: historical cohort study. *Bmj* 325:1199.

Zangen A, Solinas M, Ikemoto S, Goldberg SR, Wise RA (2006): Two brain sites for cannabinoid reward. *J Neurosci* 26:4901-7.

Zetterström T, Sharp T, Marsden C, Ungerstedt U (1983): In vivo measurement of dopamine and its metabolites by intracerebral dialysis: Changes after d-amphetamine. *J. of Neurochemistry* 41:1769-1773.