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DIFFERENT ADDICTIVE DRUG EXPOSURE INDUCES SELECTIVE ALTERATIONS OF THE ENDOGENOUS OPIOID SYSTEM : MODULATION OF TRANSCRIPTION AND EPIGENETIC MECHANISMS

Presentata da:

FRANCESCA FELICIA CAPUTI

Coordinatore Dottorato: Relatore:

CHIAR.MO PROF. GIORGIO CANTELLI-FORTI   PROF.SSA PATRIZIA ROMUALDI

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ABSTRACT

Over the centuries people have developed different forms of addiction to various substances.

Drug addiction manifests clinically as compulsive drug seeking, drug use, and cravings that can persist and recur even after extended periods of abstinence. From a psychological and neurological perspective, addiction is a disorder of altered cognition. The brain regions and processes that underlie addiction overlap extensively with those that are involved in essential cognitive functions, including learning, memory, attention, reasoning, and impulse control. Drugs alter normal brain structure and function in these regions, producing cognitive shifts that promote continued drug use through maladaptive learning and hinder the acquisition of adaptive behaviors that support abstinence.

All addictive drugs have in common that they enhance (directly or indirectly or even trans synaptically) dopaminergic reward synaptic function in the nucleus accumbens (NA) and for this reason are used self-administration's drugs to maintain a high level of dopamine (DA) and thus a high level of hedonic. In some classes of addictive drugs (e.g. opiates), the tolerance to the euphoric effects develops with chronic use.

The fundamental principle that unites addictive drugs is that each one enhances synaptic DA by means that dissociate it from normal behavioral control, so that they act to reinforce their own acquisition.

Within this context, our attention has focused on the study of phenomena associated with the consumption of alcohol and heroin.

Alcohol is the drug of abuse certainly more socially accepted and alcoholism, a condition that occurs following chronic use of alcohol, is the cause of many deaths.

To figure out what dose would be most suitable for the treatment we began our studies by analyzing the blood alcohol levels (BALs), and also to understand which binge alcohol paradigm was chosen to more closely resemble human drinking behaviour.

The data showed that single or repeated EtOH binge intoxication induce BALs in the range of 350–450 mg/dl and thus it was clearly pharmacologically active. During binge treatment, the highest BAL (450 mg/dl) was reached after 30 minutes following the third administration and decreased to the level 120 mg/ml after 9 hours. A single dose

caused a BAL of 350 mg/dl 1 hour after the administration and decreased to non-pharmacologically level after 9 hours .

After determining the time required to reach the highest levels of alcohol in the blood, the animals received daily intragastric administrations of alcohol (20% in water) at a dose of 1.5 g/kg or equal volume of water in their home cages following this binge pattern regimen: three times daily at 1.5-hour intervals (10:00 am, 11:30 am, and 1:00 pm), for a total daily dose of 4.5 g/kg/day. They were sacrificed at different time point.

- 1 day group (1D) treated for 1 day and sacrificed 24h after last administration
- 5 days group (5D) treated for 5 days and sacrificed 24h after last administration
- 1-WD treated for 5 day and maintained 24h in withdrawal, then sacrificed
- 3-WD treated for 5 day and maintained for 3 days in withdrawal, then sacrificed
- 7-WD treated for 5 day and maintained for 3 days in withdrawal, then sacrificed

Behavioral observations of intoxication and withdrawal were carried out on the animals, under acohol exposure, by two operators independently and in blind fashion.

EtOH-induced intoxication in animals starting from the first binge administration and reached the maximum peak at Day 2. Intoxication signs decreased over Day 3 and disappeared over Day 5.

EtOH-treated rats showed significant withdrawal signs measured 20 hours after each final daily EtOH treatment. The sum of the observation scores progressively increased from 0 on Day 1 to 12.5 ± 0.4 on Day with significant differences between the experimental groups on Day 4, Day 7, Day 9 and Day 10 confirming the presence of significant overall withdrawal severity.

Although alcohol has long been considered an unspecific pharmacological agent, recent molecular pharmacology studies have shown that acts on different primary targets, including ion channels and receptors, kicking off a cascade of synaptic events involving many neurotransmitter systems.

Through gene expression studies conducted in recent years, it has been shown that the classical opioid receptors are differently involved in the consumption of ethanol and, furthermore, the system nociceptin / NOP, recently included in the family of endogenous opioid system, and both appear able to play a key role in the initiation of alcohol use in rodents. What emerges is that manipulation of the opioid system,

nociceptin in particular, may be useful in the treatment of addictions and there are several evidences that support the use of this strategy. The PFCx is a region of especial interest in opiate addiction because it plays an important role in cognitive control over drug intake, and also because it is directly related with the meso-corticolimbic dopaminergic system that mediates the rewarding and addictive properties of opiates. Based on these informations we have performed our studies of gene expression to evaluate if the same experimental conditions could lead changes in another brain area. The second area studied was the AM, which is a major substrate for neuronal behavior associated with the consumption of alcohol and anxiety.

In the AM, gene expression studies revealed a significant increase in the levels of PDYN in the group of animals treated for 1 day and in the W-1D group. In the PFCx after 1 day of treatment with EtOH, PDYN mRNA was increased, whereas no changes were evident in the other groups.

In the AM levels of the PNOC mRNA transcript in the AM were significantly increased in the 1D group and continues to increase with the progression of the treatment (5D group) becoming even higher. When the animals were kept in abstinence, the levels of peptide mRNA were still significant high in theW-1D group. Conversely, in the PFCx we did not observe significant changes in the expression of PNOC.

Alterations in the expression of the receptors are visible only for the receptor KOP in the AM area in 5D group; no alteration are visible in the PFCx for both receptors analyzed. Recently, an increasing number of experimental evidence suggest that changes in gene expression induced by drugs of abuse may be mediated by epigenetic mechanisms.

It is already known that many histone modification act like marker to identify active/ or inactive chromatin structure in the regulation of gene transcription.

In our study we used H3K27me3 which is a repressive marker; H3K9Ac and H3K4me3 which are active marker. In the AM, we found in 1D group of animals a significant decrease of H3K27me3 in both PDYN and PNOC promoter regions as well as a significant increase in H3K9Ac for PDYN promoter and for PNOC promoter. In the AM of animals treated for 5 days (5D group) a significant increase of H3K9Ac in PNOC promoter but no significant alteration we for other histone modification analyzed and in PDYN promoter region were found. In the AM, in contrast, we did not observe any histone modifications changes in the W-1D group in both genes promoter.

Moreover, EtOH exposure did not induce any statistically significant alteration of H3K27me3, H3K9ac and H3K4me3.

The analyses of DNA methylation in PDYN and PNOC promoters didn't show a significative alteration.

The data, about alcohol exposure, confirm a role of the PDYN/KOP system in the negative hedonic state associated with alcohol addiction and the hypothesis that the NOC system could function as a 'brake' to limit EtOH intake.

The linkage between gene expression alterations and epigenetic modulation in PDYN and PNOC promoters following alcohol treatment confirm the possible chromatin remodeling mechanism already proposed for alcoholism.

Our results could be important to partially fill the lack of knowledge of how EtOH by itself affects the opioid system in the brain, and suggest the possibility of using drugs acting on these systems for the treatment of withdrawal symptoms and alcohol dependence.

In the second part of present study, we also investigated alterations in signaling molecules directly associated with MAPK pathway in a unique collection of postmortem brains from heroin abusers.

Several studies have implicated ERK1/2 and p38 MAPKs in the modulation of various forms of synaptic plasticity, including the neuroplastic changes induced by drugs of abuse. In addition to PKA-mediated protein phosphorylation, extracellular signal-regulated kinase (ERK) activity may also be regulated after heroin exposure. Moreover, previous studies has shown that amphetamine activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) resulting in cAMP response element-binding protein (CREB) and Elk-1 phosphorylation in striatal neurons.

On basis of these findings, our interest was focused on understanding the effects that prolonged exposure of heroin can cause in an individual, over the entire MAPK cascade and consequently on the transcription factor ELK1, which is regulated by this pathway.

In the putamen of heroin addicts, the protein expression of MEK1 was significantly reduced, whereas MEK2 was also lower but not significantly changed. Of the two known targets of the MEK1/2, we observe a significant decrease in the protein level of ERK1; Conversely, there is a significant increase in the protein levels of ERK2. Dual phosphorylations of the ERKs are required for their full activation and for their ability to mediate a variety of cellular functions, including activation of downstream transcription factors. In the putamen of heroin addicts, the level of phosphorylated

ERK1 and phosphorylated ERK2 was not significantly changed when compared with control subjects. Elk-1 is directly phosphorylated by ERK1/2, and once activated forms a ternary complex on serum response elements (SREs) that activate immediate early genes such as c-fos. In the putamen of heroin addicts, the protein expression of Elk-1 was significantly increased. However, there is a significant reduction in the level of phosphorylated Elk-1 in the putamen of heroin addicts.

The data, about heroin exposure, confirm the presences of perturbations in protein levels of the MAPK pathway as a consequence of chronic heroin abuse.

Moreover, we have shown that the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) resulting in Elk-1 phosphorylation in striatal neurons supporting the hypothesis that prolonged exposure to substance abuse causes a dysregulation of MAPK pathway, in the areas delegated to the reward.

Our results could be important to clarify the role of ELK1 in the regulation of several gene expression, for changes in neuronal size, in the synaptic connectivity and in behavioral plasticity.

Knowledge of the neuroanatomy, neurophysiology, neurochemistry and neuropharmacology of addictive drug action in the brain is currently producing a variety of strategies for pharmaco-therapeutic treatment of drug addiction, some of which appear promising.

Abbreviations

5-HT	5-hydroxytryptamine
ADH	Alcohol dehydrogenase enzyme
CNS	Central nervous system
CP	Caudate putamen
CPP	Conditioned place preference
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
DA	Dopamine
DDCt	Delta-delta Ct
DNMT	DNA methyltransferases
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAT	Histone acetyltransferase
HDACs	Histone deacetylase
ICSS	Intracranial self-stimulation
KOP	Kappa opioid receptor
L-DOPA	L-3,4-dihydroxyphenilalanine
MAO-B	Monoamine oxidase B
MCI	Mild cognitive impairment
MFB	Medial forebrain bundle
Mn-EBDC	Manganese ethylenebis-dithiocarbamate
NFTs	Neurofibrillary tangles
NMDA	N-methyl-$_D$-aspartate
N/OFQ	neuropeptide nociceptin/orphanin FQ
NOP	Nociceptin opioid receptor
PKA	Protein kinase A
PDYN	prodynorphin
PNOC	pronociceptin Orphanin FQ
ppN/OFQ	Pre-pro nociceptin Orphanin FQ
RNAPII	RNA polymerase II
ROS	Reactive oxygen species
SN	Substantia nigra
SNPs	Single nucleotide polymorphism
SRE	Serum response element
SRF	Serum response factor
TCF	Ternary complex factor
UCH-L1	Ub carboxyl-terminal hydrolase
VTA	Ventral tegmental area

1. GENERAL BACKGROUND

1.1   Concept and Identification of Addictive Drugs

Defining the set of addictive drugs is complicate in part as it depends on whether society considers them to be destructive. For example, evidence for clinical opium use extends to 5000 B.C. in Sumeria, where it was known as ''joy plant''. Arabic pharmacologists who wrote on opium, including Galen, mention opium's clinical use and its toxicity but don't speak about the habit (Tibi, 2006).

The first scientific article on opium is dated in 1701 by Dr. John Jones, from the London College of Physicians (Jones, 1701), who discusses clinical uses, as well as withdrawal symptoms, including death, after ''lavish use.'' Subsequently, studies at the Royal Hospital in Greenwich of Dr. John Awsiter have compared opium's effects to those of drunkenness and the outlines features of withdrawal and overdose.

He further discussed tolerance of large doses by those with opium habit, writing the dire consequences for the society (Awsiter, 1763).

Other researchers, in 1990, had already foreshadowed to be able to treat one habit-forming drug by prescribing nervous stimulants, which would have included ethanol or camphor (Sneader, 1990). It may be that the contemporary concept of addiction begins here.

Over the centuries people have developed different forms of addiction to various substances.

Drug addiction manifests clinically as compulsive drug seeking, drug use, and cravings that can persist and recur even after extended periods of abstinence. From a psychological and neurological perspective, addiction is a disorder of altered cognition.

The brain regions and processes that underlie addiction overlap extensively with those that are involved in essential cognitive functions, including learning, memory, attention, reasoning, and impulse control.

Drugs alter normal brain structure and function in these regions, producing cognitive shifts that promote continued drug use through maladaptive learning and hinder the acquisition of adaptive behaviors that support abstinence.

In a 2005 review, Steven Hyman stated the current neurological conception of drug abuse concisely: characterizing addiction as a disease of "pathological learning". This article reviews the knowledges on the cognitive effects of drugs and their neurological underpinnings.

These effects may be particularly disruptive when individuals are exposed to drugs during brain development, which lasts from the prenatal period through adolescence, and in individuals with mental disorders.

All addictive drugs have in common that they enhance (directly or indirectly or even trans synaptically) dopaminergic reward synaptic function in the nucleus accumbens (NA) and for this reason are used self-administration's drugs to maintain a high level of dopamine (DA) and thus a high level of hedonic. In some classes of addictive drugs (e.g. opiates), the tolerance to the euphoric effects develops with chronic use.

The fundamental principle that unites addictive drugs is that each one enhances synaptic DA by means that dissociate it from normal behavioral control, so that they act to reinforce their own acquisition.

This occurs via the modulation of synaptic mechanisms that can be involved in learning, including enhanced excitation or disinhibition of DA neuron activity, blockade of dopamine reuptake, and altering the state of the pre-synaptic terminal to enhance evoked over basal transmission.

Questions about the molecular actions of addictive drugs remain unresolved and this explains why the study of addiction treatment has been challenging.

1.2 How the Addictive drugs acts on the Presynaptic Dopamine Neurotransmission

1.2.1. Identification of a role for Dopamine Neurotransmission in Addiction

The midbrain dopamine neurons, that project to the forebrain, were initially identified as a single continuous layer (Dahlstrm *and* Fuxe, 1964), arising from a single embryological cell group (Seiger *and* Olson, 1973).

However, perhaps because the lateral and medial portions were largely restricted to established brain regions the dorsal substantia nigra (SN) and the ventral tegmental area (VTA), the lateral and medial portions of this layer were given different labels (A9 and A10, respectively) and eventually became identified with two distinct nominal systems (a nigrostriatal system and a mesolimbic system (Ungerstedt, 1971)(Figure 1).

These two systems in turn became identified with different functions: the nigrostriatal system known to degenerate in Parkinson's disease, with motor function, and the mesolimbic system, important for the habit-forming effects of cocaine and for approach behaviors, with motivation and reward function.
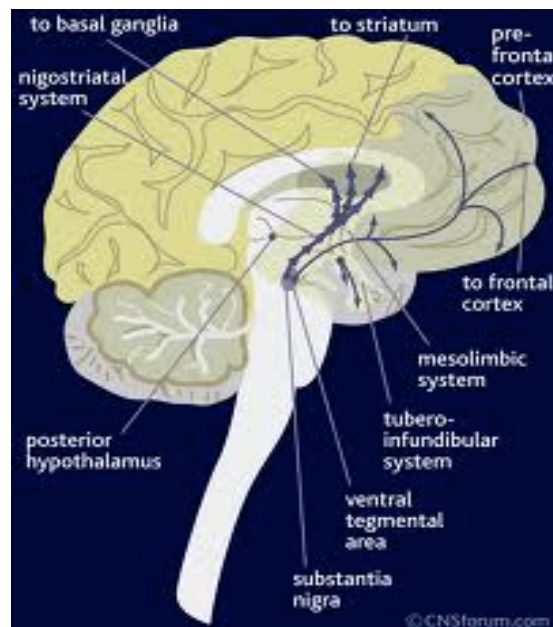
Fig. 1. Mayor Dopaminergic pathways in the central nervous system

The nigrostriatal and mesolimbic dopamine ''systems'' are not simply differentiated anatomically, and significant functional interactions between the two systems have been widely suggested (Haber *et al.,* 2000). A large body of evidences shows that the SN dopamine neurons, and not just those of the VTA, play a significant role in reward and addiction (Everitt *and* Robbins, 2005).

As was noted previously, there is no clear boundary between the two nominal midbrain dopamine systems (Figure 2).
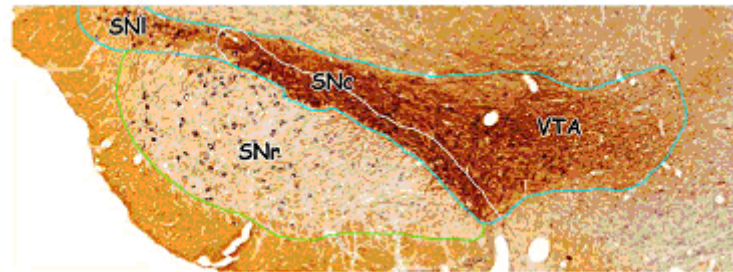
Fig. 2. Coronal sections of the ventral midbrain of the rat. The blue line outlines the layer of dopaminergic cell bodies and dendrites (dark brown) as revealed by tyrosine hydroxylase immunohistochemistry. The green line outlines the GABAergic cell bodies (purple) of the substantia nigra pars reticulata (SNr) as revealed by in situ hybridization. The spacing of the cells in the dorsal and ventral tiers is evident from in situ hybridization

Anterograde and retrograde tracing studies show that the SN and VTA dopamine cells have overlapping, not distinct, projection fields. The discovery of dopamine terminals in prefrontal cortex (PFCx) initially thought to arise uniquely from the VTA, prompted postulation of a third (mesocortical) or an expanded (mesocorticolimbic) system, but projections to the PFCx were subsequently found to arise from the medial SN as well as from the VTA (Fallon *and* Loughlin, 1995). Thus it is no longer possible to think of the limbic and striatal dopamine systems as arising from anatomically distinct lateral SN and medial VTA.

Thus the midbrain DA neurons are currently seen as a final common path for the rewarding effects of medial forebrain bundle stimulation (MFB). The mesocorticolimbic dopamine system is most frequently associated with brain stimulation reward and the reward sites are found in both SN and VTA (Routtenberg *and* Malsbury, 1969; Crow, 1972).

In this region are activated activated directly the sensitive terminals of the DA afferents rather than the relatively insensitive DA neurons.

1.2.2. Dopamine Action : the Reward

The term "reward", in the more general meaning, refers to a stimulus or event that is rewarding. The effects of a reward are short-lived and not stored in long-term memory, but they can influence the probability and vigor of the next response in a series when animals are responding rapidly or when they are responding for slowly decaying rewards such as addictive drugs (Gallistel *et al.,* 1974; Pickens *and* Harris, 1968).

One of the principal neuronal system involved in processing reward information appears to be the Dopamine System. The DA projections from the VTA to the NA are the key component of the brain reward circuitry. This circuitry provides a common way for the valuation of diverse rewards by the brain (Montague *and* Berns, 2002)(Figure 3).

Fig. 3. Main neuronal circuits for mesolimbic dopamine reward system

Within the VTA/NA circuit, DA is required for natural stimuli, such as food and opportunities for mating, to be rewarding; similarly, DA is required for the addictive drugs to produce reward (Kelley *and* Berridge, 2002; Di Chiara, 1998; Koob *and* Bloom,1988; Wise *and* Rompre, 1989).

The most obvious difference between natural goal objects, such as food, and addictive drugs is that the latter have no intrinsic ability to serve a biological need. However, because both addictive drugs and natural rewards release DA in the NA and other forebrain structures, addictive drugs mimic the effects of natural rewards and can thus shape behavior (Montague *et al.,* 2004; Kelley *and* Berridge, 2002; Berke, 2003).

Indeed, it has been hypothesized that addictive drugs have a competitive advantage over most natural stimuli in that they can produce far greater levels of DA release and more prolonged stimulation.

An early view of DA function was that it acted as a hedonic signal (signaling pleasure), but this view has been called into question by pharmacological blockade, lesion (Berridge *and* Robinson, 1998), and genetic studies (Cannon *and* Palmiter, 2003) in which animals continued to prefer ("like") rewards such as sucrose despite DA depletion.

Moreover, the actions of nicotine have always remained a mystery on this account, because nicotine is highly addictive and causes DA release but produces little if any euphoria. Instead of acting as a hedonic signal, DA appears to promote reward-related learning, binding the hedonic properties of a goal to desire and to action, thus shaping subsequent reward-related behavior (Berridge *and* Robinson, 1998).

Berridge and Robinson showed that DA is not required for the pleasurable (hedonic) properties of sucrose, which, in their investigation, continued to be "liked" by rats depleted of DA.

Instead they have proposed that NA-DA transmission mediates the assignment of "incentive salience" to rewards and reward-related cues, such that these cues can subsequently trigger a state of "wanting" for the goal object as distinct from "liking." In their view, an animal can still "like" something in the absence of DA transmission, but the animal cannot use this information to motivate the behaviours necessary to obtain it. Overall, it can be concluded that DA release is not the internal representation of an object's hedonic properties; the experiments by Schultz (1993) suggest instead that DA serves as a prediction-error signal that shapes behavior to most efficiently obtain rewards (Schultz *et al.,* 1993).

This view of DA function is consistent with computational models of reinforcement learning (Montague *et al.,* 2004; Sutton *and* Barto, 1998). Reinforcement learning

models are based on the hypothesis that the goal of an organism is to learn to act in such a way as to maximize future rewards.

When such models are applied to the physiological data described earlier, pauses and phasic spiking of DA neurons can be conceptualized as the internal representation of reward prediction errors by which the planned or actual actions of the monkey ("agent") are "criticized" by reinforcement signals (i.e., rewards that turn out to be better, worse, or as predicted).

DA release can thus shape stimulus-reward learning to improve prediction while it also shapes stimulus-action learning, i.e., the behavioral response to reward-related stimuli (Schultz *et al.,*1997).

Given the likelihood that the addictive drugs exceed natural stimuli in the reliability, quantity, and persistence of increased synaptic DA levels, a predicted consequence of these hypotheses would be profound over-learning of the motivational significance of cues that predict the delivery of drugs. At the same time, much remains unclear.

If the activation of DA neurons plays a significant role in reward function, these neurons should be responsive to the presentation of rewarding stimuli. Indeed this has been well established by electrophysiological studies (Schultz, 1997).

When, after many repeated trials, an animal learns that some distal environmental stimulus reliably predicts the presentation of reward, DA neurons come to respond to the distal predictive stimulus and cease responding to the proximal reward signal itself. The distal signal, in effect, becomes the reward signal.

1.3 How the Addictive drugs acts on the Endogenous Opioid System

Endogenous opioids are small molecules naturally produced in the body that resemble morphine and have long been implicated in the actions of opiate drugs and alcohol. There are three classes of endogenous opioids: endorphins, enkephalins, and dynorphins (Figure 4).
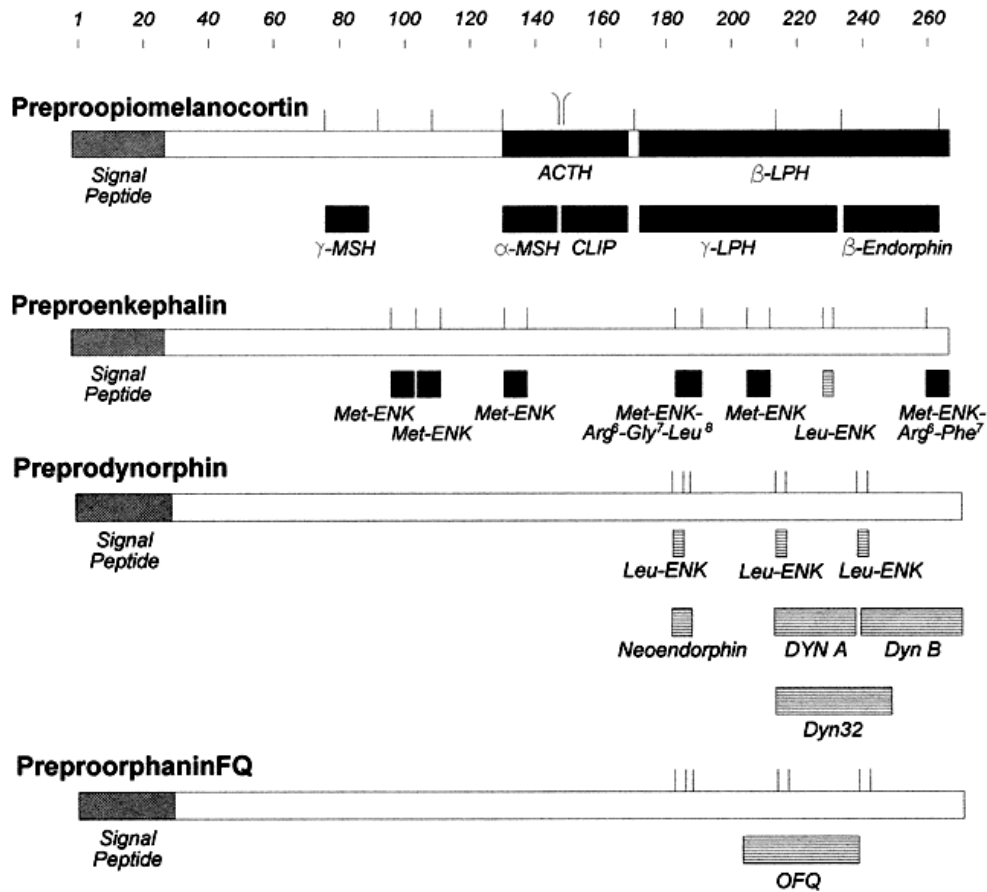
Fig. 4. Proopiomelanocortin, preproenkephalin A, preproenkephalin B, and preproorphanin FQ (prepronociceptin) precursor molecules and their related cleavage products

They all exert their effects by interacting with three subtypes of opioid receptors: μ (MOP), δ (DOP), and κ (KOP).

1.3.1 Role of Opioid System in Dopamine release

The relations between exogenous opioids and functions of neurotrasmettor and neuromodulators are very complex, and act, as is known, through different receptor types (Sbrenna *et al.,* 2000, 1999): the exposure of opioid receptors to the opiate, produces, as you might expect, tolerance, which is selective for the various receptors: the morphine, for example, induce the tolerance at the level of MOP, and cross-tolerance compared to other μ-agonists, but this

tolerance is selective and does not involve the KOP (Picker *et al.,* 1991). The main types of opioid receptors are μ, δ and κ (Manallack *et al.,* 1986) (Figure 5) and they belong to the family of G protein-coupled receptors have been have been isolated and characterized using molecular and pharmacological techniques (Evans *et al.,* 1992; Uhl *et al.,* 1994; Knapp *et al.,* 1995).
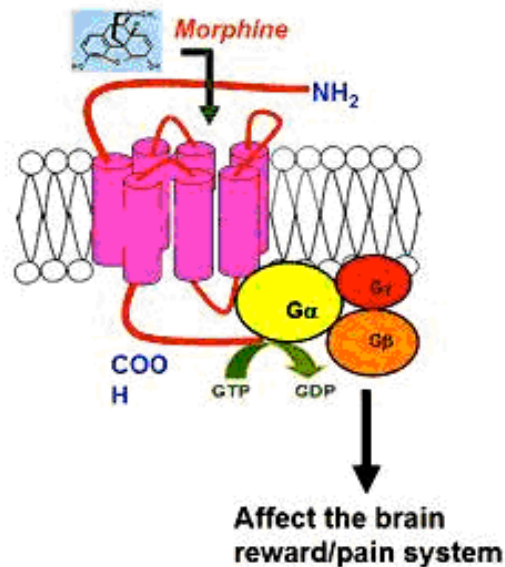
Fig. 5. Mu opioid receptor: located on the membrane of neuronal cells

Endogenous opioid ligands exhibit different preferences for each receptor. β-Endorphin binds with a higher affinity to μ than δ or k-opioid receptors, naturally indicating that it is the endogenous ligand for this opioid receptor type (Khachaturian *et al.,* 1993; Simon *et al.,* 1973). The affinity of enkephalins for DOP is 20-fold greater than that for MOP receptors, and dynorphin is presumed to be the endogenous ligand for KOP (Chavkin *et al.,* 1982; Simon, 1991). Of the other novel endogenous opioids isolated, orphanin FQ appears to be an endogenous ligand for the nociceptin opioid receptor (NOP) which has a high degree of homology with the opioid receptors (Meunier *et al.,* 1995; Reinscheid *et al.,* 1995; Zadina *et al.,* 1997). The involvement of opioid receptors in drug dependence has been further examined using μ- and δ-opioid receptor knockout mice; μ knockout mice display impaired self administration and conditioned place preference for morphine, heroin, alcohol, THC, and nicotine (Contet *et al.,* 2004).

Recently, reduced locomotor activating effects (Chefer *et al.,* 2004), conditioned place preference (CPP) (Hall *et al.,* 2004), and self-administration (Mathon *et al.,* 2005) of cocaine were shown in MOP knockout mice as well.

Since different drugs of abuse have different primary loci of action, a prominent and general role of the MOP in the rewarding effects of various substances is indicated.

Several studies show that activation of the opioid system can modulate the secretion of DA, which as we have seen is much involved in the reward phenomena. When μ- and δ-opioid receptor agonists are self-administered in animal models (Devine *and* Wise, 1994), DA release in the NA increases (Devine *et al.,* 1993). This increase occurs via the action of the agonists on GABAergic neurons in the VTA , which naturally inhibit DA neurotransmission.

Indeed some reduction in the levels of DA seems be induced by agonists of the MOP (Yonehara *and* Clouet, 1984) and morphine has shown itself capable of reducing the concentration of DA in the portal circulation pituitary action that is reversible under the influence opioid antagonist naloxone (Gudelesky *and* Porter, 1979).

While the general action of opiates on DA may play a inhibitory character, the other the specific stimulatory effect on certain areas of supports the activation of brain reward circuit (Sell *et al*., 1999): in experimental animals seems to be well documented that the release of dopamine in the NA represents the biochemical substrate capable of determining the additive behavior, both for opiates, and for other substances that induce dependence (Herz, 1998; Leshner *and* Koob, 1999) although this mechanism is not yet fully verified in humans (Gratton, 1996), and also a significant reduction of DA would occur during withdrawal from opiates (Lichtigfeld *and* Gillman, 1996).

Ventricular infusions of β-endorphin were shown to increase dopamine release in the NA via μ- and δ-opioid receptors (De Vries *and* Shippenberg, 2002). This increased in DA release is likewise due to the inhibitory effect of β-endorphin on GABA blocking DA neurons (Di Chiara *and* North, 1992).

Furthermore, β-endorphin-induced reinforcement, as tested by the CPP paradigm, correlates positively with an increase in DA release in the NA (Spanagel *et al.,* 1991). Interestingly, the reinforcing effect of β-endorphin occurs only at doses that stimulate DA release, which suggests that β-endorphin is an endogenous mediator of reinforcement, especially for addictive drugs that increase mesolimbic DA neurotransmission as a secondary target (Figure 6).
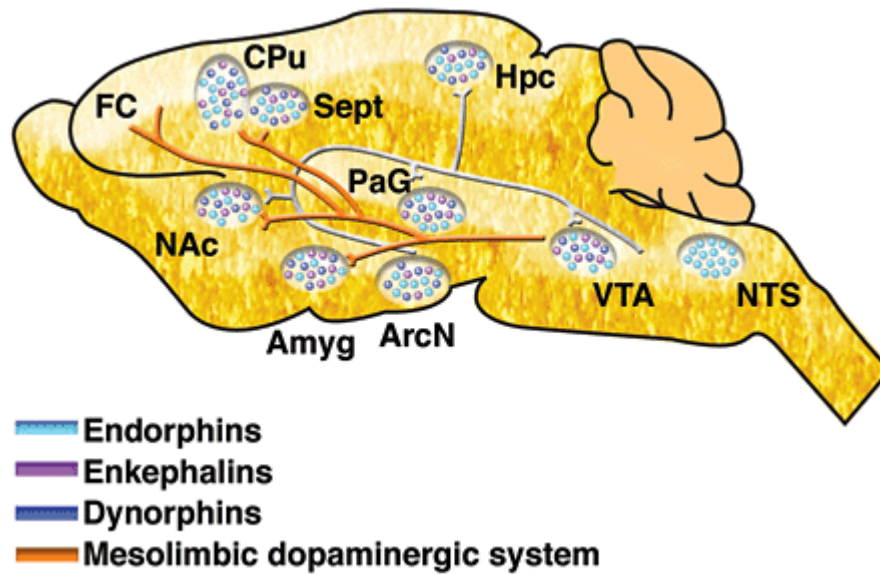
Fig. 6. Lengthwise view of the rat brain showing the distribution of endogenous opioid

Involvement of β-endorphin in drug-induced reward and reinforcement is supported by several evidence from studies performed during acquisition, maintenance and withdrawal stages of addictive drug usage, i.e. cocaine, alcohol, nicotine and THC; whether the endorphins are also involved in drug reinstatement has yet to be determined.

The reward pathways for different addictive drugs converge to a common pathway in which the β-endorphin is a modulator element. As such, the observed elevations in brain levels of β-endorphin during drug self-administration and extinction of the drug-reinforced behavior would be functionally significant.

For example, β-endorphin in the NA may activate appetitive rewarding. This possibility is supported by opioid receptor blocker-mediated reduction of the conditioned reinforcing effects of addictive drugs.

Although drugs of abuse have different acute mechanisms of action, their brain pathways of reward exhibit common functional effects upon both acute and chronic administration (Nestler, 2005), including the involvement of β-endorphin as a common mechanism underlying the behavioral effects of substances of abuse was reviewed.

Currently, evidence supports a prominent role for β-endorphin in the reward pathways of cocaine and alcohol.

1.3.2 Addictive behavior mediated by Endogenous Opioid System

Animal models for drug dependence, where the characteristic aspects of human drug dependence can be mimicked, have provided a useful tool to study the neurobiological substrates underlying the dependence-creating properties of drugs of abuse.

Several animal models based on operant conditioned behavior have been used to monitor the reinforcing and rewarding effects of addictive drugs. The underlying principle of operant conditioning is that consequences control behavior. An animal performs since it is reinforced for doing so. Reinforcement can be positive (food, water or addictive drugs) or negative (electric shock that can be avoided by making the correct response). Animals learn to obtain rewards and avoid punishment by responding correctly. In this context, the role of endogenous opioid system seems to be very important.

Several physiological and behavioral effects of enkephalins and β-endorphin, such as rewarding sensations and addictive properties, are similar to those displayed by morphine (Belluzzi *and* Stein, 1977; Goeders *et al.*, 1984; Van Ree *et al.,* 1979).

Involvement of opioids, such as β-endorphin, in the reinforcing and dependence-creating properties of drugs has been shown indirectly in pharmacological studies. Most of these studies focused on the relation between opioid receptors and drug-seeking behavior.

Much evidence exist demonstrating alterations in cocaine-seeking behavior upon blockade of μ- and δ-opioid receptors, which are favored by β-endorphin, hence indirectly indicating that this endogenous opioid is involved in certain aspects of cocaine addiction.

Low doses of the non-specific opioid antagonists, naloxone and naltrexone, or the specific μ-antagonist, CTAP, are sufficient to inhibit cocaine-induced CPP (Gerrits *et al.,* 1995; Kim *et al.,* 1997; Kuzmin *et al.,* 1997).

It may be stated that the vast majority of literature indicates that blockade of opioid receptors in the brain decreases both the reinforcing and conditioned motivational effects of drug in both animals and humans, depending on a critical dose used (Roth-Deri *et al.,* 2008).

1.4 Addiction as a multistage process

Addiction is defined as compulsive drug use despite negative consequences. The goals of the addicted person become narrowed to obtaining, using, and recovering from drugs, despite failure in life roles, medical illness, risk of incarceration, and other problems. An important characteristic of addiction is its stubborn persistence (Hser *et al.,* 2001; McLellan *et al.,* 2000).

Although some individuals can stop compulsive use of tobacco, alcohol, or illegal drugs on their own, for a large number of individuals rendered vulnerable by both genetic and non genetic factors (Merikangas *et al.,* 1998; Rhee *et al.,* 2003), addiction proves to be a recalcitrant, chronic, and relapsing condition (McLellan *et al.,* 2000).

The central problem in the treatment of addiction is that even after prolonged drug-free periods, well after the last withdrawal symptom has receded, the risk of relapse, often precipitated by drug-associated cues, remains very high (Wikler *and* Pescor, 1967; O'Brien *et al.,* 1998).

A recent reviews characterize addiction as a two-stage process.

In the first stage, the individual's occasional drug taking becomes increasingly chronic and uncontrolled.

The neurological source of these symptoms is drug-induced deregulation of the brain's reward system (Feltenstein *and* See, 2008).

Normally, increased DA signaling within this system, specifically, in the ventral striatum or NA, produces pleasurable feelings. Drugs of abuse hyper-activate this system, triggering abrupt and large increases in NA dopamine signaling, producing intense sensations that motivate additional drug taking, and promoting the formation of maladaptive drug-stimulus associations (Feltenstein *and* See, 2008).

Individuals in the second stage of the addictive process present additional clinical features, including withdrawal symptoms during early abstinence, persistent vulnerability to relapse, and alterations in decision making and other cognitive processes. Although modification of the dopaminergic reward system remains important at this stage, it probably is not sufficient to maintain these complex and long-lasting changes.

Kalivas and Volkow (Kalivas *and* Volkow, 2005) summarize evidence implicating drug-induced alterations in signals carried by the neurotransmitter glutamate from the brain area that is primarily associated with judgment, the PFCx, to the NA.

Moal and Koob (Koob *and* Moal, 2008) emphasize changes in brain stress circuits and negative reinforcement (i.e., effects that motivate drug taking by causing discomfort during abstinence, such as the onset of withdrawal symptoms).

Thus, whereas early drug use fosters maladaptive drug stimulus associations that contribute to drug seeking and use, later stages disrupt cognitive and other processes that are important for successful abstinence.

1.4.1 Reinforcement as Consolidation

The term ''reinforcement'' is unambiguous. It refers only to the seemingly retroactive ''stamping-in'' of recent associations between stimuli, stimuli and responses, and responses and outcomes that occurs when the reinforcer is given after the stimulus or response in question. Reinforcing actions of a reward depend on this temporal sequence.

In its most fundamental sense, reinforcement refers to any treatment that enhances the permanence or ''consolidation'' of a memory trace in the nervous system (Landauer, 1969).

In both the rapid time frame of cellular models of synaptic potentiation and the more prolonged time frame of consolidation of behavioural learning and memory, the DA has an important and frequently necessary role and at each of these levels of analysis, DA in the nigrostriatal system and DA in the mesolimbic system have each been implicated (Figure 7).
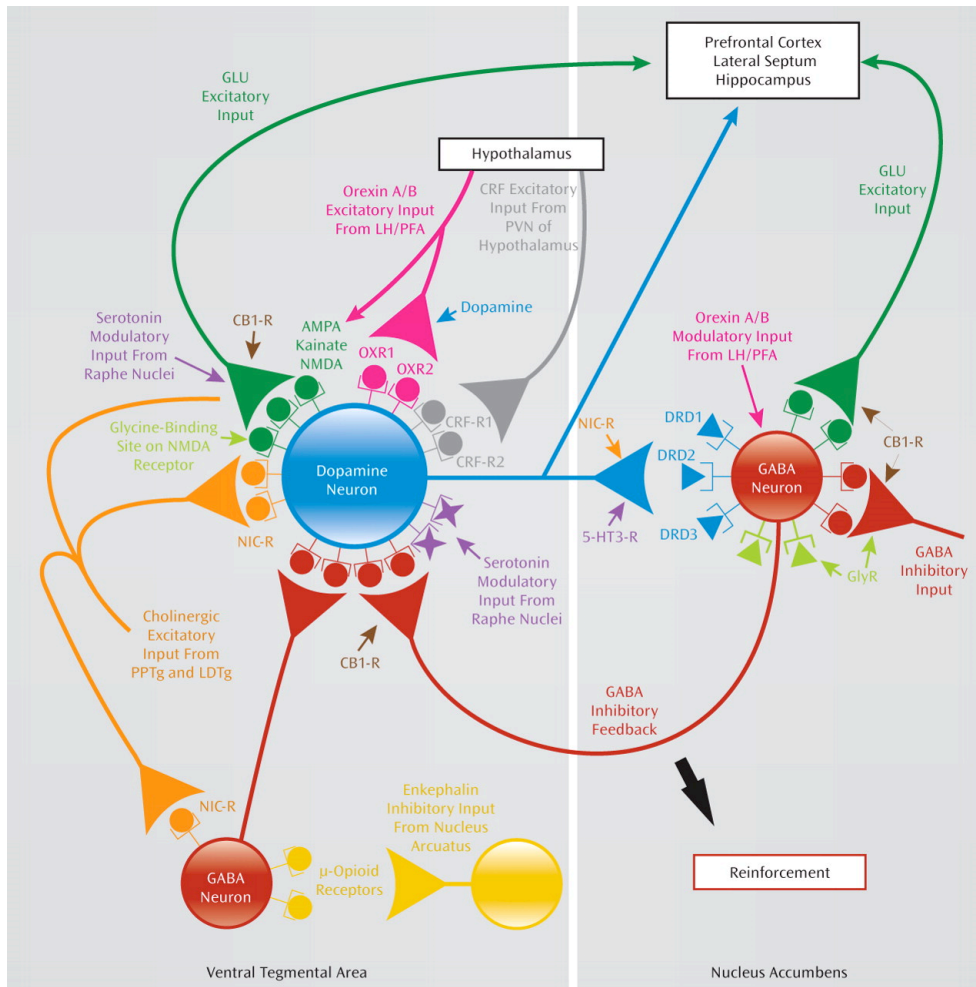
Fig. 7. Neuronal Pathways Involved With the Reinforcing Effects of different Drugs of Abuse

One approach for the study of reinforcement in animal models of alcoholism is a procedure called operant conditioning. With this approach, animals are trained to perform a response (e.g., press a lever or nose-poke a hole) that results in delivery of a stimulus (e.g., a small amount of alcohol) the animals are motivated to obtain. Operant conditioning procedures can be fine-tuned to include different work requirements for stimuli with varying degrees of motivational value for the individual tested. In this procedure models, how in humans, are shown varying degrees of willingness to work for alcohol and other drugs under many different conditions.

1.4.2 Tolerance and Withdrawal

Tolerance in an individual, is the process whereby increasing doses of a substance are required over time to give the same effect. It is mediated by a combination of post-synaptic receptor down-regulation and reduced receptor sensitivity.

There may also be changes in pre-synaptic auto-receptors affecting tonic levels of DA. In addition there are often compensatory increases in opposing pharmacological systems to help maintain a homeostatic balance within brain neural circuits (Taylor *and* Fleming, 2001; He *et al.,* 2002).

A consequence of these processes of tolerance is that on abrupt cessation of taking an addictive substance, homeostasis is lost and the opponent processes dominate, giving rise to withdrawal symptoms.

The neurotransmitter systems that are affected will vary from substance to substance.

For instance, with opioids, reduced function of opioid receptors likely occurs through altered second messenger systems since few changes in receptor number have been found (Nestler, 2001a; 2001b; Nestler *and* Aghajanian, 1997).

While increases in phasic DA have a role in mediating initial pleasure, neuro-adaptations occur in chronic use, resulting in a hypodopaminergic state.

These changes, occurring in withdrawal and early abstinence, probably underlie symptoms such as dysphoria, anhedonia and irritability, and may contribute to craving and drug-seeking behavior.

For some substances, such as stimulants, such symptoms predominate with no or few physical symptoms. By contrast, for other substances of abuse such as opioids or alcohol, physical withdrawal symptoms can be prominent and contribute significantly to their dependency since drug use is to stave off or combat withdrawal. This is 'negative reinforcement', i.e. taking a drug to overcome an aversive state.

1.4.3 Addiction a disease of Learning and Memory

The most distinctive attribute of long-term memory is persistence over time. New studies have uncovered many aspects of the molecular and cellular biology of synaptic plasticity, and the acquisition and consolidation of memory, which are thought to depend on synaptic plasticity. Much less, however, is known about the

molecular and cellular biology of long-term memory persistence. Recent findings in the field are construed within the conceptual framework that proposes that consolidation and persistence of long-term memories require modulation of gene expression, which can culminate in synaptic remodeling.(Figure 8).
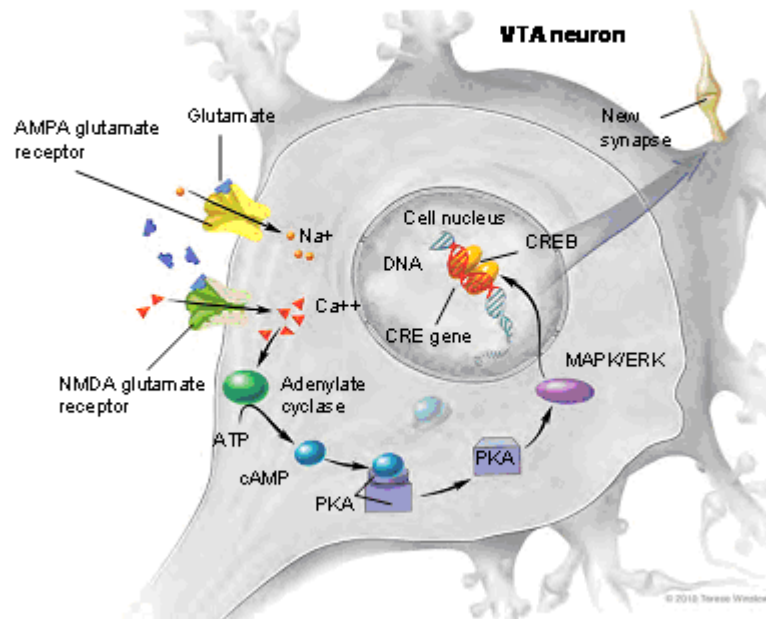
Fig. 8. A Cell Signaling Cascade in Learning and Memory.

Glutamate binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-d-aspartic acid (NMDA) receptors in the neuron membrane, opening channels for sodium and calcium to flow into the cell; calcium influx induces adenylate cyclase to convert adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP triggers activation, sequentially, of protein kinase A (PKA), mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), and cAMP response element-binding (CREB). CREB attaches to DNA , increasing DNA production of protein for the construction of new synapses

The synaptic plasticity is complex, but it can be divided into mechanisms that change the strength or "weight" of existing connections and those that might lead to synapse formation or elimination and remodeling of the structure of dendrites or axons (Chklovskii *et al.,* 2004). The specificity of drug and their relationship to specific behavioral sequences suggest that at least some of the mechanisms underlying addiction must be associative and synapse specific.

The best-characterized candidate mechanisms for changing synaptic strength that are both associative and synapse specific are long-term potentiation and long-term depression.

These mechanisms have been hypothesized to play critical roles in many forms of experience-dependent plasticity, including various forms of learning and memory (Martin *et al.,* 2000; Malenka, 2003).

1.5 Vulnerability, risk factors and relapse

The brain circuits mediating the pleasurable effects of addictive drugs are anatomically, neuro-physiologically and neuro-chemically different from those mediating physical dependence, and from those mediating craving and relapse. There are important genetic variations in vulnerability to drug addiction, yet environmental factors such as stress and social defeat also alter brain-reward mechanisms in such a manner as to impart vulnerability to addiction. In short, the 'bio-psycho-social' model of etiology holds very well for addiction (Figure 9).
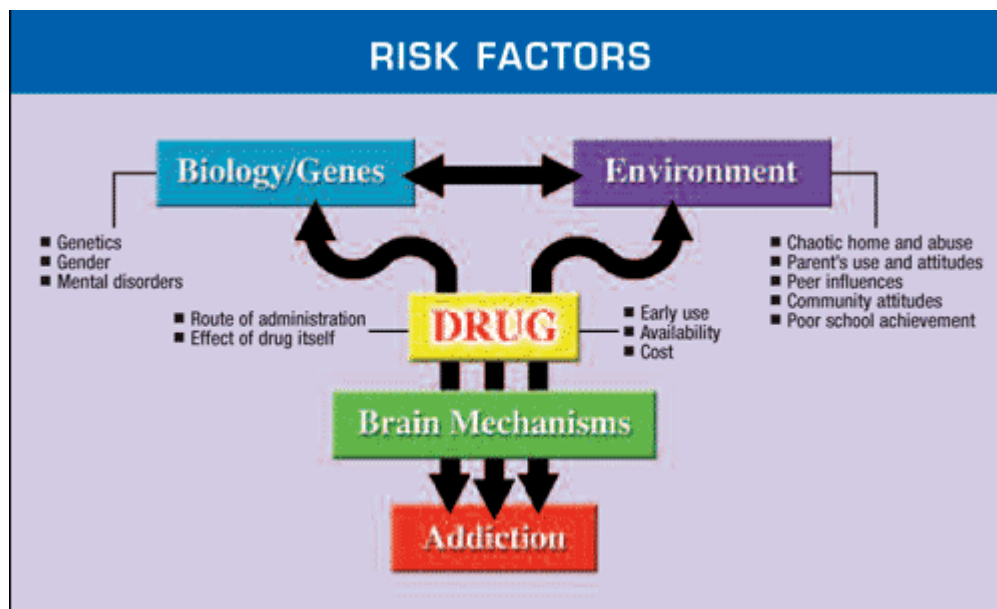
Fig.9. Factors determine whether a person will become addicted to drugs

Because addiction relapse is a common phenomenon, research in the past decade has focused on whether there is a biology underlying relapse susceptibility, and if so, whether it is possible to develop new treatments to decrease relapse risk (Sinha, 2001; Shaham *et al.,* 2003).

The most common reasons for relapse given by substance-abusing patients include stress, negative mood and anxiety, drug-related cues, temptations and boredom, and lack of positive environmental contingencies (eg, job, family relationships, responsibilities) (McKayn *et al.,* 1995).

To understand how and why recovering addicted individuals succumb to relapse, particularly in the context of external environmental stimuli and interoceptive cues, it is important to examine the psychobiological consequences of chronic drug use and assess whether such changes are involved in increasing relapse risk.

High levels of stress and trauma exposure are commonly associated with substance use disorders (Sinha, 2008; Enoch, 2011).

Increases in irritability, anxiety, emotional distress, sleep problems, dysphoria, aggressive behaviors, and drug craving are common during early abstinence from alcohol, cocaine, opiates, nicotine, and marijuana (Sinha, 2008).

The dependent state is marked by negative affect, distress, and anhedonia during early abstinence, which relates to neuroadaptations in brain reward and stress pathways (Sinha, 2001; 2008; Kalivas *and* Volkow, 2005).

We can argue that often the same phenomena that can lead to initiation of drug use, are often also the cause of relapses.

1.6 Ethanol

Ethanol (EtOH) is a psychoactive substance which act with multiple effects on the human body, such as: energetic, nutritional, pharmacological, toxic and psychic. EtOH , after being ingested, is rapidly metabolized into the stomach by the alcohol dehydrogenase enzyme (ADH), and then mostly in the liver by a series of oxidation reactions (Figure 10).
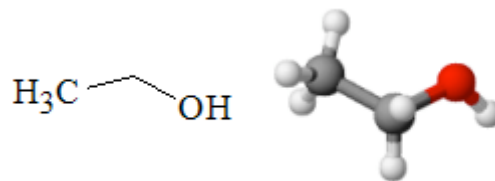
Fig. 10. Ethanol structure

The intermediate metabolite, acetaldehyde, is a reactive and toxic compound, contributing to the damage induced by ethanol (Figure 11).
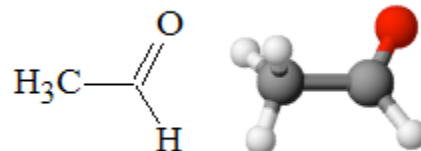
Fig.11. Acetaldehyde structure

EtOH is oxidized to acetaldehyde through the actions of various alcohol ADH enzymes (e.g., enzymes encoded by the ADH1B and ADH1C genes), through the microsomal enzyme cytochrome P450 2E1 (CYP2E1), and by microbes living in the human gastrointestinal tract (e.g., mouth and colon) (Figure 12).

The relative contributions of these pathways and the differences in activity between enzymes encoded by different ADH1B and ADH1C alleles is represented by the thickness of the arrows. Acetaldehyde is oxidized to acetate primarily by the enzyme aldehyde dehydrogenase 2 (ALDH2). Again, the thickness of the arrows indicates the rate of acetaldehyde oxidation in people carrying two active ALDH2*1 alleles, one

active ALDH2*1 and one inactive ALDH2*2 allele, or two inactive ALDH2*2 alleles, respectively.

The reaction catalyzed by ADH require a consumption of NAD +, whose availability is a limiting factor in the metabolism of EtOH, which is why the conversion rate of EtOH in the liver is fixed (approximately 8 g or 10 ml per hour for a man of 70 kg).

The role of CYP2E1 enzyme, which belongs to the cytochrome P450, occurs when the amount of EtOH taken exceeds the capacity of the catabolic dehydrogenase. In a state of chronic alcoholism, the activity of this enzyme is induced causing an alteration in the ability of the liver in detoxification.
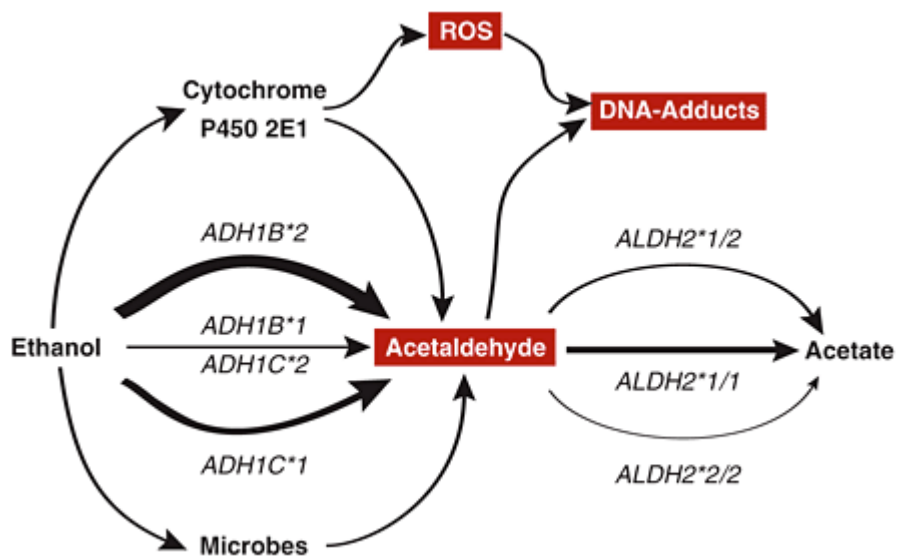
Fig. 12. Ethanol metabolism

1.6.1 Neurobiology of Alcohol Dependence

Alcoholism is a debilitating disorder for the individual and very costly for society. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), more than 17 million people in the United States either abuse or are dependent on alcohol (NIAAA, 2007), with a cost to U.S. society of over $180 billion annually (NIAAA, 2004). A major goal of alcohol research is to understand the neural underpinnings associated with the transition from alcohol use to alcohol dependence. Positive reinforcement is important in the early stages of alcohol use and abuse.

Negative reinforcement can be important early in alcohol use by people self-medicating coexisting affective disorders, but its role likely increases following the transition to dependence. Chronic exposure to alcohol induces changes in neural circuits that control motivational processes, including arousal, reward, and stress. These changes affect systems utilizing the signaling molecules DA, opioid peptides, γ-aminobutyric acid, glutamate, and serotonin, as well as systems modulating the brain's stress response.

These neuro-adaptations produce changes in sensitivity to alcohol's effects following repeated exposure (i.e., sensitization and tolerance) and a withdrawal state following discontinuation of alcohol use. Chronic alcohol exposure also results in persistent neural deficits, some of which may fully recover following extended periods of abstinence. However, the organism remains susceptible to relapse, even after long periods of abstinence. Recent research focusing on brain arousal, reward, and stress systems is accelerating our understanding of the components of alcohol dependence and contributing to the development of new treatment strategies.

1.6.1.1 Reinforcement and the Transition from Alcohol use to Dependence

Reinforcement is a process in which a response or behavior is strengthened based on previous experiences. Positive reinforcement describes a situation in which a presumably rewarding stimulus or experience (e.g., alcohol-induced euphoria) increases the probability that the individual exhibits a certain response (e.g., alcohol-seeking behavior) (Gilpin *and* Koob, 2008).

Negative reinforcement occurs when the probability of an instrumental response (e.g., alcohol-seeking behaviour) increases if this response allows the individual to circumvent (i.e., avoidance response) or alleviate (i.e., escape response) an aversive stimulus. In alcohol dependence, the aversive stimulus often is composed of motivational/affective symptoms (e.g., anxiety, dysphoria, irritability, and emotional pain) that manifest in the absence of alcohol (i.e., during withdrawal) and which result from prior discontinuation of alcohol consumption. Thus, people may drink to prevent or alleviate the anxiety they experience during alcohol withdrawal. In conditioned positive and negative reinforcement, stimuli that become associated with either alcohol or withdrawal can motivate subsequent alcohol-seeking behavior.

Alcohol-drinking behavior is driven by both positive and negative reinforcement, although their relative contributions change during the transition from alcohol use to abuse to dependence (Gilpin *and* Koob, 2008).

1.6.1.2 Positive reinforcement

The positive reinforcing effects of alcohol generally are accepted as important motivating factors in alcohol-drinking behavior in the early stages of alcohol use and abuse. These effects most often are examined using animal models of self-administration. With different operant conditioning procedures, researchers can determine the time course, pattern, and frequency of responding for alcohol.

Operant procedures most often are used to examine oral self-administration of alcohol, but they also can be used to assess self-administration of alcohol via other routes. For example, rats will respond for alcohol infusions directly into the stomach (Fidler *et al.,* 2006), blood stream (Grupp, 1981), or brain (Gatto *et al.,* 1994). But the reinforcing properties of alcohol can be assessed using different procedures, such as the intracranial self-stimulation (ICSS) in specific region hat are important in mediating the rewarding properties of alcohol.

In this procedure, rats are implanted with electrodes in discrete brain regions and then are allowed to self-administer mild electrical shocks. if only mild electrical stimulation of a certain brain region is required to maintain responding, ICSS is said to have a high reward value; if, by contrast, a stronger electrical stimulation of a given brain region is required, then ICSS is said to have a lower reward value.

Alcohol increases the reward value of ICSS because in the presence of alcohol, weaker electrical stimulation is required to maintain responding (Lewis *and* June, 1990).

Some recently developed animal models mimic binge drinking in humans. This pattern of self-administration, defined in humans as an excessive pattern of alcohol drinking that produces blood alcohol levels greater than 0.08 percent within a 2-hour period, may be associated with dependence (NIAAA, 2004). Models of binge drinking have been developed for both adult (Ji *et al.,* 2008) and adolescent (Truxell *et al.,* 2007) rats and intend to mimic drinking behavior motivated primarily by the positive reinforcing effects of alcohol early in the transition to dependence. For

example, sweeteners often are added to the alcohol solution in these models, a procedure that is thought to reflect the situation in humans because people tend to begin drinking alcohol in sweetened beverages (Gilbert, 1978; Samson *et al.,* 1996). Other approaches successfully have used genetic selection to produce animals that readily self-administer alcohol in a binge-like pattern (Grahame *et al.,* 1999; Lumeng *et al.,* 1977). For this approach, can be used models of specific subtypes of alcoholism.

1.6.1.3 Negative reinforcement

As mentioned above, the early stages of alcohol use and abuse mainly are associated with alcohol's positive reinforcing effects. However, alcohol's negative reinforcing effects may contribute to alcohol-drinking behavior at this stage in people who suffer from coexisting psychiatric disorders and use alcohol to self-medicate these disorders.

Comorbidity of alcohol problems (i.e., abuse or dependence) with anxiety and depressive/ bipolar disorders is high (44 percent and 50 percent, respectively) (Kushner *et al.,* 1990; Weissman *et al.,* 1980).

Thus, these people may use alcohol to alleviate the symptoms of the coexisting disorders. Generally, however, the negative reinforcing effects of alcohol become a critical component of the motivation to drink alcohol during the transition to dependence, when withdrawal symptoms occur following discontinuation of alcohol use and the individual drinks to avoid those withdrawal symptoms.

In animal models, the negative reinforcing properties of alcohol often are studied during periods of imposed abstinence after chronic exposure to high doses of alcohol. Such studies have identified an alcohol deprivation effect, that is, a transient increase in alcohol-drinking behavior following long-term alcohol access and a period of imposed abstinence (Sinclair *and* Senter, 1967).

Similarly, chronic inhalation of alcohol vapor can reliably produce large elevations in alcohol self-administration (Roberts *et al.,* 1996, 2000a), an effect that is amplified when animals repeatedly are withdrawn from the alcohol vapor (O'Dell *et al.,* 2004) and which lasts well into protracted abstinence (Gilpin *et al.,* 2008). In general, studies using these approaches have demonstrated that the pattern of alcohol exposure (i.e., the frequency of withdrawals) appears to be as important as the

cumulative alcohol dose in revealing alcohol's negative reinforcing properties. Changes in the reinforcing value of alcohol during the transition from alcohol use and abuse to dependence reflect (counter) adaptive neural changes resulting from chronic exposure to high alcohol doses.

As stated above, during the early stages of non-dependent alcohol use, drinking behavior largely is motivated by alcohol's positive reinforcing effects, whereas in the dependent state it likely is driven by both the positive and negative reinforcing effects of the drug (Figure 13).
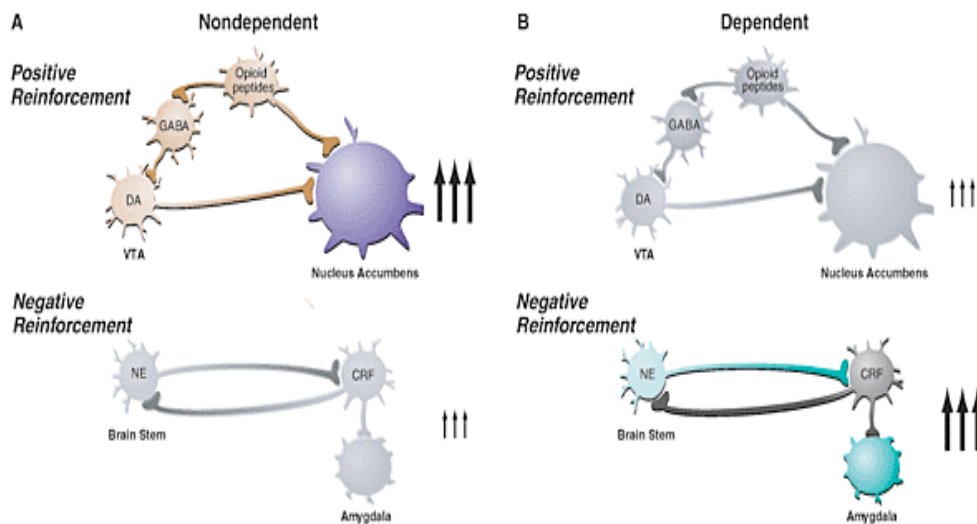
Fig. 13. Changes in the activity of the reward circuit mediating the acute positive reinforcing effects of alcohol and the stress circuit mediating negative reinforcement of dependence during the transition from nondependent alcohol drinking to dependent drinking. Key elements of the reward circuit are DA and opioid peptide neurons that act at both the VTA and the NA and which are activated during initial alcohol use and early stages of the progression to dependence (i.e., the binge/intoxication stage). Key elements of the stress circuit are corticotrophin-releasing factor (CRF) and norepinephrine (NE)-releasing neurons that converge on γ-aminobutyric acid (GABA) interneurons in the central nucleus of the AM and which are activated during the development of dependence.

1.6.1.4 Brain circuits Mediating Alcohol Reinforcement

Alcohol interacts with several neurotransmitter systems in the brain's reward and stress circuits (Figure 14).

These interactions produce alcohol's acute reinforcing effects. Following chronic exposure, these interactions result in changes in neuronal function that underlie the development of sensitization, tolerance, withdrawal, and dependence. Research using pharmacological, cellular, molecular, imaging, genetic, and proteomic techniques already has elucidated details of some of these alcohol effects.
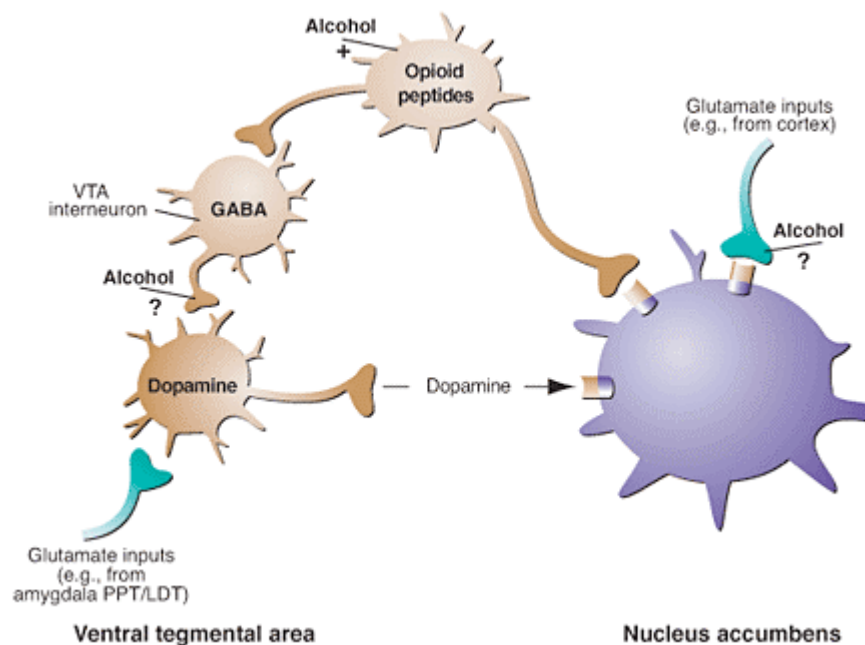
Fig. 14. Alcohol's effects on neurotransmitter systems involved in the brain's reward pathways. Alcohol, by promoting γ-aminobutyric acid (GABA) subtype GABA$_A$ receptor function, may inhibit GABAergic transmission in the VTA, thereby disinhibiting (i.e., activating) VTA dopamine. As a result, these neurons release dopamine in the nucleus accumbens, activating reward processes there. Similarly, alcohol may inhibit release of the excitatory neurotransmitter glutamate from nerve terminals that act on neurons in the NA. Many additional mechanisms (not shown) are proposed, through which alcohol may act on these pathways. Some evidence suggests that alcohol may activate endogenous opioid pathways and possibly endogenous cannabinoid pathways (not shown).

Dopamine Systems. DA is a neurotransmitter primarily involved in a circuit called the mesolimbic system. Studies suggest that DA also has a role in the incentive motivation associated with acute alcohol intoxication. For example, alcohol consumption can be blocked by injecting low doses of a compound that interferes with dopamine's normal activity (i.e., a DA antagonist) directly into the NA (Hodge *et al.,* 1997; Rassnick *et al*., 1993). Furthermore, alcohol ingestion and even the anticipation that alcohol will be available produce DA release in the NA as determined by increased DA levels in the fluid outside neurons (Weiss *et al*., 1993). However, lesions of the mesolimbic dopamine system do not completely abolish alcohol-reinforced behavior, indicating that DA is an important, but not essential, component of alcohol reinforcement (Rassnick *et al.,* 1993). Finally, alcohol withdrawal produces decreases in DA function in dependent individuals, and this decreased DA function may contribute to withdrawal symptoms and alcohol relapse (Melis *et al.,* 2005; Volkow *et al.,* 2007) (Table 1).

Opioid Systems. Researchers have hypothesized that positive alcohol reinforcement is mediated at least in part by the release of endogenous opioids in the brain. This hypothesis is supported by numerous studies demonstrating that opioid antagonists acting either at all opioid receptor subtypes or only at specific subtypes suppress alcohol drinking in a variety of species and models (Ulm *et al.,* 1995). Moreover, complete inactivation (i.e., knockout) of the μ-opioid receptor blocks alcohol self-administration in mice (Roberts *et al.,* 2000b).

The agent naltrexone, a subtype-nonspecific opioid receptor antagonist, currently is approved as a treatment for alcoholism in humans and is particularly effective in reducing heavy drinking. Opioid systems influence alcohol drinking behavior both via interaction with the mesolimbic Da system and also independent of the mesolimbic DA system, as demonstrated by alcohol-induced increases in extracellular endorphin content in the NA (Olive *et al.,* 2001) (Table 1).

Opioid receptor antagonists interfere with alcohol's rewarding effects by acting on sites in the VTA, NA, and central nucleus of the AM (Koob, 2003).

"Light Side" of Addiction: Positive Reinforcement

	Baseline alcohol self-administration	"Binge"-like alcohol self-administration	Progressive ratio/second-order reinforcement schedules	Alcohol priming–induced reinstatement	Alcohol-conditioned cue-induced reinstatement
Dopamine antagonist	↓	↓			↓
Opioid antagonist	↓	↓	↓	↓	↓

"Dark Side" of Addiction: Negative Reinforcement

	Baseline alcohol self-administration or place preference	Withdrawal-induced anxiety-like or aversive responses	Dependence-induced increases in self-administration	Stress-induced reinstatement
Corticotropin-releasing factor antagonist	——	↓	↓	↓
Neuropeptide Y	——	↓	↓	

Table 1. Summary of Neurobiological Mechanisms of Alcohol During the Phases of the Addiction Cycle Dominated by Positive Reinforcement Versus Negative Reinforcement.

γ-Aminobutyric Acid Systems. γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. It acts via two receptor subtypes called GABA-A and GABA-B. Alcohol can increase GABA activity in the brain through two general mechanisms:

- It can act on the GABA-releasing (i.e., presynaptic) neuron, resulting in increased GABA release; or
- It can act on the signal-receiving (i.e., postsynaptic) neuron, facilitating the activity of the GABA-A receptor.

Alcohol drinking is suppressed by compounds that interfere with the actions of the GABA-A receptor (i.e., GABA-A receptor antagonists) as well as compounds that stimulate the GABA-B receptor (i.e., GABA-B agonists) in the NA, ventral pallidum, bed nucleus of the stria terminalis, and AM (Koob, 2004). Of these, the central nucleus of the AM, a brain region important in the regulation of emotional states, is particularly sensitive to suppression of alcohol drinking by compounds that act on the GABA systems (i.e., GABAergic compounds) (Hyytia *and* Koob, 1995). Indeed, acute and chronic alcohol exposure produce increases in GABA transmission in this brain region (Roberto *et al.,* 2003, 2004) (Table.1).

Compounds targeting the glutamate systems also are being used in the treatment of alcohol dependence. For example, the agent acamprosate modulates glutamate transmission by acting on NMDA and/or metabotropic glutamate receptors (Littleton, 2007). Thus, by dampening excessive glutamate activity, acamprosate blocks excessive alcohol consumption. Acamprosate's ability to suppress alcohol drinking has been observed across species, and the drug has been approved for the treatment of alcoholism in humans, primarily for its perceived ability to reduce alcohol craving and negative affect in abstinent alcoholics (Littleton, 2007).

Serotonin Systems. The neurotransmitter serotonin (also known as 5-hydroxytryptamine or 5-HT) has long been a target of interest for potential pharmaco therapies for alcoholism because of the well-established link between serotonin depletion, impulsivity, and alcohol-drinking behavior in rats and humans (Myers *and* Veale, 1968; Virkkunen *and* Linnoila, 1990) (Table1).

Pharmacological compounds that target the serotonin system by inhibiting neuronal reuptake of serotonin, thereby prolonging its actions, or by blocking specific serotonin receptor subtypes have been shown to suppress alcohol-reinforced behavior in rats (Johnson, 2008). However, some researchers are debating whether these compounds can affect alcohol-reinforced behavior without affecting consummatory behavior in general. During alcohol withdrawal, serotonin release in the NA of rats is suppressed, and this reduction is partially reversed by self-administration of alcohol during withdrawal (Weiss *et al.,* 1996).

1.6.2 Epigenetics

1.6.2.1 General consideration

Epigenetics (from the Greek, epi-: *επί-* over, above; and -genetics) correspond to the study of heritable changes in gene expression or in the cellular phenotype caused by mechanisms other than changes in the DNA sequence (Feinberg *and* Tycko, 2004). Cellular differentiation is a well know example of relevance of epigenetic mechanism. If all cells within an organism have the same DNA (Nestler, 2009) then the ability to have different cells with different functions must be due to a selective activation or silencing of particular genes within genome (Grewal, 2003). Actually, it has been demonstrated that epigenetic events, altogether with genetic events, plays a crucial role in tumor progression (Jordà *and* Peinado, 2010).

Three epigenetic mechanisms are considered the most important ones: genomic imprinting, histone modifications and DNA Methylation (Feinberg *and* Tycko, 2004) (Figure 15). Genomic Imprinting refers to the relative silencing of one parental allele compared with the other parental allele as consequence of differentially methylated regions within or near imprinted genes.

Histone modifications, principally acetylation, methylation and phosphorylation, are important in transcriptional regulation due the ability to induce chromatin structure modification, altering DNA accessibility (Feinberg *and* Tycko, 2004). DNA methylation is the most common epigenetic mechanism (Jordà *and* Peinado, 2010) and consists in a covalent modification of DNA, in which a methyl group is transferred from S-adenosylmethionine to the C-5 position of cytosine by a family of cytosine (DNA-5)-methyltransferases (Feinberg *and* Tycko, 2004) and occurs predominantly in the cytosines that precede guanines (CpG) (Bird, 1986) (Figure 16).
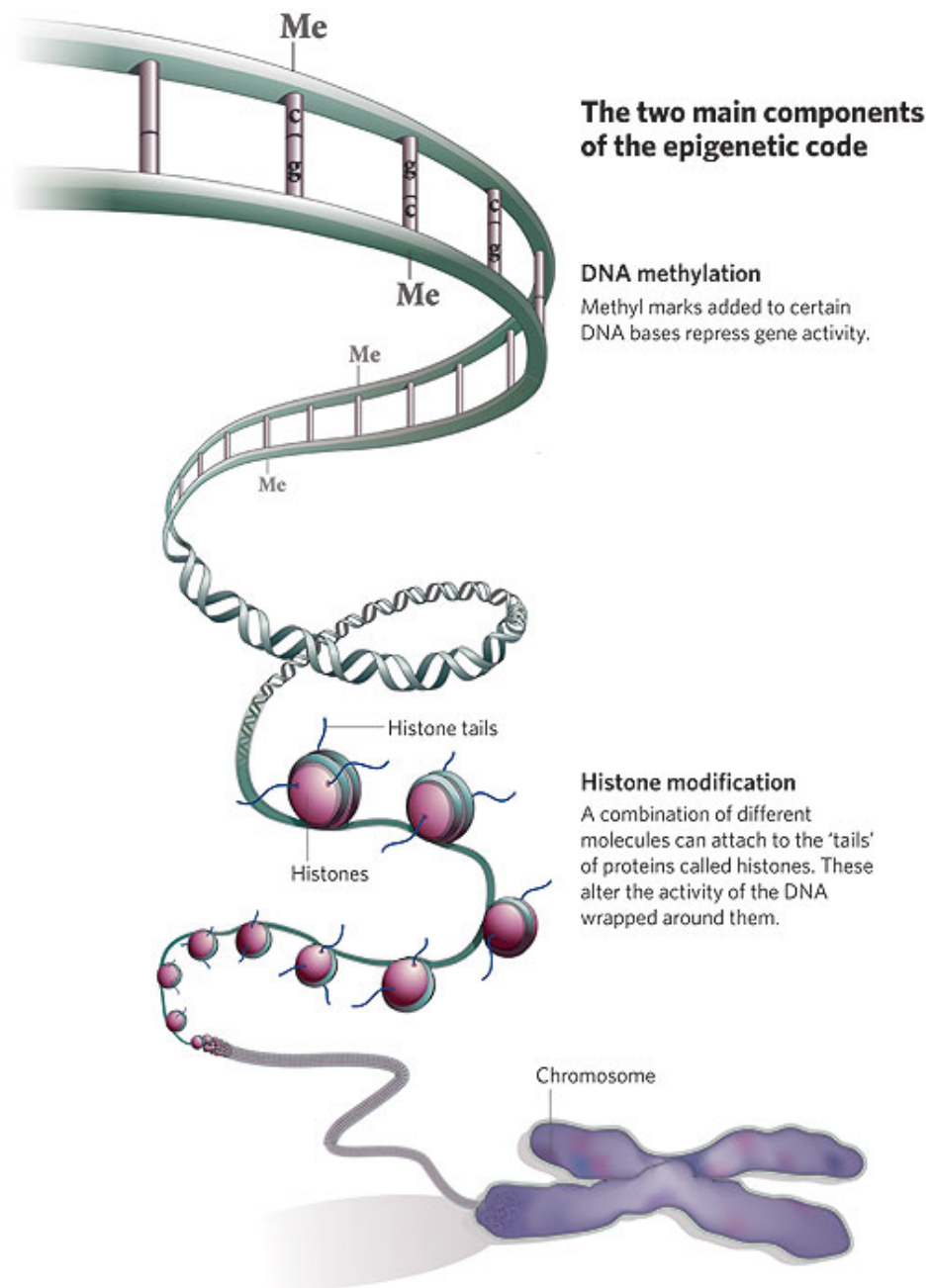
Fig. 15. Pattern of principal components of epigenetic code, DNA Methylation and Histone modification.

1.6.2.2 Histone modifications

Gene expression can also be modulated by the chromatin state. DNA is packed in the nucleus of eukaryotic cells through its chromatin organization. The nucleosome, the fundamental unit of chromatin structure, consists of 146 base pairs of DNA wrappped around an octamer of histone made up of two copies of each of the core histone (H2A, H2B, H3 and H4) (Kouzarides, 2007) (Figure 16).
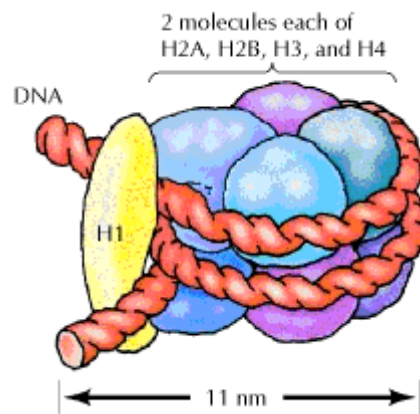
Fig. 16. DNA wrapped around histone octamers to form nucleosomes

Each core histone is composed of a structured domain and an unstructured amino-terminal tail of varying lengths from 16 amino acid residues for H2A, 32 for H2B, 44 for H3 and 26 for H4, protruding outward from the nucleosome (Taniura *et al.,* 2007). These proteins provide not only a solid structure; N-terminal regions of histones which protrude from the nucleosome are susceptible to interactions with other proteins. Chromatin can exist either in a decondensated, active arrangement, termed euchromatin, or in a condensated, inactive state, i.e. heterochromatin.

The post-translational modification of the residues at histone tails are: methylation of lysines and arginines, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. Two widely studied histone modifications are histone acetylation and phosphorylation.

Histone acetylation is linked with transcriptional activation, while deacetylation is related to transcriptional repression (Berger, 2007).

Histone acetylation is a reversible modification of lysine residues within the amino-terminal tail domain of core histone; histone acetyltransferase (HATs) transfers an

acetyl-group from acetylcoenzyme A to the ε-amino group of the lysine residue, while histone deacetylase (HDACs) acts in the reverse to remove the acetyl group. Also histone can be methylated by histone methyltransferases, inducing changes in the chromatin structure.

Methylation may create binding sites for other regulatory proteins thus influencing the chromatin structure, either condensating or relaxing the structure (Chouliaras *et al.,* 2010).

Although DNA methylation and histone modifications can act independently, they can also interact with each other. DNA methylation is associated with histone modifications through methyl CpG binding proteins interaction with dynamic complexes containing histone-modifying enzymes that promote gene repression and DNA replication and repair (Klose *and* Bird, 2006).

The binding of some deoxy-methylcytosine binding proteins to methylated sequences attracts complexes containing co-repressors and histone deacetylases, leading to a change in the chromatin structure from an open, trancriptionally active form to a more compact, inactive form, inaccessible to the transcription machinery (Richardson, 2003).

1.6.2.3 DNA Methylation

DNA methylation appears to be one of the most important epigenetic mechanisms used by the cell, for the establishment and manteinance of the correct patterns of gene expression. Indeed, alterations in the patterns of genomic methylation are strongly associated with several human diseases, making the use of specific inhibitors of the processes involved a common practise in their treatment (Egger *et al.,* 2004).

DNA methylation patterns are stablished during differentiation, and serve to suppress genes unnecessary for the function of the mature cell. Demethylation of DNA also occurs and involves at least two mechanisms: the first is the mechanism by which 5-azacytidine (an irreversible DNA Mtase inhibitor) hypomethylates DNA, and a second mechanism is that may involve DNA demethylase (Richardson, 2003).

DNA methylation in mammals occurs in the cytosin of the CpG dinucleotide via a reaction catalysed by enzymes named DNA methyltransferases (DNMTs) and the recognition of methylated cytosines is done by proteins that posses a specific binding domain, the so-called methyl-CpG binding domain. DNMTs are expressed throughout neural development, and in the adult brain in selective regional and cell-specific patterns including mature stem cell generative zones mediating ongoing neurogenesis (Feng *et al.*, 2007).

Moreover, DNMTs are actively regulated by physiological and pathological states and interactions, and they promote neuronal survival, plasticity and stress responses (Ooi *et al.,* 2007). In mammals, the DNMT family includes five proteins: DNMT1, DNMT2, DNMT3, DNMT3B, and DNMT3L (DNMT3-like).

On the other hand, the proteins that binds to and recognises 5-methylcytosines are the methyl-CpG binding domain proteins in mammals are MeCP2, MBD1, MBD2, MBD3 and MBD4. MeCP2 was the first of these proteins to be characterised.

The methylation of CG sequences can affect nearby gene expression. Hypomethylation of regulatory sequences usually correlates with gen expression, while methylation results in transcriptional suppressor. In general, the more CpG islands located in the promoter of a gene, the more the trancription level is dependent on DNA methylation (Graff *and* Mansuy, 2008).

Methylation of CpG units disrupts the binding of transcription factors and attracts proteins known as methyl-CpG binding domain proteins that are associated with gene silencing and chromatin compaction (Antequera *and* Bird, 1993).

The CpG islands, regions with more than 500 bp and a G + C content larger than 55%, are localized in the promoter regions of 40% of all the genes in mammals and are normaly maintaind in the non-methylated form (Bird *and* Wolffe, 1999), but the CpGs located outside the CpG islands are usually methylated (Urdinguio *et al.,* 2009).

The importance of DNA methylation in the function of normal cells is evidenced by its role in differentiation, X chromosome inactivation, genomic imprinting maintenance of chromatine structure, and suppression of "parasitic" DNA. Methylated citosines can serve as binding platform for specific proteins. On the other hand, this modification can also prevent binding of proteins to DNA.

It has been observed that multiple exogenous agents can affect DNA methylation, and it is possible that transient exposure to a DNA methylation inhibitor can have long term effects on DNA methylation.

1.6.3 Relationship between Alcohol effects and Chromatin Remodeling

Recently, experimental evidences suggest that the variations of gene expression induced by drugs of abuse may be mediated by epigenetic mechanisms (Renthal *and* Nestler, 2008). Several studies have demonstrated that intermittent ethanol treatment in the rats induces PFCx and hippocampal damage by inflammatory processes, and causes important short and long-lasting cognitive and behavioral deficits (Pascual *et al.,* 2007). Human epidemiological studies have also demonstrated that early-onset alcohol use is associated with an increased risk of subsequent alcohol abuse and the development of alcohol disorders, including dependence (Grant *and* Dawson 1997; Hawkins *et al.,* 1997; De Wit *et al.,* 2000). Other studies have also demonstrated that the age of first encounter with psychoactive drugs is critical, given the greater probability to shift from use to abuse and to develop addiction (Anthony *and* Petronis, 1995; Breslau *and* Peterson 1996; Patton *et al.,* 2004). Although these studies suggest that adolescence is a stage that is vulnerable to the consequences of alcohol and other psychoactive drugs abuse, the pathogenic process leading to drug addiction is still far from being completely understood.

The mesolimbic DA system is known to be involved in the reward and reinforced effects of drugs of abuse, including alcohol (Koob *and* Weiss, 1992; Robbins *and* Everitt, 2002).

Recent evidence suggests that histone modification and chromatin-remodelling events are involved in drug-related behavioural sensitization and reward (Schroeder *et al.,* 2008), and in particular that alcohol exposure can induce possible mechanism for long-term neuro-chemical alterations and recently the chromatin remodeling has been reported as a plausible mechanism for alcoholism (Pandey *et al.,* 2008).

Pandey et al. (2008) have revealed a novel epigenetic mechanism suggesting that, the increased histone acetylation and decreased HDAC activity in the AM may be involved in the anxiolytic effects of acute EtOH and conversely, decreased histone acetylation and increased HDAC activity in the AM may be involved in the

development of anxiety during withdrawal after chronic EtOH exposure. The cessation of chronic EtOH consumption has been shown to lead to the development of withdrawal symptoms both in human alcoholics and animal models (Weiss *and* Rosenberg, 1985; Wilson, 1988; Lal *et al.,* 1993; Pandey *et al.,* 2003).

These evidence presented here suggests that decreased histone acetylation, attributable to increased HDAC activity in the AM during alcohol withdrawal, may be associated with withdrawal symptoms, such as anxiety, and that acute EtOH exposure may produce anxiolytic effects because of its ability to increase histone acetylation through HDAC inhibition (Pandey *et al.,* 2008).

Moreover, specific studies conducted in our laboratories have shown that exposure to ethanol and its metabolite induce chromatin modifications in neuroblastoma cell line on the endogenous opioid system (D'Addario *et al.,* 2011a).

They have showed a link between gene expression alterations evoked by EtOH and acetaldehyde and epigenetic modulation in the promoter region of the PDYN gene, proposing a temporal relationship between transcriptional PDYN silencing and chromatin alterations.

The reactivation of the PDYN gene by the same agents on prolonged exposure may be related to preferential methylation of H3K4 and acetylation of H3K9 while keeping H3K27 un-methylated. This hypothesis is also supported by temporal changes in RNA polymerase II (RNAPII) recruitment and activation consistent with epigenetic changes. In fact, the increase observed in RNAPII, either total and S5P, at 72 h after EtOH exposure correlates with the increase at this time point of the two activating marks investigated, H3K4me3 and H3K9Ac, but not of the repressive one, H3K27me3. In addition, the alteration on H3K9Ac at 48 h becomes more relevant, since this histone modification seems to precede not only the reactivation of gene expression evident just after 96 h, but also the recruitment and activation of the RNAPII (D'Addario *et al.,* 2011a).

1.6.4 Pharmacological Treatment for Alcoholism

The treatment of alcoholism, seeks a full psycho-social rehabilitation of the subject and also provides a possible psychosocial intervention, a pharmacotherapy. In this case we can identify various stages. A phase of detoxification, preceded, if necessary, by treatment of withdrawal syndrome and followed by treatments aimed to the relapse prevention. In fact, about half of alcoholics go relapsed after a shorter or longer abstaining period (Maisto *et al.,* 2000).

The main drugs used in the treatment for alcohol-addiction are: disulfiram, naltrexone, the acamprosato. Drugs acting on the system dopaminergic (tiapride, amisulpride and flupenthixol), and serotonergic (buspirone, fluoxetine, nefazodone, ritanserina and ondansetron) have been and are currentlysubject of clinical studies. Moreover, the mood-stabilizing drugs (Salloum *et al.,* 2005), as well as those acting sedative-hypnotic (Kranzler *et al.,* 1994), have demonstrated their effectiveness in the presence of psychiatric comorbidity.

Disulfiram: The disulfiram causes irreversible inhibition of both, cytosolic and mitochondrial,

the enzyme aldehyde dehydrogenase form (Figure 17). This molecule, by inhibiting aldehyde dehydrogenase, causes accumulation of acetaldehyde which is formed by oxidation of ethanol by the alcohol dehydrogenase.
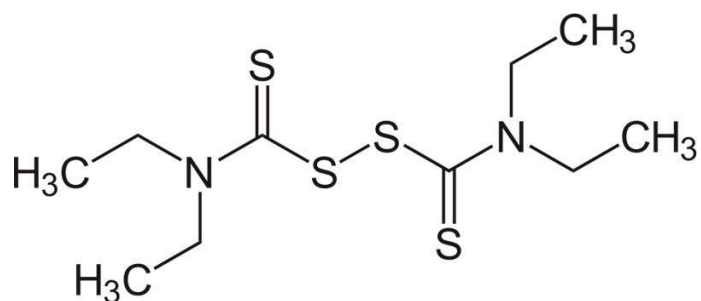
Fig. 17 Disulfiram

In the presence of disulfiram, alcohol intake is then accompanied the onset of toxic effects acetic aldehyde, rather unpleasant and alarming on the subjective level (facial flushing, throbbing headache, sweating, nausea, vomiting, tachycardia, dyspnea, hypotension, dizziness, collapse). Therefore the goal of treatment with disulfiram is to create an aversion alcohol, rather than to modulate the neuropsycho pharmacological effects.

Naltrexone : Neuropharmacological assumption of the efficacy of opioid antagonists is that the endorphins contribute to the effects of positive reinforcement of alcohol interacting with the mesolimbic dopamine system, as demonstrated experimentally by the fact that the decrease of the self-administration of alcohol is associated with a decrease of dopamine release (Gonzales *and* Weiss, 1998). Indeed, preclinical studies, performed in different experimental conditions, agree to observe that the administration of μ-receptor antagonists such as naloxone and naltrexone attenuate the consumption of alcohol (Herz, 1997; Stromberg *et al.,* 1998) (Figure 18).
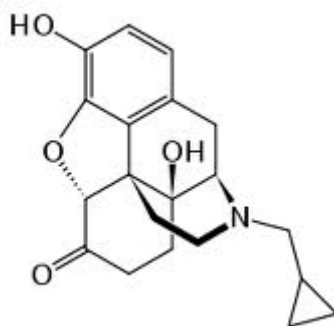
Fig. 18. Naltrexone

Clinical studies (Volpicelli *et al.,* 1992; O'Malley *et al.,* 1992) have demonstrated the ability of the naltrexone to reduce relapses in heavy drinkers [= heavy drinkers taking more than 5 units (1 unit = 10g of alcohol) alcoholic / day], as well as to reduce the craving and the frequency of alcohol intake. Unfortunately, not all clinical trials subsequently conducted on relapse, have confirmed these positive results.

Acamprosate

The modulatory effects of this molecule on glutamatergic transmission (in particular, its action of depression of the transmission and the same Activation of NMDA receptors) largely explain its use in therapy alcoholism. As known, in fact, one of the bases of the neuropharmacological alcohol dependence is established right rising both the number of functionality of NMDA receptors (Figure 19).

Fig. 19. Acamprosate

1.7 Heroin

Opiates are among the most commonly abused illegal drugs (Kreek, 1997; Hughes *and* Rieche, 1995).

Currently, heroin stands out as the most addictive and destructive illegal drug on the planet. It has considerably grave side effects and devastating implications for delicate organs of the body like the brain, the CNS and respiratory structure for those who take it. Its effects on the brain cause it to lose the capacity to manage normal, standard bodily functions thus rendering the body incapable of performing even the simplest of tasks.

1.7.1 Heroin pathways

Heroin is an opioid, regardless of chemical structure, that produces effects similar to those of opium and morphine (Wickler, 1980). Heroin has the scientific name diacetylmorphine; its chemical structure is similar to that of 6-acetyl morphine and morphine (Figure 20) and is a chronic relapsing disease that is associated with a high level of mortality and crime (Hulse *et al*., 1999).

The blood brain permeability of heroin is about 10 times that of morphine (Washington *et al*., 2001; Bao *et al*., 2006).

Fig. 20. Heroin structure after blood brain barrier crossing

Once heroin crosses the blood brain barrier, it is hydrolyzed into 6-acetyl morphine and morphine, which then quickly bind to opioid receptors (Bao *et al*., 2006). The "rush" felt by heroin users is the sensation caused by the rapid entry of heroin into the brain and the attachment of 6-acetyl morphine and morphine to opioid receptors. Opioids in general can change the neurochemical activity in the brain stem causing a depression in breathing. In the limbic system opioids cause an increase in feelings of pleasure, and have the ability to block pain signals sent through the spinal cord (NIDA, 2005) (Figure 21).
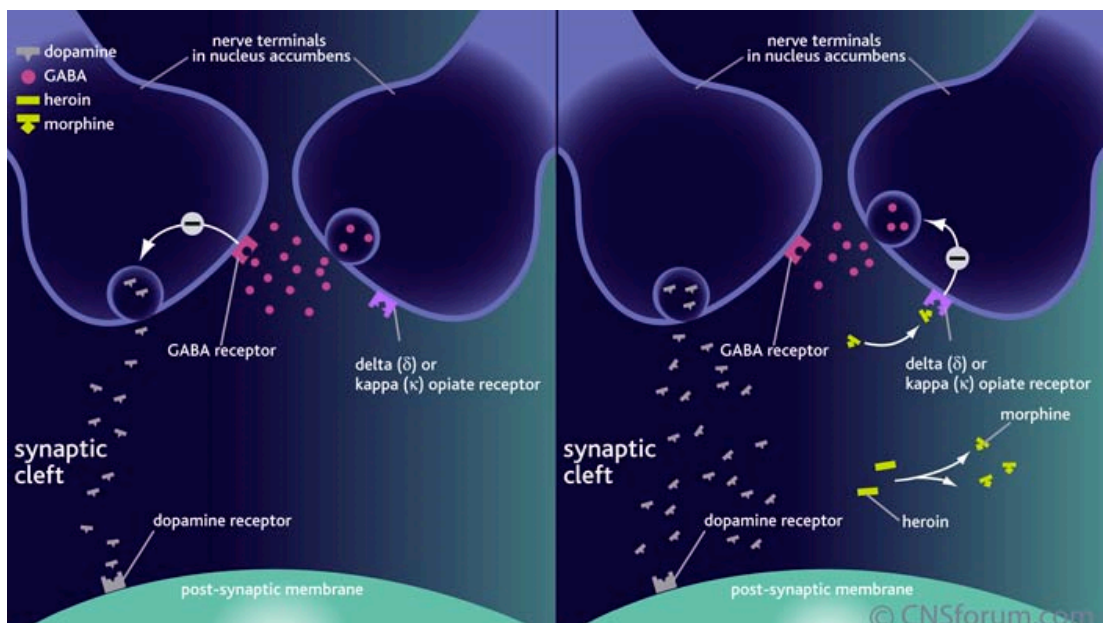
Fig. 21. Brain under heroin exposure

Several reports suggest that chronic exposure to opiates, such as morphine and heroin, can result in cognitive deficits (Guerra *et al*., 1987; Ciopolli *and* Galliani, 1987; Spain *and* Newsom, 1991).

For example, heroin users have poorer performance on attention, verbal fluency, and memory tasks than controls (Guerra *et al*., 1987), and rats chronically exposed to morphine show impaired acquisition of reference memory (Spain *and* Newsom, 1991). Such findings suggest that long-term opiate use may produce maladaptive plasticity in brain structures involved in learning and memory.

The rewarding effects of heroin, like prescription opiate-based drugs, are mediated via morphine-dependent activation of the μ-opioid receptor (MOR; Matthes *et al.,*

1996; Sora *et al.,* 2001). The opioid receptors, μ, κ and δ, belong to the superfamily of G protein-coupled receptors (GPCR).

Activation of the opioid receptors can modulate multiple effectors, including the inhibition of adenylyl cyclise and voltage-gated Ca2+ channels (Gross *and* Macdonald, 1987; Tallent *et al.*, 1994) and the activation of inward rectifying K+ channels, phospholipase C (PLC; Spencer *et al.*, 1997) and mitogen-activated protein kinase (MAPK) components ERK1 and ERK2 (Fukuda *et al.*, 1996, Cheng *et al.,* 1996). Activation of the MAPK pathway can occur through the Gαi/o associated Gβγ subunits in a Ras-dependent manner as well as by the process of opioid receptor phosphorylation by GRK3 and association with β-arrestin2 (Macey *et al.*, 2006). Once activated, ERK1/2 can regulate many downstream cytoplasmic and nuclear targets to alter gene expression and, as a consequence, events such as synaptic plasticity in the brain.

The physiological output of opioid receptors is dependent primarily on coupling to the inhibitory Gαi and Gαo subunits of the heterotrimeric G proteins. However, persistent activation of opioid receptors can alter downstream signaling events by increasing the responsiveness of Gβγ as well as Gαs subunits.

In addition, this long-term exposure to opiates also results in receptor desensitization, an event which is related to opioid tolerance and dependence. Other compensatory measures, such as changes in gene transcription and protein synthesis, which contribute to long-term alterations in synaptic function, exist to counter sustained activation of the MOR.

A large meta-analysis recently performed identified a hypothetical common network of molecules that underlie addiction to a number of different drugs of abuse (Li *et al.*, 2007).

Genetic components contribute significantly to susceptibility to heroin addiction (40-60%) and gene variants such as those encoding opioid receptors have been shown to be associated with this disease. The most widely-studied single nucleotide polymorphism (SNPs) in the OPRM1 gene are located within exon 1 (A118G and C17T variants) and encode amino acid substitutions in the extracellular N terminus of the receptor.

In particular, the A118G variant has been associated with an enhanced response to therapies for alcohol and nicotine dependence and positive susceptibility to opioid addiction (Kroslak *et al.*, 2007).

In contrast, other studies, including two meta-analyses of case-controlled subjects, failed to detect an association of the A118G variant with opioid dependence. These inconsistencies are likely a reflection of the high linkage disequilibrium the A118G SNP has with other causative variants.

There are also conflicting reports in the literature that describe the impact the amino acid substitution (N40D) has on receptor binding in in vitro studies. However, the 118G variant is associated with reduced OPRM1 mRNA levels in human brains and in decreased receptor levels in cells expressing the same variant (Kroslak et al., 2007). Furthermore, a mouse model possessing the equivalent SNP in the OPRM1 gene (A112G) also showed reduced mRNA expression and receptor protein levels, as well as reduced morphine-mediated hyperactivity and anti nociception (Mague et al., 2009).

Over 100 SNPs have been identified in the OPRM1 gene and a number of these variants have been associated with a varied response to alcohol (Ehlers et al., 2008), intiation of smoking (Zhang et al., 2006a) and the initial positive response to heroin (Zhang et al., 2006b).

The signaling and transcriptional reprogramming that underlies adaptations of the brain to chronic heroin exposure is currently poorly understood.

When heroin is injected or smoked, users typically feel two types of euphoric effects, a "rush" and a "high."

The rush usually lasts one to two minutes and occurs right after the drug is administered. It is described as an intense feeling that is felt throughout the body, especially in the abdomen.

Following the rush is a high that can last four to six hours. The feeling is described as pleasant, with indifference to internal and external stimuli. The following characterizations may occur during a high (Stimmel, 1992). Timing of Effects : injecting heroin intravenously can produce a feeling of euphoria in seven to eight seconds. The peak effects of smoking heroin are similar to those obtained from intravenous injection (Cone, 1998). In contrast, injecting intramuscularly, leads to a slower onset of euphoria, taking five to eight minutes (NIDA, 2005).

The peak effects of snorting heroin occur in 10 to 15 minutes (NIDA, 2005) whereas the oral administration has little effect.

1.7.2 Priming, Addiction and Withdrawal

A large body of evidence indicates that the reinforcing effects of heroin are mediated by the drugs actions in the VTA, (the cell body region of the mesolimbic dopamine system) and in the NA (a terminal region of this system) (Wise, 1996). Results from studies using systemic drug injections indicate that dopamine neurotransmission mediates cocaine-induced reinstatement (Self *and* Nestler, 1998; Shalev *et al.*, 2002; Spealman *et al.*, 1999). Reinstatement induced by cocaine and heroin priming is mediated by activation of dopamine D2-like, but not dopamine D1-like, receptors (Alleweireldt *et al.*, 2003; De Vries *et al.*, 1999; Khroyan *et al.*, 2000; Marinelli *et al.*, 2003; Self *et al.*, 1996; Wise *et al.*, 1990).

In earlier studies, Stewart (1984) found that intra-VTA infusions of morphine, which increases dopamine cell firing and release, reinstate heroin or cocaine seeking, and intra-accumbens infusions of amphetamine, which increases local DA release, reinstate heroin seeking. Based on these studies and studies using systemic drug injections, there was, until recently, a consensus that accumbens DA activity mediates reinstatement induced by heroin priming. In another study, Luo *et al.* (2004) trained rats to self-administer heroin or saline (a control condition) and after extinction of the drug-reinforced responding they examined in different groups the effect of heroin priming injections on reinstatement or fMRI signal in several brain areas, including components of the mesocorticolimbic system. The main finding was that rats with a history of heroin self-administration showed profound tolerance to the effect of heroin priming on the Functional magnetic resonance imaging (fMRI) signal. The significance of these findings, however, is not clear because previous studies have shown that the effects of heroin and dopaminergic drugs on reinstatement of heroin seeking is highly correlated with their ability to induce locomotor sensitization (De Vries *et al.*, 1998; De Vries *and* Shippenberg, 2002).

1.7.3 Heroin Relapse

The central problem for treatment of heroin addiction remains the return to drug use after periods of abstinence (relapse) (Mendelson *and* Mello, 1996; O'Brien, 1997; Wallace, 1989). Studies in humans provide evidence that relapse to heroin or cocaine use or craving for these drugs can be triggered by exposure to the self-administered

drug (De Wit, 1996; Meyer *and* Mirin, 1979), drug-associated cues (Carter *and* Tiffany, 1999; Childress *et al*., 1993) or stress (Sinha, 2001).

Others have argued that this clinical scenario can be modelled in a reinstatement model using laboratory rats and monkeys (Epstein *and* Preston, 2003; Shaham *et al.*, 2003; Spealman *et al.,* 1999; Stewart, 2000).

In the operant version of the reinstatement model, laboratory animals are trained to self-administer drugs and are then subjected to extinction training during which lever presses are not reinforced with drugs.

Reinstatement of extinguished lever responding (the operational measure of drug seeking) is determined after such manipulations as non contingent priming injections of the drug (deWit *and* Stewart, 1981; Stretch *et al.,* 1971), exposure to cues associated with drug intake (Davis *and* Smith, 1976; Meil *and* See, 1996) or exposure to stress (Erb *et al.,* 1996; Shaham *and* Stewart, 1995).

During testing for reinstatement, extinction conditions remain in effect (drug is not available). In the conditioned place preference (CPP) variation of the reinstatement model, laboratory animals are trained to associate a distinct environment with drug injections and are then subjected to extinction training during which they are exposed to the same environment in the absence of drug.

Resumption of preference for that environment is then determined after non-contingent priming injections of the drug (Mueller *and* Stewart, 2000; Parker *and* McDonald, 2000) or exposure to stress (Sanchez *and* Sorg, 2001).

The effects of drug-associated cues on relapse to drug seeking can also be examined in extinction tests that are administered at different days after the termination of drug self administration (Di Ciano *and* Everitt, 2004; Lu *et al*., 2004; Tran-Nguyen *et al.,* 1998). This permits characterization of the time course of susceptibility to relapse to drug seeking.

1.7.4 The MAP Kinase Pathway

The MAP kinases (MAPK) are phosphorylated enzyme activities that regulate various biological mechanisms such as gene expression, metabolism, proliferation and programmed cell death and cell motility. These enzymes, once activated, they act on different target proteins that other proteins may be kinases, transcription factors, phospholipases, and cytoskeletal proteins and determining the level of

phosphorylation at specific serine and threonine residues with activation or inhibition of their activities (Figure 22).
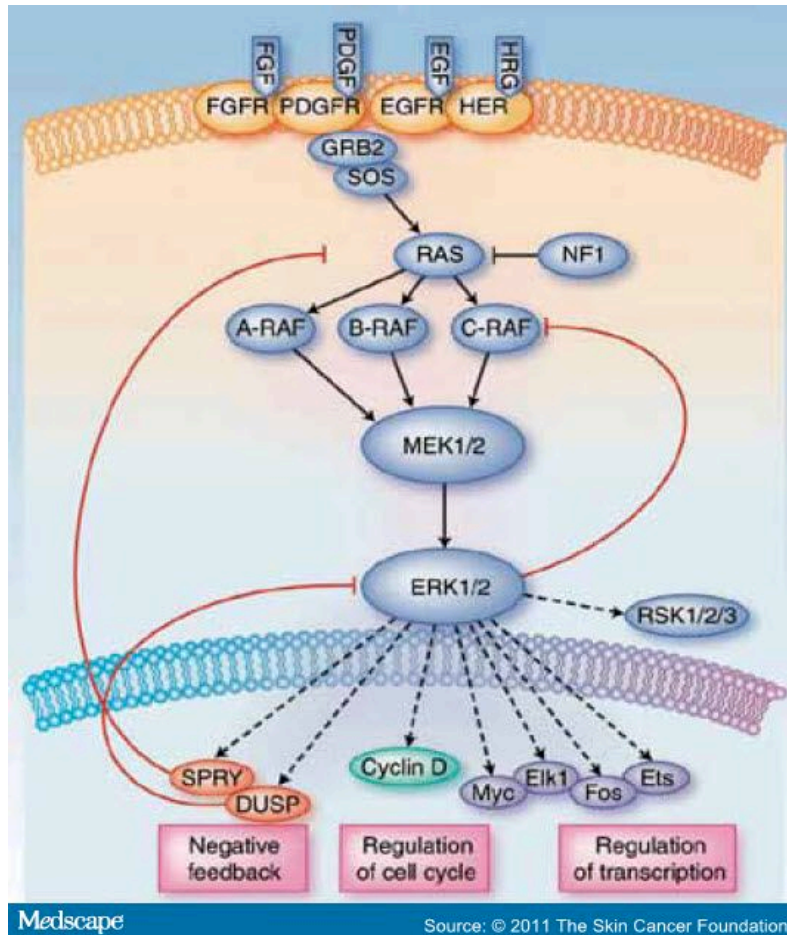
Fig. 22. The Mitogen-Activated Protein Kinase Pathway

Molecular Machanisms The activation of various MAPK provides a sequence of phosphorylations by different kinases activated in succession. Starting from an extracellular stimulus is coming, following the activation cascade of different substrates, the MAPK phosphorylation of the final will go to that then phosphorylate target proteins localized in different cellular regions (Chang *and* Karin, 2001).

In particular, the cascade of activation generally begins at the hands of a small G protein (Ras) that transmits a signal activating a MAPKKK that phosphorylates, at specific serine residues and \ or threonine, a MAPKK which, in turn, phosphorylates

and active, through a dual tyrosine and threonine phosphorylation, the MAPK itself (Yoav *et al.*, 2006).

In particular, an example of activation mechanism involving tyrosine kinase receptors for growth factors.

The cascade begins with activation of receptor tyrosine phosphorylation in response to the link with growth factors. The tyrosine phosphorylated binds directly or through the adapter Shc, Grb2 protein, responsible for the recruitment of Sos and subsequent activation of Ras, which, through Raf and MEK kinase, phosphorylates the MAPK (including ERK) that are thus activated. Another activation mechanism is the involvement of G protein-coupled receptors (G protein coupled receptors, GPCRs). In the case of Gq protein-coupled receptors, diacylglycerol (DG) of phosphoinositide hydrolysis, which forms the membrane, activates protein kinase C (PKC) that phosphorylates Raf, MEK and therefore responsible for the activation of ERK. The MAPK phosphorylation site recognized by the always consists of a tyrosine or a threonine followed by proline. The phosphate groups that both the MAPK substrate proteins are then removed by specific phosphatase resulting signal is switched off (Yoav *et al.,* 2006).

1.7.4.1 ERK 1 / 2

The ERK 1 \ 2 are widely expressed and are involved in the regulation of proliferation and cell differentiation and neuronal plasticity. ERK proteins are products of two genes, ERK 1 (Mapk3) and ERK2 (Mapk1) (Boulton *et al.*, 1991).

Besides the two major proteins ERK1 (44 kDa) and ERK 2 (42 kDa) protein isoforms are known to other members of the same family including ERK1b (46 kDa) typical of rodents (Yung *et al.,* 2000) and ERK2c ERK1b and their primate (Aebersold *et al.,* 2004; Gonzalez *et al.,* 1992). The involvement of ERK 1 / 2 in long-term memory (long-term memory, LTM) has been demonstrated by various behavioral and biochemical studies.

With the test of Morris has been shown that, following administration of inhibitors of ERK, treated animals took a much longer time to perform the test compared to control animals.

When learning was also observed an increased activity of hippocampal ERK. In neuronal cells, neurotransmitters such as DA and norepinephrine, through their receptors coupled to adenylate cyclase, or acetylcholine through muscarinic receptors coupled M1 type of phosphoinositide hydrolysis, can stimulate the' activities of ERK, and then , influence the neuronal plasticity.

The ERKs are also able to facilitate gene transcription by modifying chromatin structure through the activation of kinases that determine the relaxation of the DNA double helix by increasing the access of transcription initiation factors.

Once activated by various extracellular stimuli or internal processes of the ERK pathway are involved in the processes of proliferation, cell differentiation, regulating the expression of genes necessary for cell growth and division, and the effect of the enzyme-dependent translocation to the nucleus, determine the increase in the synthesis of nucleotides necessary for the formation of new molecules of DNA and RNA and cellular proteins.

All ERKs are activated by a double phosphorylation at residue tyrosine and threonine regulators. This dual phosphorylation appears to be mediated by MEK. Following mutational analysis has discovered a region in the C-terminal portion of the ERK (amino acids 312 to 320 of ERK2) important for interaction with MEK (Rubinfeld *et al.,* 1999; Chuderland *and* Seger, 2005).

The most important residues for interaction in this region are 3 amino acids (Asp316, Asp819, Glu320 in ERK2). This region is able to interact with three and two basic residues located in the hydrophobic N-terminal portion of MEK called domain D (Zhou *et al.,* 2006).

Crystallization studies have shown that the binding of the domain D with the ERK pathway induces a conformational change that exposes the threonine and tyrosine residues outside regulators allowing the attack of MEK and phosphorylation of these residues (Pulido *et al.,* 1998).

1.7.4 ETS family and ELK1

The ETS-domain transcription factor Elk-1 was first discovered in a fraction of HeLa cell nuclear extract that formed ternary complexes with the serum response factor (SRF) on the c-fos serum response element (SRE)(Shaw *et al.,* 1989). This protein was thus named p62TCF, based on its properties of being a 62 kDa protein and being a ternary complex factor (TCF).

The TCFs represent a subfamily of the ETS domain transcription factor family (Graves *and* Petersen, 1998; Sharrocks, 2001). These transcription factors are characterized by the presence of the ETS DNA-binding domain, and sequence conservation within this domain alone is sufficient to classify ETS-domain proteins into subfamilies. Elk-1 is the best studied of the TCFs, and several functional domains/motifs have been identified (Figure 23).
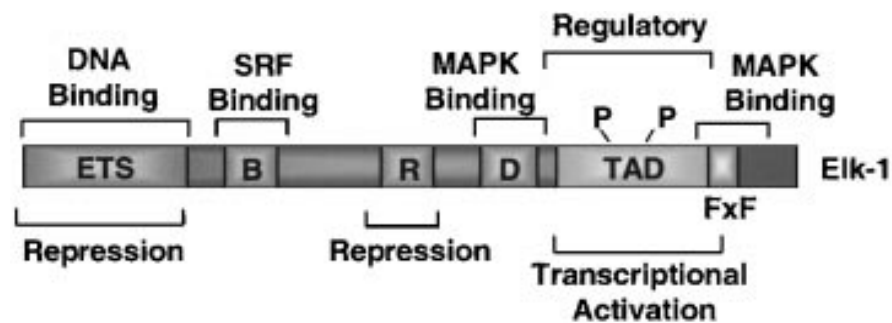
Fig. 23. Domain structure of Elk-1

The locations of the ETS domain, B-box (SRF interaction) motif, Rmotif, D-domain, FxF motif [MAP kinase (MAPK) binding] and TAD are indicated. The ETS domain and the R-motif exhibit transcriptional repression activity. The TAD also overlaps the regulatory region of the protein that is phosphorylated (P) by MAP kinases.

Elk-1 was initially identified as part of a ternary complex with SRF on the c-*fos* SRE. Since then, the molecular and structural details of how this complex is assembled and regulated have been elucidated. It is recruited to the SRE by a combination of protein-DNA and protein-protein interactions (Figure 24).
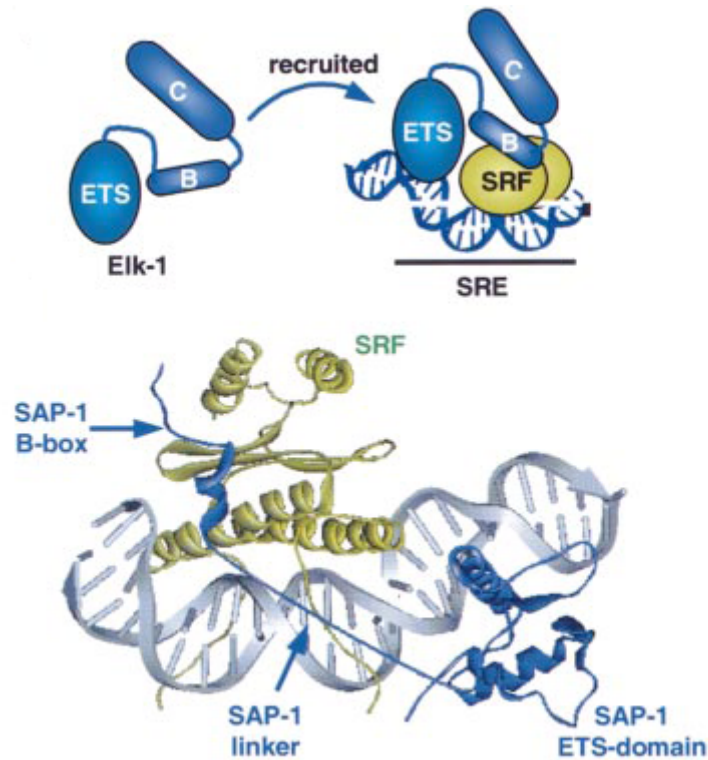
Fig. 24. Structure of the TCF–SRF–SRE ternary complex

The formation of the ternary complex can be regulated at several levels. For example, phosphorylation stimulates ternary complex formation by Elk-1 (Janknecht *et al.,* 1994; Gille *et al.,* 1992).

Binding of Elk-1 to the c-*fos* SRE in the absence of SRF cannot be detected, suggesting that protein-protein interactions with SRF are a pre-requisite for its recruitment (Hipskind *et al.,* 1991). But at the same time, this stimulation can also be observed with high-affinity ETS binding sites in the absence of SRF (Sharrocks, 1995), indicating that this enhancement is mediated, at least in part, by promotion of protein/DNA interactions via the ETS domain. Conversely, ternary complex formation and autonomous DNA binding by Elk-1 and other TCFs can be inhibited by interaction with members of the Id subfamily of helix-loop-helix proteins.

The TCFs are direct targets of the MAP kinases, and Elk-1 was the first TCF shown to be regulated by ERKs (Hill *et al.,* 1993; Marais *et al.,* 1993).

Phosphorylation of Elk-1 both enhances its recruitment to DNA (either in ternary complexes or autonomously) and potentiates its transcriptional activation activity.

ERK-mediated Elk-1 phosphorylation is also thought to promote the formation of quaternary complexes containing two Elk-1 molecules (Gille *et al.,* 1996).

Recently, the mechanism by which MAP kinases recognize and interact with the TCFs has been elucidated in detail. While the local context of the phosphoacceptor motifs plays an important role in specificity determination for a particular type of MAP kinase, docking modules also play a major role in specificity determination.

In Elk-1, the D-domain and FxF motif constitute a bipartite docking module that dictates its selective phosphorylation by ERK and JNK MAP kinases, but selects against phosphorylation by p38 (Yang *et al.,* 1998; Jacobs *et al.,* 1999).

In Elk-1 the FxF motif is responsible for directing the preferential phosphorylation of the critical Ser-383 residue (Fantz *et al.,* 2001).

However, while it should be emphasized that Ser-383 is clearly the most important residue for Elk-1 function, Elk-1 contains multiple MAP kinase sites that are phosphorylated to a high stoichiometry *in vivo* (Cruzalegui *et al.,* 1999). The function(s) regulated by the other MAP kinase sites in Elk-1 are currently unknown.

Phosphorylation of the TCFs regulates a number of key activities. A sequential series of events is triggered by phosphorylation by MAP kinase of the C-terminal regulatory domain of Elk-1 (Figure 25).
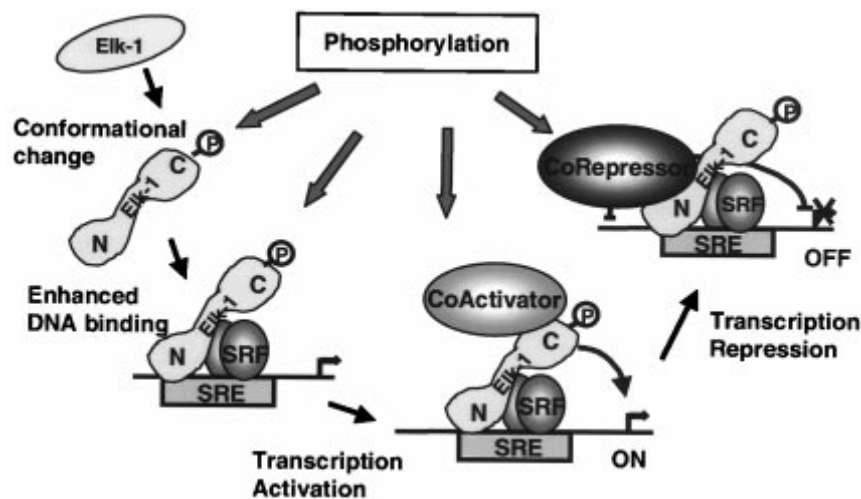

Fig. 25. Role of phosphorylation in Elk-1 regulation

4. AIM OF RESEARCH

The research project conducted during these years, is focused on the effects evoked by drugs of abuse in the central nervous system (SNC).

Within this context, our attention has focused on the study of phenomena associated with the consumption of alcohol and heroin.

Alcohol is the drug of abuse certainly more socially accepted and alcoholism, a condition that occurs following chronic use of alcohol is the cause of many deaths, just think that in Italy every year there are approximately 50,000 new alcoholics and 40,000 deaths related to alcohol. Recent data from the Italian Institute of Health indicate that about 1 and a half million young people (aged between 11 and 24 years) are at high risk of alcoholism.

Although alcohol has long been considered an unspecific pharmacological agent, recent studies of molecular pharmacology have shown that acts on different primary targets, including ion channels and receptors, kicking off a cascade of synaptic events involving many neurotransmitter systems. However, it should be noted that prolonged use at high doses induces alterations on all systems and thus becomes difficult to define which of these is the most involved.

Despite considerable progress in explaining the contribution of the susceptibility factors to the development of alcohol dependence, the exact mechanism of this phenomenon remains unclear. In particular there are two big questions to be answered:

- what are the genetic and environmental factors that cause the initiation and maintenance of alcohol consumption
- what are the changes in the brain behind the transition from control to a compulsive use

Genetically, we can determine non-specific susceptibility factors that increase the risk of developing alcohol dependence, including contemporary psychiatric disorders and depressive disorders more serious, while on a personal level or passive dependence and antisocial impulsive behavior may lead to different individual responses (Cloninger, 1987).

These factors have been proposed to reflect the hypothesis of differences in neurotransmitter systems of the brain, which in turn can influence the

pharmacodynamics of alcohol, and also determine, minimally, an individual variability in search of substance.

A large number of studies suggest that the endogenous opioid system appears to be an important target for the mediation of the mechanisms associated with alcohol consumption (Koob *et al.,* 1998).

Through studies conducted in recent years on gene expression, it has been shown that the classical opioid receptors are differently involved in the consumption of ethanol (Roberts *et al.,* 2001) and, furthermore, the system nociceptin / NOP, recently included in the family of endogenous opioid system, and both appear able to play a key role in the initiation of alcohol use in rodents (Ciccocioppo *et al.,* 2004).

In particular, naltrexone, known opioid antagonist, if taken daily in alcohol abusers, following a period of detoxification, can reduce the frequency of relapse in some groups of patients (Volpicelli *et al.,* 1992) because it is able to revert the increased release Dopamine in the NA (Gonzales *and* Weiss, 1998).

Several studies have also shown that nociceptin exhibits high structural similarity with the DYN and can interact with a G protein coupled receptor, NOP, whose sequence is very similar to that of the KOP. Despite the high degree of similarity between these two systems, they appear to exert opposing physiological functions, in particular, there is a real functional antagonism of the nociceptin system against the endogenous opioid system (Reinscheid *et al.,* 1998).

Genetic studies have shown the presence of polymorphisms in genes coding for KOP and for the precursor of its ligand, the PDYN, which is associated with the risk of developing alcohol dependence (Xuei *et al.,* 2006).

It was also observed that variations in genes coding for the nociceptin system, for NOP receptor and for the precursor of its ligand, the pronociceptin/Orphanin FQ (PNOC), are associated with alcoholism (Xuei *et al.,* 2008).

What emerges is that manipulation of the opioid system, nociceptin in particular, may be useful in the treatment of addictions and there are several evidences that support the use of this strategy (Sakoori *and* Murphy, 2004; Kotlinska *et al.,* 2003).

Based on these findings, the primary aim was to evaluate the effects on gene expression induced by ethanol precursors of opioid peptides (PNOC and PDYN) and their respective receptors in different regions of the brain associated with addiction and to understand the physiological function of N / OFQ in endogenous reward and the development of addiction.

Our interest has focused in particular on the analysis of the PFCx where there is a high density of opioid receptors (Mansour *et al.,* 1987).

The PFCx (Fuster, 2001; Miller *and* Cohen, 2001) is a region of considerable interest in opiate addiction because it plays an important role in cognitive control over drug intake (Moghaddam *and* Homayoun, 2008), and also because it is directly related with the meso-corticolimbic dopaminergic system that mediates the rewarding and addictive properties of opiates (Volkow *et al.,* 2003; Everitt *et al.,* 2007; Schoenbaum *and* Shaham, 2008). The PFCx is involved in the processes of response to sensory stimuli (Devinsky *et al.,* 1995) and alcoholism is also often accompanied by signs of dysfunction in charge of PFCx (Lyvers, 2000).

Based on these results we have continued studies of gene expression to evaluate if the same experimental conditions could lead other modifications in the AM area, an important substrate for neuronal behavior associated with the consumption of alcohol and anxiety (Fadda *and* Rossetti, 1998; McBride, 2002).

In fact, anxiety is one of the first symptoms linked to the abstinence and it's also an important factor in negative reinforcement, which leads to excessive consumption of alcohol (Weiss *et al.,* 2001; Pandey, 2003).

Recently, an increasing number of experimental evidence suggest that changes in gene expression induced by drugs of abuse may be mediated by epigenetic mechanisms (Renthal *and* Nestler, 2008). There are two types of chromatin modifications involved in the regulation of gene transcription: histone modifications and DNA methylation.

Other mechanisms such as RNA interference and prion proteins may contribute to epigenetic regulation. Chromatin is the form in which nucleic acids are organized in the nucleus of the eukaryotic cell and is composed of DNA, RNA and proteins. It has a particularly dynamic structure, responds to extracellular signals and controls gene expression (Kouzarides, 2007; Bernstein *et al.,* 2005).

The histone modifications are posttranslational events and occur on the N-and C-tails which protruding from the surface of the chromatin polymer. They include acetylation, phosphorylation, methylation and ADP-ribosylation.

These changes end up altering chromatin structure by influencing histone-DNA and histone-histone contacts.

In contrast to the transient nature of phosphorylation and acetylation, methylation of histones seems to be a relatively stable signature for the long-term maintenance of

the state of chromatin (Schubeler *and* Elgin, 2005). Increasing evidences on the relationships between changes in methylation of lysine on histone 3, and alteration of gene expression are evident (Boggs *et al.,* 2002).

Epigenetic mechanisms seem to play a role in the cellular effects induced by EtOH (Shukla *and* Aroor, 2006) and, recently, the chromatin remodeling has been reported as a plausible mechanism for alcoholism (Pandey *et al.,* 2008).

With this purpose, studies have been performed using epigenetic Chromatin Immuno Precipitation assays (ChIP) in order to observe different changes in the promoter regions of several target genes, related to the alterations in gene expression, evoked by alcohol exposure.

Moreover, many findings suggest that hypo-methylation of the DNA surrounding the promoter region is a prerequisite for gene activation, and that a high methylation status may mead to the loss of transcriptional activity (Hsieh, 1994; Furuta *et al.,* 2008).

In the second part of the present study, I also investigated alterations in signaling molecules directly associated with MAPK pathway in a unique collection of post-mortem brains from heroin abusers.

The reinforcing effects of opiate drugs, like heroin, are mediated by opiate receptors in the VTA (Bozarth *and* Wise, 1981), NA (Olds, 1982), and hippocampus (Stevens *et al.,* 1991) through both DA-dependent and, independent mechanisms. Following chronic heroin self-administration, neuro-adaptations in mesolimbic DA neurons and their target regions are thought to play a central role in the maintenance of opiate addiction (Koob *and* Le Moal, 2001; Nestler *and* Aghajanian, 1997; Self *and* Nestler, 1995).

In this context, chronic morphine exposure in rats resulted in structural changes of neurons compatible with the induction of synaptic plasticity (Sklair-Tavron *et al.,* 1996; Robinson *and* Kolb, 1999; Spiga *et al.,* 2003; Liao *et al.,* 2005; Ballesteros-Yáñez *et al.,* 2007).

Although some signaling pathways have been associated with morphine-induced changes in neuronal size, synaptic connectivity and behavioral plasticity, increasing evidences indicate that proteins of the apoptotic pathways can also play relevant roles in promoting various forms of synaptic plasticity (Gilman *and* Mattson, 2002; Mattson *and* Gleichmann, 2005; Mattson, 2007).

Given that opiate receptors are coupled to inhibitory G proteins that reduce cyclic AMP formation (Childers, 1991), a compensatory up-regulation in cyclic AMP-dependent protein kinase (PKA) has been reported in the NA following chronic heroin self-administration (Self *et al.,* 1995). Increased PKA activity in the NA causes elevated drug and alcohol intake (Self *et al.,* 1998; Wand *et al.,* 2001) and exacerbates the aversive aspects of opiate withdrawal.

Several studies have implicated ERK1/2 and p38 MAPKs in the modulation of various forms of synaptic plasticity (Derkinderen *et al.,* 1999; Thomas *and* Huganir, 2004), including the neuroplastic changes induced by drugs of abuse (Girault *et al.,* 2007).

In addition to PKA-mediated protein phosphorylation, extracellular signal-regulated kinase (ERK) activity may also be regulated after heroin exposure. All ERKs are activated by a double phosphorylation at residue tyrosine and threonine regulators and among their molecular targets we find ELK1, the best members studied of the TCFs. We already know that TCFs are direct targets of the MAP kinases, and Elk-1 was the first TCF shown to be regulated by ERKs (Hill *et al.,* 1993; Marais *et al.,* 1993). Previous studies have shown that amphetamine activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) resulting in cAMP response element-binding protein (CREB) and Elk-1 phosphorylation in striatal neurons (Choe *and* Wang, 2002).

On basis of these findings, my interest is focused on understanding the effects that prolonged exposure of heroin can cause in an individual, over the entire MAPK cascade and consequently on the transcription factor ELK1, which is regulated by this pathway.

3. MATERIALS AND METHODS

3.1 Ethanol studies in animals model

3.1.1 Animals and Treatments

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National (Ministry of Health) laws and policies (authorization no. 204/2008-B). Carewas taken to minimize the number of experimental animals and to take measures to minimize their suffering.

Adult male SpragueDawley rats (Harlan, Udine, Italy) weighing 330–360 g at the beginning of the experiment were housed two per cage in standard Macrolon cages (Tecniplast Gazzada, Buguggiade, Italy) in a temperature- and humidity-controlled room with a constant 12-hour light/dark cycle (lights on at 7am).The rats were allowed to acclimatize for at least 1 week before the start of the experiments.

Free access to standard lab chow and tap water were available ad libitum. Alcohol or water was administered intragastrically by oral gavage (Figure 26) by using a slightly bent stainless steel feeding needle. No restraint was used. The procedure was finished within 10 seconds.
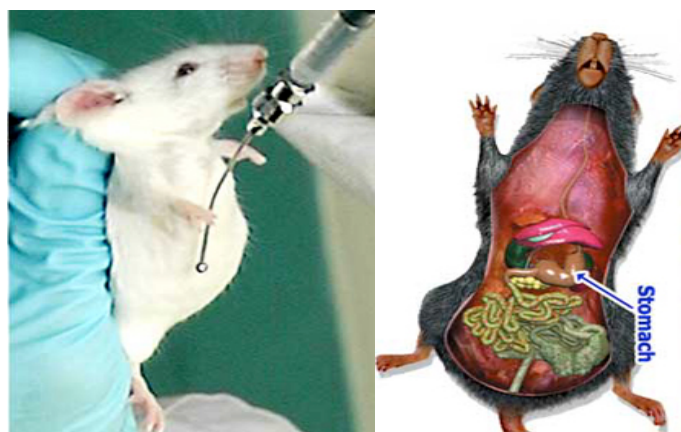
Fig. 26. Procedure for Oral Gavage

Preliminary results from our laboratory showed that a single intragastric administration of 20% EtOH at a dose of 3 g/kg induced a long lasting loss of righting reflex (LORR) and eventually coma.

This dose induced an up-regulation of PDYN and PNOC gene expression in the AM dissected 30 minutes after the treatment; whereas no changes were observed after 2 hours, the other timepoint investigated. Moreover, a low dose of EtOH (0.75 g/kg) at both the timepoints (30 minutes and 2 hours) did not induce any changes in opioid genes expression (data not shown).

On the basis of these results and previous report (Zhou *et al.*, 2000), a binge alcohol paradigm was chosen to more closely resemble human drinking behavior with several hours of heavy alcohol exposure by the oral route.

Hence, animals received daily intragastric administrations of alcohol (20% in water) at a dose of 1.5 g/kg or equal volume of water in their home cages following this binge pattern regimen: three times daily at 1.5-hour intervals (10:00 am, 11:30 am, and 1:00 pm), for a total daily dose of 4.5 g/kg/day.

Two groups of rats received EtOH (1D group; n = 7) or water (control group; n = 7) for 1 day, were killed by decapitation and brain regions of interest were rapidly dissected and frozen on dry ice 30 minutes after the treatment. This timepoint was chosen based on preliminary studies as mentioned above.

EtOH or water (control) were also administered to other groups of animals (n = 7 for each group: three groups administered with EtOH and three groups with water) for 5 days and, depending on the end-point and based on the behavioral studies described below, they were named as 5D (rats killed 30 minutes after the last EtOH or water administration), 1 day withdrawal (W-1D), 3 days withdrawal (W-3D) or 7 days withdrawal (W-7D) (rats killed 1, 3 or 7 days after the last dose of EtOH or water), in accordance with the behavioural parameters assessed (see Table 2 for synopsis of the experimental design).

Brains were placed into an ice-cold matrix, and then sliced with a razor blade into coronal sections. Slices containing the PFCx (1 mm starting at 3.20 anterior the bregma) or AM (2 mm starting at 1.5 posterior the bregma) were obtained.

Sample-punches of the PFCx (including prelimbic and infralimbic regions) and of the AM (including basolateral complex and central nucleus) were dissected under

stereomicroscope in accordance with rat brain atlas (Paxinos *and* Watson, 1986), frozen immediately on dry ice and stored at -80°C until analysis.

days	dose (g/kg)		Number and timing of p.o. administration	Time point of brain dissection after last p.o. administration	Group
	single	**total/day**	EtOH (20% in water) treatment p.o.		
1	1.5	4.5	3 (10:00, 11:30, 13:00)	30 min	1D
5	1.5	4.5	3 (10:00, 11:30, 13:00)	30 min	5D
5	1.5	4.5	3 (10:00, 11:30, 13:00)	1 day	W-1D
5	1.5	4.5	3 (10:00, 11:30, 13:00)	3 days	W-3D
5	1.5	4.5	3 (10:00, 11:30, 13:00)	7 days	W-7D

ETOH = Ethanol

Table 2. Description of the experimental design showing ETOH doses, treatments and tissue dissection timing for each group of study.

Body weight of rats have been recorded daily (Table 3).

Body Weight (g : mean ± SEM)						
Groups	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
veh	341 ± 6	336 ± 6	333 ± 7	333 ± 7	331 ± 8	329 ± 7
EtOH	343 ± 4	330 ± 4	319 ± 5	308 ± 6	303 ± 8	301 ± 9
	Day 7	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**
veh	330 ± 6	331 ± 8	347 ± 9	352 ± 9	354 ± 9	357 ± 9
EtOH	299 ± 6	297 ± 7	299 ± 9	304 ± 9	309 ± 9	315 ± 8

SEM = standard error of the mean.

Table 3. Bodyweight of rats subjected to 5-day intragastric administrations (three times per day) of water (vehicle) or EtOH, including withdrawal groups (total period 12 days). Results are expressed in grams (mean _ SEM). No significant differences between vehicleand EtOH-treated groups have been detected at any day.

3.1.2 Blood alcohol levels (BALs)

For BALs, a separate set of rats ($n = 7$) was used. Blood was collected from the tail vein (0.5 ml) 30, 60, 120, 210, 300, 420, 540 minutes following a single EtOH administration of 3.0 g/kg and the first EtOH administration of 1.5 g/kg of the cumulative dose (1.5 g/kg three times at 1.5-hour intervals) and immediately frozen. EtOH levels were determined by gas chromatography (Schroeder, 1984). Samples were mixed with an internal standard of n-butanol, incubated for 60 minutes at 40°C and a 1-ml headspace aliquot was injected into an HRGC 5160 Mega series (Carlo Erba, Milan, Italy) containing a Phenomenex Zebron ZB-624
(Phenomenex, Torrance, CA, USA). The analysis was performed isothermally at 150°C with a constant pressure of 0.2 Kg/cm2 of Helium carrier gas. The inlet and detector temperature were kept at 200°C. EtOH peaks were identified and quantitated by comparison with a known standard. The blood EtOH concentrations are expressed in mg/dl.

3.1.3 Behavioral measures

Behavioral observations of intoxication and withdrawal were carried out by two operators independently and in blind fashion.

3.1.3.1 Degree of Alcohol Intoxication

Following the second alcohol or water administration, the degree of alcohol intoxication was assessed every day by using the following rating scale described by Majchrowicz (Majchrowicz, 1975):

0 = **Neutral :** no signs of intoxication.

1 = **Sedation:** reduced muscle tone, dulled appearance and slow locomotor activity, but no impairment of gait or coordination.

2 = **Ataxia 1:** slight gait impairment and slight motor incoordination, but able to elevate abdomen and pelvis.

3 = **Ataxia 2:** clearly impaired staggering gait and impaired motor coordination, some elevation of abdomen and pelvis.

4 = **Ataxia 3:** slowed righting reflex, heavily impaired motor coordination, no elevation of abdomen and pelvis.

5 = **LORR:** unable to right itself when placed on its back, other reflexes still present.

6 = **Coma:** no signs of movement; no response to pain stimuli; no blinking reflex; spontaneous breathing.

3.1.3.2 Measurement of physical signs of alcohol withdrawal

The rats were then observed for 5 minutes 20 hours after the last EtOH administration. At each observation time, rats were assessed simultaneously for the following behavioral conditions: Each parameter was scored from 0 to 5 using a rating scale .

0= Agitation
1= Tail stiffness
2= Abnormal posture
3= Abnorml gait
4= Autonomic hyperactivity
5= Lack of exploratory behavior

3.1.4 Real-Time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

3.1.4.1 Extraction and measurement of total RNA

Total RNA was prepared according to the method previously described (Chomczynski *and* Sacchi, 1987). The RNA was extracted from single tissue samples by homogenizing with TRI Reagent solution (Ambion Inc. Italy), containing phenol and guanidine thiocyanate (Ambion), 1 mL TRI Reagent solution per 50-100 mg tissue. Then, 0.2 ml chloroform/2 ml of homogenate, and centrifuging the suspension at 12,000 x g for 10-15 minute at 4°C, and was transfered the aqueous phase to a fresh tube. A volume of 0.5 ml isopropanol was added, incubated for 15 min at 4°C and the RNA pellet was isolated by centrifugation at 12,000 x g for 25 min at 4°C.

The pellet was washed twice with 75% ethanol, dried under vacuum and then resuspended in 25 µl of Rnase-free water. Total RNA, digested with DNase RNase-free enzyme to eliminate genomic DNA content, was quantified by measurement of absorbance at 260 nm (1OD/ml = 40 µg RNA/ml). The ratio OD260/OD280 > 2 provided an estimate of the purity of the total RNA.

3.1.4.2 Reverse Transcription and PCR

RNA samples were subjected to DNase treatment and converted to cDNA with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) by using random hexamers (0.45 µg of total RNA in a final reaction volume of 20 µl). The cDNAs were subsequently diluted three times.

Relative abundance of each mRNA species was assessed by real-time RT-PCR employing 1 µl of the diluted samples in a final volume of 20 µl using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA).

To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined and the point of early log phase of product accumulation is defined by assigning a fluorescence threshold above background defined as the threshold cycle number or Ct.

Differences in threshold cycle number were used to quantify the relative amount of PCR target contained within each tube. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2-DDCt) for statistical analysis (Pfaffl, 2001; Livak *and* Schmittgen, 2001).

All data were normalized to the endogenous reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Results on RNA were normalized to results obtained on RNA from the control, vehicle treated rats. After PCR, a dissociation curve (melting curve) was constructed in the range of 60 ºC to 95 ºC to evaluate the specificity of the amplification products. The primers used for PCR amplification (Table 2) were designed using Primer 3.

Total RNA was converted to complemetary DNA (cDNA) using 50 U Superscript II Reverse Transcriptase (Invitrogen, Milan, Italy) in 20 μL of buffer containing 0.5 mM deoxynucleotide triphosphates (Invitrogen, Milan, Italy), 40 U RNase inhibitor (Invitrogen, Milan, Italy) and 0.5 μg Oligo (dT) 12-18 Primer (Invitrogen, Milan, Italy) (Table 4).

	FORWARD (5'- 3')	REVERSE (3'- 5')	PRODUCT SIZE
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT	207
BACT	ATCACGATCATGGCCCTCTACTCC	TGGTGGCAGTCTTCATCTTGGTGT	106
KOP	TTGGCTACTGGCATCATCTG	ACACTCTTCAAGCGCAGGAT	177
NOP	AGCTTCTGAAGAGGCTGTGT	GACCTCCCAGTATGGAGCAG	101
PDYN	CCTGTCCTTGTGTTCCCTGT	AGAGGCAGTCAGGGTGAGAA	157
PNOC	TGCAGCACCTGAAGAGAATG	CAACTTCCGGGCTGACTTC	170

BACT = beta-actin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; KOP = kappa opioid receptor; NOP = nociceptin opioid receptor; PDYN = prodynorphin; PNOC = pronociceptin.

Table 4. Primer sequences used for reverse-transcription-polymerase chain reaction

3.1.4.3 Statistical analysis

Intoxication and withdrawal signs data, represented as total score, were analyzed by non-parametrical analysis (Mann–Whitney U-test). EtOH effects on genes expression alterations were statistically asnalyzed using the twotailed Student's t-test. Statistical significance was set at $P < 0.05$.

3.1.5 Epigenetic studies

3.1.5.1 Analysis of histones modification by Chromatin Immunoprecipitation (ChIP) and Real Time PCR

Chromatin was prepared from frozen tissues as previously described with minor modifications (Dahl *and* Collas, 2007): proteins were cross-linked to DNA by addition of formaldehyde at a final concentration of 1% in phosphate buffer saline (PBS) containing a broad-range protease inhibitor cocktail (PIC) (Roche) and butyrate (Sigma), for 8 min at room temperature. The cross-linking reaction was quenched by adding glycine to a final concentration of 0.125 M and incubating for 5 min at room temperature, the sample washed, and lysed thorough resuspension by pipetting in 120 ul of lysis buffer.

The sample was incubated on ice and sonicated for 30 s to shear the DNA to fragments ranging in size from 150 to 700 bp, as analyzed by agarose gel electrophoresis.

The lysate was centrifuged at 12 000 g for 10 min at 4°C and the supernatant transferred into a chilled tube, leaving around 30 ul of buffer with the pellet. Another 30 ul of lysis buffer was added and the tube vortexed. After centrifugation as before, 50 ul of the supernatant was pooled with the first supernatant and sonicated for another 2 X 30 s on ice.

After removing a few µl to serve as "input" DNA, for each immunoprecipitation, 8 µg of chromatin was diluted 10-fold in RIPA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 140 mM NaCl) containing PIC and incubated overnight by rotation with either no

antibody as control or with 1 - 4 μg of antibody, previously coated with Protein A beads (Invitrogen), for 2 hours at 4°C with agitation, against either H3K4me3 (Abcam, ab8580), H3K27me3 (Millipore, 17-622), H3K9Ac (Millipore, 17-658).

The beads and associated immune complexes were washed three times with RIPA buffer and once with Tris-EDTA buffer. The immune complexes were eluted with elution buffer (20 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl) containing proteinase K (50 μg/ml) at 68°C for 2 hours, and DNA was recovered by phenol extraction, ethanol precipitated, and resuspended in 50 μl of sterile water. This procedure has been described in more detail (Dahl *and* Collas, 2007). Thereafter, real-time qPCR (RT-qPCR) quantification of the genomic sequences from regions in the rat PDYN and PNOC proximal promoter associated with the immunoprecipitated proteins were carried out. The primers used for PCR amplification were designed using Primer 3 software (Rozen *and* Skaletsky, 2000):

PDYN (from −348 to −175 bp)
Forward (5′- ctgtctcctcccatctctgc) and the antisense primer: (5′-tagctgctccaggtgatgtg).

PNOC (from −645 to −460 bp)
Forward (5′- cagacagggaggacatggat) and the antisense primer: (5′-ggactgcaaagtgcagacaa).

The relative abundance was assessed by RT-qPCR using iQ SYBR Green Supermix (Bio-Rad) on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research).

To provide precise quantification of the initial target in each PCR reaction, the amplification plot was examined and the point of early log phase of product accumulation defined by assigning a fluorescence threshold above background, defined as the threshold cycle number or Ct.

Differences in threshold cycle number were used to quantify the relative amount of the PCR targets contained within each tube. After PCR, a dissociation curve (melting curve) was constructed in the range of 60 ºC to 95 ºC (22) to evaluate the specificity

of the amplification products. The relative expression of different transcripts was calculated by the delta-delta Ct (DDCt) method and converted to relative expression ratio (2^{-DDCt}) for statistical analysis (Livak *and* Schmittgen, 2001).

All ChIP data were normalized to the input DNA amounts (Ct values of immunoprecipitated samples were normalised to Ct values obtained from 'input'). In addition, results on DNA from treated samples were normalized to results obtained on DNA from the control sample. Each ChIP experiment was repeated at least three times.

3.1.5.2 DNA methylation

In order to assess the possible methylation status of the promoter region of studied genes in the rats treated with alcohol was performed:

• DNA extraction
• Bisulfite treatment of extracted DNA
• Analysis of treated DNA by Real-Time PCR

DNA extraction and purification

DNA was extracted from samples of rat brain, which have been moved at <-20 ° C for several days before extraction to facilitate lysis leukocytes. FlexiGene (Quiagen) was used to extract genomic DNA following manufacterer's instuctions which main steps are mentioned below.

1. To FG1 Buffer (lysis), contained in 50 ml falcon tube, add 7 ml of whole blood and vortex to mix well.
2. Centrifuge 2500 rpm for 30 min at 10°C. Centrifugation separate proteins (and other macromolecules and sub cellular structures) according they dimensions (and form) through generation of gravitational forces within a sample tube.

3. Surnatant is discarded and FG2 Protease Buffer (denaturation buffer) is added and inmediatly vortexed, the resulting volume is then divided in 1,5 ml eppendorf.

4. Incubate aliquots at 65° for 10 min: color changes from red to green when proteic digestion verifies.

5. Add isopropanol (100%) and mix until DNA precipitation.

6. Centrifuge 10000 rpm for 10 min at room temperature and discard surnatant.

7. Add ethanol 95 % and vortex 5 seconds.

8. Centrifuge 10000 rpm for 10 min at room temperature and discard surnatant.

9. Dry DNA pellet at room temperature until full diluent evaporation (at least 5 min).

10. Add FG3 Buffer (hydratation buffer), vortex 5 seconds at low speed, resuspend the DNA pellet and incubate for 1 hour at 65°C bath.

Sample DNA amount was determined by spectrophotometry at 260 nm and DNA aliquots were frozen at -20°C.

Sodium Bisulfite treatment

The vast majority of DNA methylation analysis is based on using a PCR using DNA treated with sodium bisulphite as a model. Two different strategies are used in the design of the primers for these reactions:

• Methylation-indipendent PCR primers (MIP)
• Methylation-specific PCR primers (MSP)

Normally, the epigenetic information is lost during the PCR because the DNA polymerase does not distinguish between methylated and non-methylated cytosine, so the polymerase adds a guanine and then a non-methylated cytosine in both situations. After PCR, each originally methylated allele is diluted to a concentration impossible to analyze, so the DNA must be modified in a way that allows methylated information to remain preserved. Treatment with sodium bisulfite, which deaminates cytosine into uracil (Clark *et al.,* 1994) is the method of choice in most laboratories

for this type of analysis. Due the fact that the degree of deamination of 5'-methylcytosine to thymine is much slower than the conversion of cytosine to uracil, it is assumed that the only remaining cytosine after treatment with sodium bisulfite are those derived from 5'metilcitosine. Thus, during the subsequent PCR, the uracil residues are transcribed as cytosine.

The procedure is based on the chemical reaction of single-stranded DNA with sodium bisulfite (HSO3-) at low pH and high temperatures.

The chemical reaction of each step is as follows: cytosine carbon-6 sulfonation, irreversible hydrolytic deamination of carbon-4 that produces a sulfonate uracil, and finally the following desulfonation under alkaline conditions to generate uracil.

Methylation of carbon-5 prevents the carbon-6 sulfonation in the first step reaction. Although the 5-methylcytosine can react with sodium bisulfite, this reaction is extremely slow, and the balance favors the 5-methylcytosine rather than thymine (the deamination product of 5-methylcytosine).

Thus it is important the subsequent necessary purification to remove salts and other reagents used in the process. Treatment with sodium bisulfite converts unmethylated cytosine of the original strand of DNA to uracil, while methylated cytosines remain cytosines. The CpG dinucleotide is the target of methylation in human cells (Figure 27).
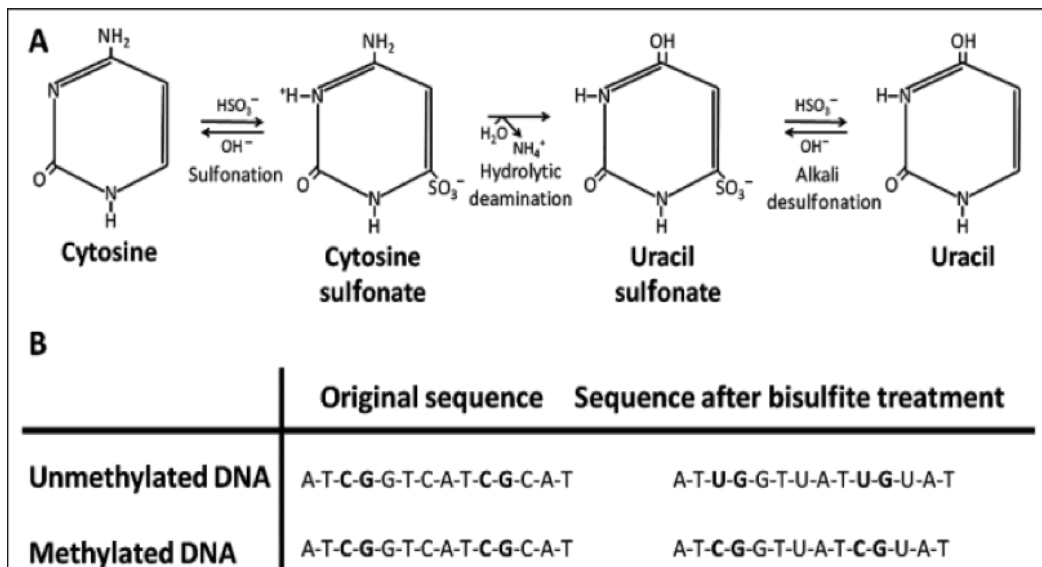
Fig. 27. Sodium Bisulftine treatment of Genomic DNA

The protocol described by Frommer et al 1992 has been widely used for the treatment with sodium bisulfite. When treatment with sodium bisulfite is conducted under appropriate conditions, the expected convertion level of unmethylated cytosines is about 99% (Taylor *et al.,* 2007).

Despite this high level of conversion, however, it is possible that a small amount of DNA have a lower conversion rate (Warnecke *et al.,* 2002) and the distribution of unconverted sites does not be random, so some promoter regions are more prone to an incomplete conversion. The conversion rate depends greatly on the quality of DNA (Warnecke *et a*l., 2002).

This is especially important to keep in mind when looking for low levels of DNA methylation with MSP primers based methods.

The antiparallel strands of DNA are no longer complementary after treatment with sodium bisulfite. Therefore, the MIP and MSP primers are designed to be both Forward and Reverse.

In mammalian DNA, the major base modification is 5-methylcytosine (5-MC), this occurs in 2-5% of all cytosine residues (generally those that are found in CpG doublets). The modification with bisulfite is a reaction between the molecule bisulfite and unmodified cytosine of single-stranded DNA. The reaction converts cytosine into uracil, while methylated cytosine (5-MC) within the CpG sites remains unchanged. The modified DNA can be amplified via PCR to understand the state of methylation.

The bisulfite modification of DNA samples was carried out with the EpiTect bisulfite kit (Zymo) (Figure 28) as described by the manufacturer. For each conversion reaction 1 ug of DNA was used. After conversion, the modified DNA was purified and eluted in 20 ul of TE (2,5 mmol / L EDTA, 10 mmol / L Tris-HCl (pH 8)), then used immediately or stored at -20 ° C for one month. Positive control (100%) and standard curves were produced using universally methylated (uDNA) and methylated (mDNA) DNA.

DNA Input: each treated sample have 500 ng of DNA

Conversion efficiency: >99% of unmethylated Cytosines are converted to Uracil

NA Recovery: >80%

- Add 5uL of M-Dilution Buffer to DNA sample. Add Nuclease-Free water to a final volume of 50μL.

- Incubate 15 min at 37°C, and add 100μL of CT Conversion Reagent to each sample. Incubate for 12 to 16 hours at 50°C.

- At the end of incubation time, transfer each sample to individual separation columns, containing 400μL of M-Binding Buffer. After the first centrifugation, the DNA remains attached to the resin present in the column while the buffer is discarded.

- Add M-Desulphunation Buffer and incubate 15-20 min.

- Add M-Wash Buffer and centrifuge to wash the DNA.

- Add 20 μL of M-Elution Buffer to release DNA from the resin.

Converted DNA can be conserved at -80° for up to three months.
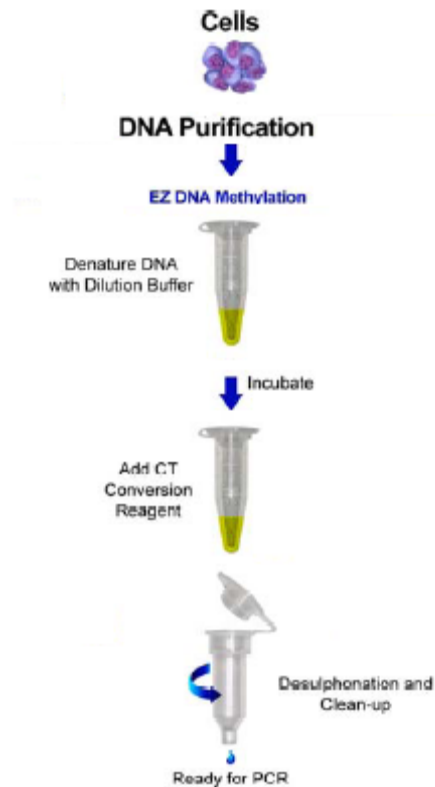
Fig. 28. EZ DNA Methylation procedure (source ZIMO RESEARCH 2010).

MSP primers

The MSP primers are designed to amplify only the methylated DNA thus the possible errors associated with MIP based methods are no longer a problem. This specificity is achieved by including a few CpG sites in the sequence of the primer, preferably at the 3' end (Figure 29) (see Table 5 for MSP primers).

If PCR strict conditions are followed, only the amplification of methylated DNA will occurs.

MSP assays are generally associated with high levels of false positives, especially when using large numbers of PCR cycles, which are often necessary to obtain high-sensitivity analytical assays.

False-priming events (in which the amplification takes place despite the mismatch between primer and sample) and not fully converted DNA molecules may be responsible for false-positive results.

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The events of false-priming can be detected through the use of an appropriate negative control and prevented by limiting the number of cycles and using higher annealing temperatures.

The present study analyzes four genes PDYN, PNOC and BACT. We also used MYOD noCpG. Of each of these genes we create their MSP primers in order to perform MSP Real-Time PCR.

After retrieving the correct sequence of special databases of the gene of interest, we look upstream in the promoter region for CpG rich areas (CpG Island). Several pairs of suitable sequences are tested (forward / reverse primers).
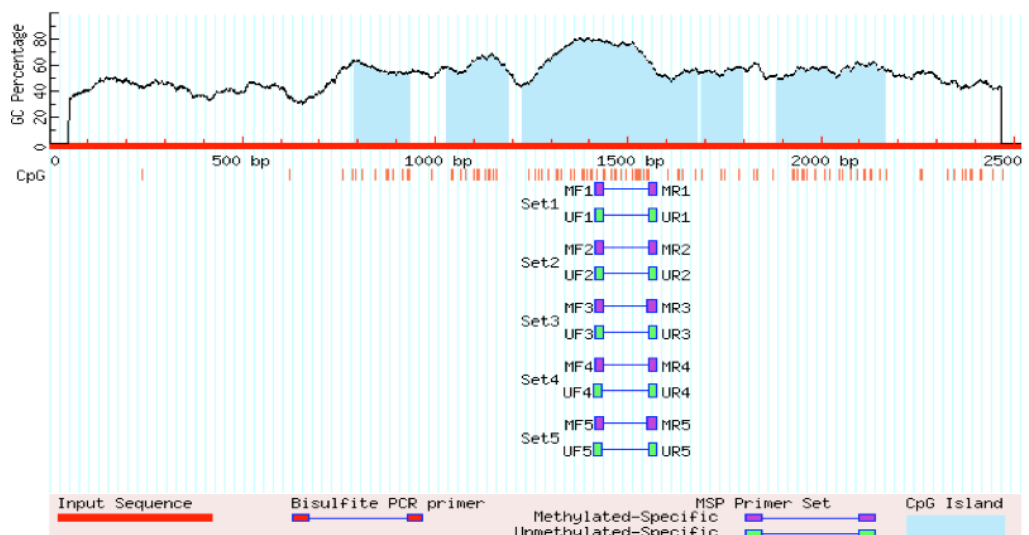
Fig. 29. Graphic representation of CpG rich areas (CpG Island) within gene promoter region.

MSP primers	
PDYN:	
Forward	5'- TTTTATAGTTTAATTACGTAAAGTCGT -3'
Reverse	5'- AACTCATTACATACCTCATACAACCG -3'
PNOC:	
Forward	5'- TTTTTGAAATTTAAGGGTTTATTTC -3'
Reverse	5'- AATTTACTACACTCCAATAACYGAT -3'
BACT:	
Forward	5'- TTTTGAATTTGGGGGTGTGT -3'
Reverse	5'- AAAACCAAACCAACTTCCTTATTCT -3'

Table 5. Sequence of MSP primers used for MSP RT-PCR.

MSP Real-Time PCR

For the methylation PCR study, a SYBR Green PCR kit was used. Each 20 ul of PCR reaction contains: 2 ul of eluted bisulfite modified DNA, 10 ul of SYBR Green PCR Master Mix, 1 ul of each of the two primers (concentrations used vary from 10 to 100 pmol / ul resulting in final concentrations of 0.3 to 3 uM), and 6 ul of DNase-free water.

To normalize for the amount of input DNA, we have chosen a pair of primers corresponding to a specific sequence of MyoD gene. Real-time PCR conditions were 95 ° C for 15 minutes followed by 45 cycles of 94 ° C for 15 s, 60 ° C for 30 s, 72 ° C for 30 s with data acquisition after each cycle. At the end, the amplification products will be verified by melting curve analysis: 95 ° C for 1 min, 55 ° C for 1 min, followed by 80 cycles of increasing incubation temperature for 10 s each, from 55 ° C to up to 95 ° C (0.5 °C increase) with data acquisition after each cycle. Two replicates for each sample were used and PCR was performed in a DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research, Waltham, MA, USA).

Ct values of each sample were recorded. Methylation percentage was calculated by 2^(-DDCT), where DDCT = (Ct Target - Ct, MyoD) sample - (Ct Target - Ct, MyoD) fully methylated DNA and multiplied by 100.

A calibration curve using the fully methylated DNA was carried out in parallel with each analysis, providing additional confirmation for each sample as methylation ratio, defined as the ratio of the fluorescence emission intensity values of target PCR product respect those of Myod PCR products.

The correct length and purity of PCR products were verified by agarose gel electrophoresis (1,5% agarose).

3.5.1.3 Statistical Analysis

EtOH effects on histone modification and DNA methylation alterations were statistically analyzed using the two-tailed Student's t- test. Statistical significance was set at $p<0.05$.

3.2 Heroine studies in human abusers

3.2.1 Reagents

The antibody directed against the MOR was obtained from GeneTex, Inc. (Irvine, CA). The antibody directed against βarrestin1 was obtained from Epitomics, Inc. (Burlingame, CA). Antibodies directed against phospho-MOR, ERK1/2, phospho-ERK1/2, Elk-1 and phospho-Elk-1 were all obtained from Cell Signaling Technology, Inc. (Danvers, MA).

The antibody directed against GAPDH was obtained from Millipore (Billerica, MA). The antibody directed against MEK1 was obtained from Invitrogen Corporation (Carlsbad, CA). Antibodies directed against β arrestin2 and MEK2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

3.2.2 Methods

3.2.2.1 Human Brain Specimens

Postmortem brains were obtained from either the Forensic Medicine Department of Semmelweis University (Budapest, Hungary) or from the Forensic Medicine Department of Karolinska Institutet (Stockholm, Sweden) under approved ethical guidelines. All cases were from Caucasians and had a postmortem interval < 24 hrs. The subjects were sorted into two case groups: those that died from heroin overdose (heroin group) and normal control subjects without head trauma (control group).

All of the subjects were evaluated for common drugs, including alcohol, barbiturates, benzodiazepines and phenycyclidine.

A total of four subjects tested positive for ethanol toxicity, three in the heroin and one in the control group, but none of these subjects showed signs of chronic alcohol abuse. Most of the subjects in the heroin group had a prior history of heroin abuse and also had physical signs of abuse such as needle track marks at time of autopsy. Furthermore, these subjects also exhibited a positive toxicology for heroin and/or its

metabolites, such as 6-monoacetylmorphine (6-MAM), morphine and morphine glucuronide. In contrast, the subjects in the control group had negative toxicology for opiates.

The heroin subjects used in this study were predominantly heroin users that had no history of methadone or buprenorphine clinical treatment and were negative for human immunodeficiency virus (HIV) infection.

These subjects therefore represent a unique population of drug abusers that can be utilized to study the long-term effects of heroin use on the biochemical and molecular alterations that occur in the human brain.

3.2.2.2 Brain Punches

The tissue (average weight of 200 mg) was punched from the putamen of 48 postmortem human brains and kept at -80°C. The tissue punches were pulverized into a fine powder using a Bio-pulverizer (Biospec Products Inc.) on dry ice, mixed and then aliquoted into eppendorf tubes that were kept at -80°C until either protein was isolated.

3.2.2.3 Western Blotting

For each brain, between 10-20 mg of pulverized human putamen brain was homogenized on ice in modified RIPA buffer (1% IGEPAL CA-630, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) with 5 mM DTT, 1 μg/ml leupeptin, complete protease inhibitor cocktail with EDTA (Roche) and Halt™ phosphatase inhibitor cocktail (Thermo Scientific) using a dounce homogenizer (Kimble-Kontes), incubated at 4°C for 20 min and then centrifuged at 15,000 g for 15 min at 4°C. The protein concentrations of the supernatants were determined by using the BCA protein assay (Thermo Scientific).

An initial test gel was performed for each of the antibodies utilized in this study to confirm that there was a linear relationship between the amount of protein loaded onto the gel and the detection of that protein. A total of approximately 12μg was

resuspended in Laemmli buffer and analyzed on SDS-PAGE gels (Bio-Rad) and transferred to Protran® nitrocellulose membranes (Whatman).

The membranes were stained for protein using Memcode (Thermo Scientific), scanned and then blocked overnight at 4°C using 50% Odyssey blocking buffer (Li-COR) diluted in PBS with gentle shaking.

Primary antibodies were diluted in Odyssey blocking buffer with 0.1% Tween-20 and incubated overnight at 4°C with gentle shaking. The membranes were washed six times, 5 min each, in PBS with 0.1% Tween-20 (PBST) at room temperature with gentle shaking. Secondary antibodies of either goat anti-rabbit or goat anti-mouse IRDye 680CW or IRDye 800CW (Li-COR) were diluted 1:5000 in Odyssey buffer with 0.1% Tween-20 and 0.01% SDS and incubated for 1 hour at room temperature with constant shaking.

The membranes were washed another six times, 5 mins each, in PBST. Membranes were imaged using the Odyssey infrared imaging machine and the blots analyzed using ImageJ software.

3.2.2.4 Statistical Analysis

Values derived from densitometric analysis using ImageJ software were normalized to total protein levels as determined by staining nitrocellulose membranes with Memcode protein stain. Data for the heroin subjects were calculated with respect to control subjects (100%). In order to determine normal distribution of the data, a Shapiro-Wilk W test was performed.

Either a square root or logarithmic transformation was performed in order to render non-normal distributions into normally distributed data.

A general linear stepwise regression analysis was used to calculate statistical significance and to identify possible covariates. Variables included in the analysis were: age, sex, PMI, brain pH, ethanol toxicity and storage time. A Student's t-test was used when no covariates were found. Spearman's correlation coefficients were calculated to assess the relationship between protein levels and also to identify correlations with toxicology. Statistical tests were carried out using JMP (v 7.0.1; SAS Institute Inc.) and all results are expressed as mean ± SEM.

4. RESULTS

4.1 Ethanol results in animals model

4.1.1 Estimation of BALs

Single or repeated EtOH binge intoxication induced BALs in the range of 350–450 mg/dl and thus was clearly pharmacologically active. During binge treatment, the highest BAL (450 mg/dl) was reached after 30 minutes following the third administration and decreased to the level 120 mg/ml after 9 hours. A single dose caused a BAL of 350 mg/dl 1 hour after the administration and decreased to non-pharmacologically level after 9 hours (Figure 30) (D'Addario *et al.,* 2011b).
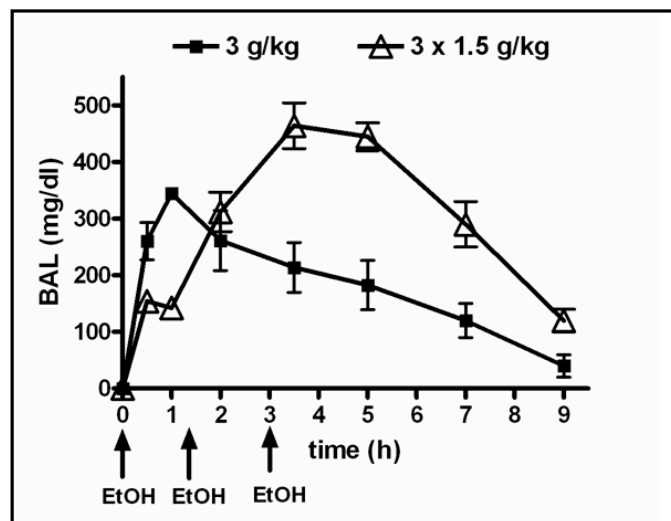

Fig. 30. Blood alcohol levels (BAL; mg/dl; mean_standard error of the mean) observed in the rat after intragastric single (3 g/kg) or repeated (3 x 1.5 g/kg) EtOH administration.

4.1.2 Behavioral changes during EtOH intoxication and withdrawal

4.1.2.1 Intoxication rating

EtOH-induced intoxication in animals starting from the first binge administration (score: 2.7 ± 0.35 overall; Mann–Withney $U = 0$, $P < 0.0002$; Figure 31) and reached the maximum peak at Day 2 (score: 4.0 ± 0.27 overall; $U = 0$, $P < 0.0002$; Figure 31). Intoxication signs decreased over Day 3 (rate: 3.27 ± 0.4 overall; $U = 0$, $P < 0.0002$; Figure 31) and 4 (score: 2.3 ± 0.3 overall; $U = 6$, $P < 0.01$; Figure 31) and disappeared over Day 5 (score: 0.4 ± 0.2 overall; $U = 13$, $P = 0.07$; Figure 31). Control animals showed no signs of intoxication in all days (score: 0.45 ± 0.09 overall) (D'Addario *et al.,* 2011b).
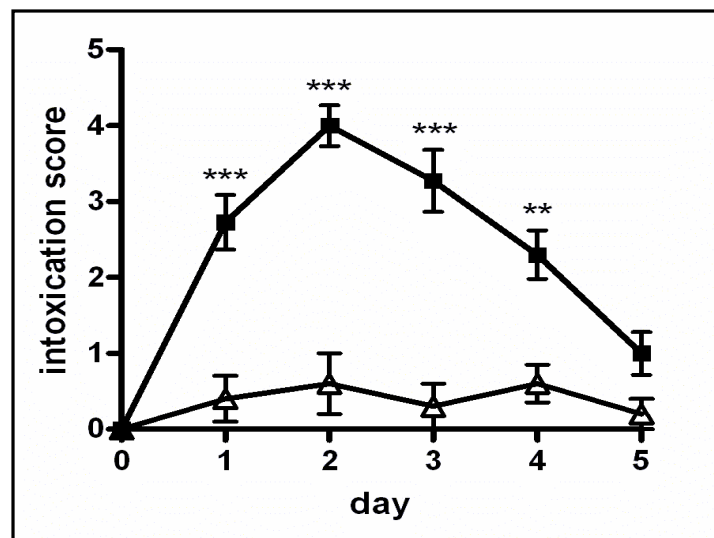
Fig. 31. Intoxication score in ethanol administered rats. Intoxication rating, assessed every day following the second alcohol or water (veh) administration, is shown across the 5 days of binge-like EtOH administration. Mann–Withney U test ***P < 0.01* and ****P < 0.001* versus control group.

4.1.2.2 Signs of alcohol withdrawal

EtOH-treated rats showed significant withdrawal signs (compared with vehicle-treated rats), measured 20 hours after each final daily EtOH treatment (Figure 32). The sum of the observation scores [agitation, tail stiffness, abnormal posture, abnormal gait, autonomic, hyper-reactivity, no rearing (Uzbay *et al.,* 1997)] progressively increased from 0 on Day 1 to 12.5 ± 0.4 on Day 6 (Mann–Withney U = 0, Z = 3.3, P < 0.001; Figure 32), with significant differences between the experimental groups on Day 4 (U = 6, Z = 2.3, P < 0.02), Day 5 (U = 0, Z = 2.4, P = 0.02), Day 7 (U = 0, Z = 2.6, P < 0.006), Day 8 (U = 0, Z = 2.7, P < 0.006), Day 9 (U = 0, Z = 2.4, P < 0.01), Day 10 (U = 0, Z = 2.6, P < 0.009) confirming the presence of significant overall withdrawal severity. Body weight of rats subjected to intragastric administrations was recorded daily up to Day 12 (W-7D group). A slight weight reduction in both vehicle- and EtOH-treated groups has been observed every day up to Day 8, recovering thereafter until the end of the experiment (Day 12). No significant differences were observed between the experimental groups (D'Addario *et al.,* 2011b).
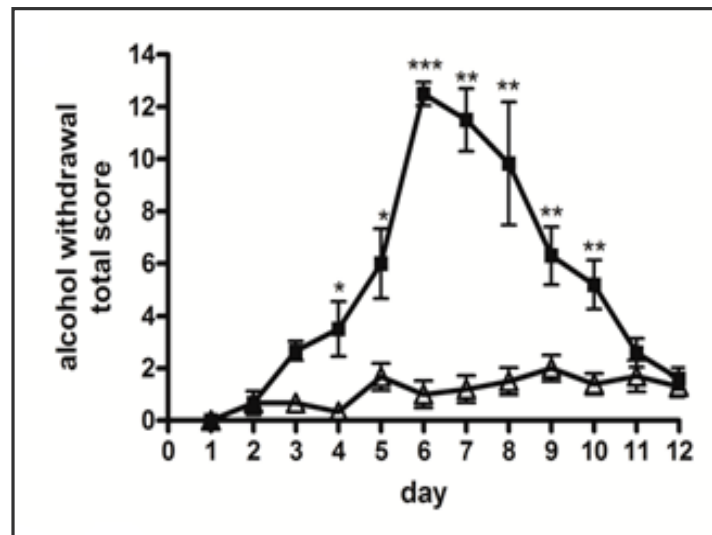
Fig. 32. Withdrawal score in ethanol administered rats. Alcohol withdrawal total score evaluated 20 hours after the last administration of EtOH or water and up to 12 days. Mann–Withney U tests confirmed that withdrawal signs occurred in EtOH treated rats starting on Day 4 up to Day 10 after EtOH binge administration. *P < 0.02 and **P < 0.01 versus control group.

4.1.3 Gene expression studies

4.1.3.1 PDYN/KOP System

In the AM, gene expression studies revealed a significant increase in the levels of PDYN in the group of animals treated for 1 day (1D group) (3.194 ± 0.865 versus control group equal to 1, unpaired t-test: **P < 0.01; F(6, 6) = 2.430) returning to values comparable with control in the 5D group, and showing again an up-regulation in the W-1D group [1.7 ± 0.07, unpaired t-test: *P < 0.05; F(5, 4) = 7.061] (Figure 33a) (D'Addario *et al.,* 2011b).

a) AM
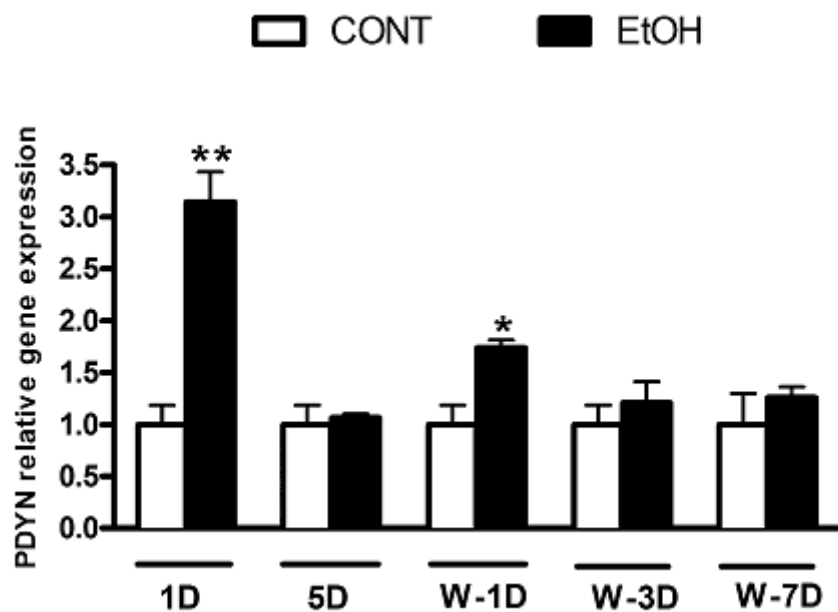
Fig. 33a. Levels of PDYN mRNA in the AM of rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 1 day (1D group) or 5 days (W-1D,W-3D,W-7D groups) in comparison with their respective control groups (CONT), see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of seven rats for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to control rats_standard error of the mean. Differences among treatments were estimated by *t*-test. *P < 0.05 and **P < 0.01 versus control group.

In the PFCx after 1 day of treatment with EtOH, PDYN mRNA was increased [1D group: 1.760 ± 0.262, unpaired t-test: $*P < 0.05$; $F(5, 6) = 4.248$], whereas no changes were evident in the other groups (Figure 33b) (D'Addario *et al.*, 2011b).

b) PFCx
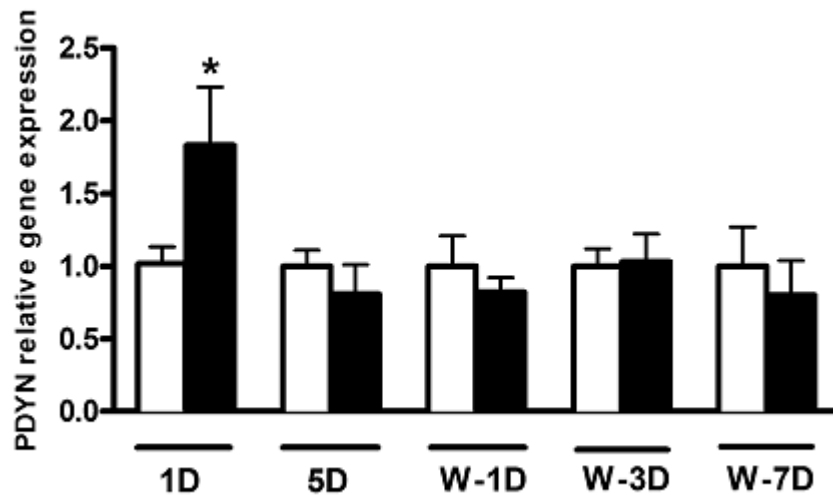
Fig. 33b. Levels of PDYN mRNA in the PFCx of rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 1 day (1D group) or 5 days (W-1D,W-3D,W-7D groups) in comparison with their respective control groups (CONT), see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of seven rats for each group. Expression was normalized to glyceraldehyde-3-phosphate

dehydrogenase and means of mRNA levels are expressed relative to control rats_standard error of the mean. Differences among treatments were estimated by *t*-test. $*P < 0.05$ and $**P < 0.01$ versus control group.

Levels of the KOP mRNA transcript in the AM show an increase after 5 days of treatments [1.71 ± 0.16, unpaired t-test: *P < 0.05; F(6,6) = 1.690] (Table 6). In the PFCx alterations in KOP gene expression were not observed (Table 6) (D'Addario *et al.,* 2011b).

	KOP				
	1D	**5D**	**W-1D**	**W-3D**	**W-7D**
AM	1,04 ± 0,33	1,71 ± 0,16 **	0,83 ± 0,29	1,08 ± 0,26	0,88 ± 0,15
PFCx	0,89 ± 0,16	0,62 ± 0,13	0,6 ± 0,05	0,91 ± 0,13	1,01 ± 0,10

Table 6. Quantitative reverse-transcription-polymerase chain reaction results. Relative KOP gene expression levels in AM and PFCx of rats orally administered with ethanol up to 5 days. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to control animals ± standard error of the mean. ** Indicate a significant difference from control group, P< 0.01 (t-test).

4.1.3.2 PNOC/NOP System

In the AM levels of the PNOC mRNA transcript in the AM were significantly increased in the 1D group [2.298 _ 0.479, unpaired t-test: *P < 0.05; F(6, 6) = 5.325] and continues to increase with the progression of the treatment (5D group) becoming even higher [2.827 ± 0.403, unpaired t-test: **P < 0.01; F(6, 6) = 3.778]. When the animals were kept in abstinence, the levels of peptide mRNA were still significant high in the W-1D group [2.211 ± 0.259, unpaired t-test: *P < 0.05; F(5, 5) = 1.558] (Figure 34a) (D'Addario et al., 2011b).

a) AM
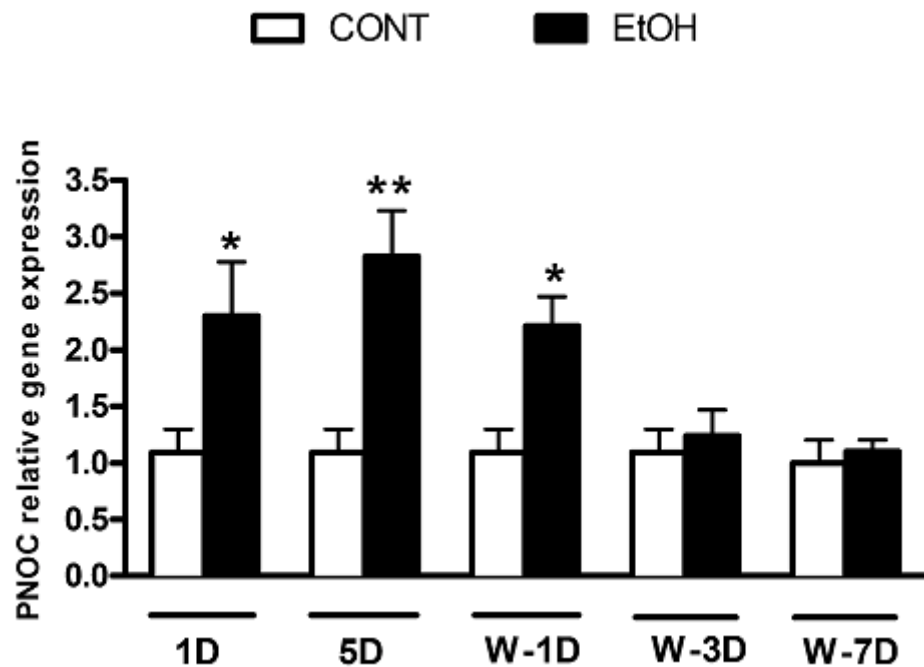
Fig. 34a. Levels of PNOC mRNA in the AM of rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 1 day (1D group) or 5 days (5D,W-1D,W-3D,W-7D groups) in comparison with their respective control groups (CONT), see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of seven rats for each group. Expression was normalized to glyceraldehyde-3-phosphate

dehydrogenase and means of mRNA levels are expressed relative to control rats_standard error of the mean. Differences among treatments were estimated by *t*-test. *P* < 0.05 and **P* < 0.01 versus control group.

Conversely, in the PFCx we did not observe significant changes in the expression of PNOC (Figure 34b) (D'Addario *et al.,* 2011b).

b) PFCx
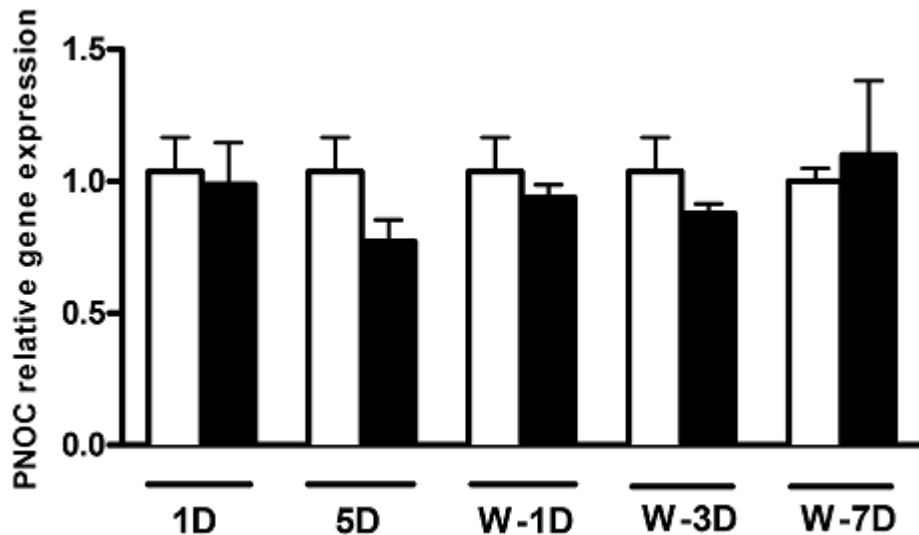
Fig. 34b. Levels of PNOC mRNA in PFCx of rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 1 day (1D group) or 5 days (5D,W-1D,W-3D,W-7D groups) in comparison with their respective control groups (CONT), see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of seven rats for each group. Expression was normalized to glyceraldehyde-3-phosphate
dehydrogenase and means of mRNA levels are expressed relative to control rats_standard error of the mean. Differences among treatments were estimated by *t*-test. *$P < 0.05$ and **$P < 0.01$ versus control group.

Moreover, no changes in NOP mRNA were detected in both the AM and PFCx (Table 7) (D'Addario *et al.,* 2011b).

	NOP				
	1D	5D	W-1D	W-3D	W-7D
AM	1,02 ± 0,18	1,35 ± 0,13	1,32 ± 0,15	0,87 ± 0,19	0,84 ± 0,11
PFCx	1,27 ± 0,11	1,07 ± 0,05	1,06 ± 0,10	1,12 ± 0,12	0,78 ± 0,17

Table 7. Quantitative reverse-transcription-polymerase chain reaction results. Relative NOP gene expression levels in AM and PFCx of rats orally administered with ethanol up to 5 days. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to control animals ± standard error of the mean.
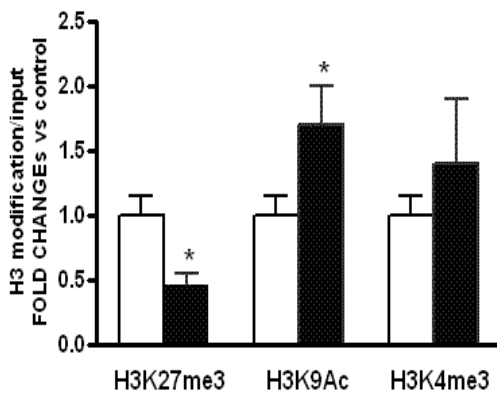
4.1.4 Histone modifications

Sprague Dawley rats have administered with repeated binge EtOH intoxication to investigate whether alcohol could evoke epigenetic changes (histone modifications) in the PDYN and PNOC promoter regions associated with the different previously demonstrated genes up-regulation in the AM complex (Figure 35).

a) Rattus norvegicus prodynorphin gene promoter region (NM_019374.3)

```
                                                        -320
Actttctttctgcctttcgattggactctctacgtctctctatctctctctctgtctcctcccatctctgcctttctcctacttt

tgtctctgttttcctgtgagactctcctggaaatgctcaaagtggccgaatttgaagtgacaaacagcgctacaccgagaatca

ccccggactgcgcgagagcatcctcctccacatcacatcacctggagcgctattgtgtttcactcaggcccagggagttgagt

Ggaaggacccgggggtctgagctctgactgccactcccccattggctcctggcagcctgtgctctgtgagcgagcgctgagcggg

                              TATA box                    +1
ctgagcgacagtggggggagactctcttctataaaaggggagggagtaaggctcacagaGAGTAAGGCTCACAGAACTGCCATAG

GGGGATTTGGTAGCCTTCAAGGCTTCCTCTGTGGCACTTCTCTGAGCTAGCGTCAGGGCTCCTTCTGAATCTTGGATCGGCCATC

CTATCACCTGATCAGCCAGAAGCCTGCCAGCGACAAAGCAGCACGCAG
```

b) Rattus norvegicus pronociceptin gene promoter region (NW_047454.2)

```
                              CRE    -645
Aaacattcaaactatggcagcaactccattctgacatgactgacagacagggaggacatggatgcttcctaggagggagagttga

Gggacagcactcccagccaggccagagggacttgagaggctgagaggaagcactttagttgttcattcacctgtctctccctccc

Gagaaatgagatgagagatggataccaccactcggtgacttgtctgcactttgcagtccttacagctctcacttcagcttccaaa

tgaaagtacatggctacaAa.................................................................................................c

aaactcttccttcccaggtgaacctcagacctgggaatcacctcagggaggaatggtgcttggccctttgtgttgtggtcctcac

                                                         +1
agctgactaaccctctccccagaccgtactgactggcatgtgccccttatgtgtttCAGCCTCTTGCTTCCCACGGCTGCACCAT

GAAAATCCTGTTTTGTGATGTCCTGCTGCTCAGCCTGCTCTCCAGCGTGTTCAGCAGCTGTCCCGAGGACTGCCTCACCTGCCAC

GAGAGGCTCCACCCGGCTCCGGGCAGCTTCAACCTGAAG
```

Fig. 35. Sequences of rattus norvegicus PDYN and PNOC promoter regions. The transcriptional start site (+1) is indicated. The TATA box on PDYN promoter and the CRE sequence on PNOC promoter are also indicated and highlighted. Primer sequences are underlined indicating also the starting positions.

Group 1D

In the AM, we found in the animals treated for 1 day with EtOH (1D group) a significant decrease of H3K27me3 in both PDYN (0,45 ± 0,09 versus CONT group = 1, p< 0,05) and PNOC (0,46 ± 0,11 versus CONT group = 1, p < 0,05) promoter regions as well as a significant increase in H3K9Ac for PDYN promoter (1,7 ± 0,3, p < 0,05) and for PNOC promoter (1,8 ± 0,26, p < 0,05) (Figure 36).

1 D Group

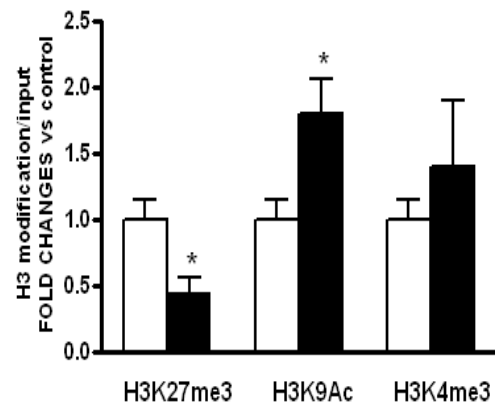**a) PDYN promoter**   **b) PNOC promoter**

Fig. 36. RT-qPCR analyses of H3K27me3, H3K9Ac and H3K4me3 immunoprecipitated DNA fragments at a)PDYN and b)PNOC promoters. ChIP showing the levels of specific histone modification normalized to total input DNA in rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 1 day (1D group, n = 7) or vehicle (cont, n =7). Data are expressed as means ± SE of triplicate independent samples.*P < 0.05 vs. control; t-test.

Group 5D
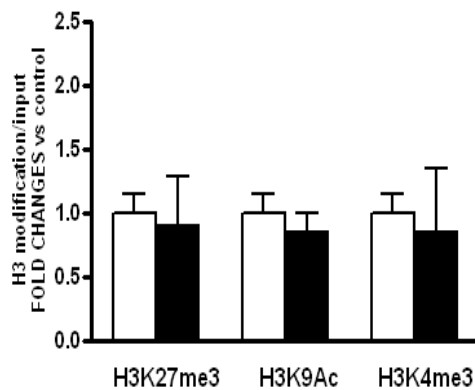
In the AM, we found in the animals treated for 5 days (5D group) a significant increase of H3K9Ac in PNOC promoter (2,4 ± 0,67, p < 0,05) (Figure 37); no significant alteration we found for other histone modification analyzed.
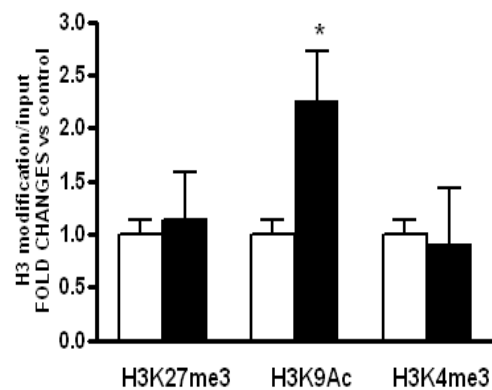
No changes we observed in PDYN promoter region.

5 D Group

a) PDYN promoter   **b) PNOC promoter**

Fig. 37. RT-qPCR analyses of H3K27me3, H3K9Ac and H3K4me3 immunoprecipitated DNA fragments at a) PDYN and b) PNOC promoters. ChIP showing the levels of specific histone modification normalized to total input DNA in rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 5 day (5D group, n = 7) or vehicle (cont, n =7). Data are expressed as means ± SE of triplicate independent samples.*P < 0.05 vs. control; t-test.
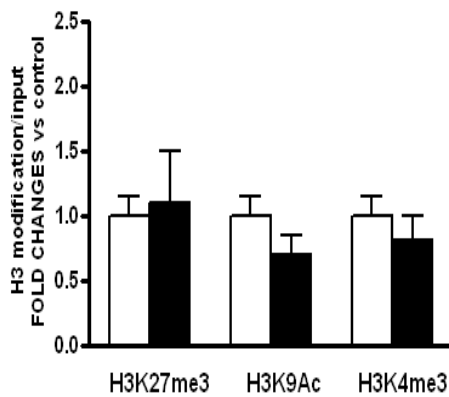
Group W-1D

In the AM, in contrast, we did not observe any histone modifications changes in the W-1D group in both genes promoter. Moreover, EtOH exposure did not induce any statistically significant alteration of H3K27me3, H3K9ac and H3K4me3 (Figure 38).

W-1D Group

a) PDYN promoter   **b) PNOC promoter**
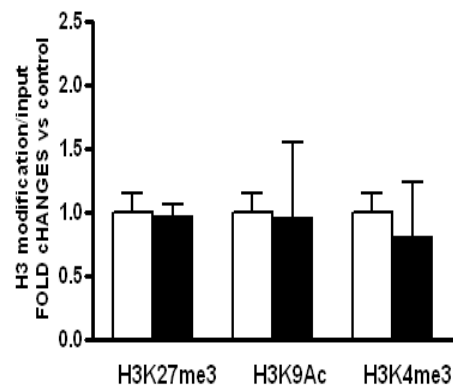
Fig. 38. RT-qPCR analyses of H3K27me3, H3K9Ac and H3K4me3 immunoprecipitated DNA fragments at a) PDYN and b) PNOC promoters. ChIP showing the levels of specific histone modification normalized to total input DNA in rats treated with EtOH intragastrically (total daily dose: 4.5 g/kg/day) for 5 day and 1 day of withdrawal (W-1D group, n = 7) or vehicle (cont, n =7). Data are expressed as means ± SE of triplicate independent samples.

4.1.5 DNA Methylation

Sprague Dawley rats have administered with repeated binge EtOH intoxication to investigate whether alcohol could evoke epigenetic changes (DNA methylation) in the PDYN and PNOC promoter regions associated with the different previously demonstrated genes up-regulation in the AM complex

The analyses of DNA methylation in PDYN and PNOC promoters didn't show a significative alteration (Table 8).

1D	CONTROL	EtOH
PDYN	46 ± 4	44 ± 5
PNOC	56 ± 9	49 ± 9
5D	CONTROL	EtOH
PDYN	41 ± 12	48 ± 18
PNOC	58 ± 6	64 ± 15
W-1D	CONTROL	EtOH
PDYN	39 ± 12	30 ± 14
PNOC	58 ± 22	63 ± 36

Table 8. DNA METHYLATION LEVELS AT GENE PROMOTERS : % of DNA methylation in promoter regions of PDYN and PNOC in rats treated with vehicle or EtOH intragastrically (total day dose : 4.5g/kg/day) for 1 day (1D group) or for 5 days (5D group, W-1D).

4.1 Heroin results in Human abusers

4.1.1 Regulation of MAPK signaling components in the brains of heroin addicts

One of the key intracellular pathways activated by MOR is the MAPK pathway. To determine whether there are perturbations in protein levels of the MAPK pathway as a consequence of chronic heroin abuse, we focused on the core components of the pathway including MEK1/2 and ERK1/2. In the putamen of heroin addicts, the protein expression of MEK1 was significantly reduced (91.28 ± 2.55%, $p < 0.05$; $F_{1,41} = 4.33$; Figure 39a), whereas MEK2 was also lower but not significantly changed (91.43 ± 4.29%; Figure 39b).
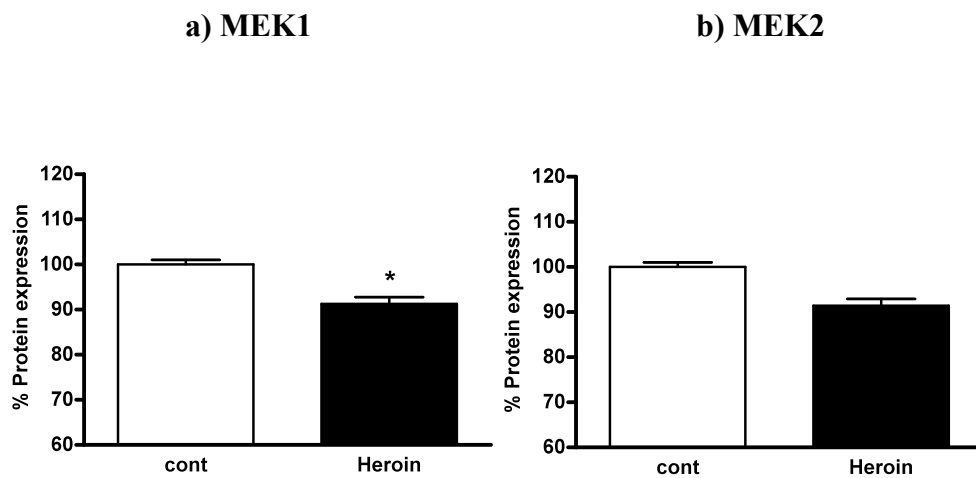
a) MEK1   b) MEK2

Fig. 39. Protein levels of a) MEK1 b) MEK2 in the putamen of control subjects and heroin abusers. Results are expressed as mean ± SEM with respect to control subjects (100%). * P < 0.05.

Of the two known targets of the MEK1/2, we observe a significant decrease in the protein level of ERK1 ($84.78 \pm 3.83\%$, $p < 0.05$; $F_{1,46} = 4.40$; Figure 40a). Conversely, there is a significant increase in the protein levels of ERK2 ($111.78 \pm 2.32\%$. $p < 0.005$; $F_{1,44} = 9.33$; Figure 40b).
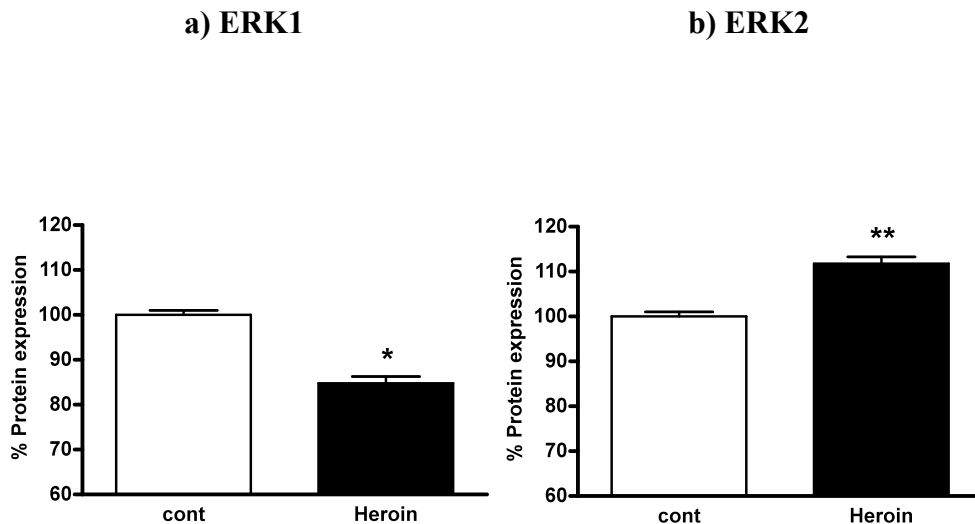
a) ERK1   **b) ERK2**

Fig. 40. Protein levels of a) ERK1 b) ERK2 in the putamen of control subjects and heroin abusers. Results are expressed as mean $\pm$ SEM with respect to control subjects (100%). * P < 0.05, ** P < 0.01.

Dual phosphorylations of the ERKs are required for their full activation and for their ability to mediate a variety of cellular functions, including activation of downstream transcription factors. In the putamen of heroin addicts, the level of phosphorylated ERK1 ($105.67 \pm 13.01\%$; Figure 41a) and phosphorylated ERK2 ($120.1 \pm 11.4\%$; Figure 41b) was not significantly changed when compared with control subjects.

a) pERK1   **b) pERK2**

Fig. 41. Protein levels of a) pERK1 b) pERK2 in the putamen of control subjects and heroin abusers. Results are expressed as mean ± SEM with respect to control subjects (100%).
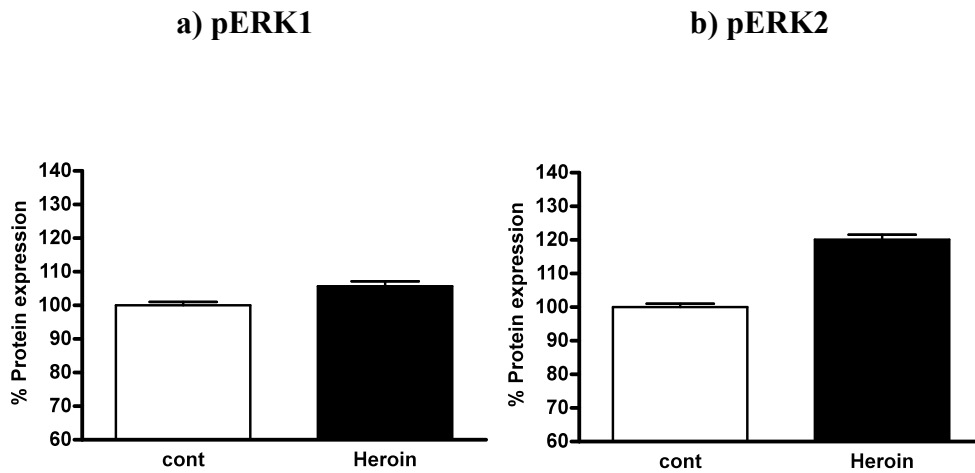
4.1.2 Regulation of Elk-1 in the brain of heroin addicts

The well-characterized transcription factors Ets-like protein-1 (Elk-1) has been implicated in the cellular responses of opioids and is a nuclear targets for activated ERK1/2. Elk-1 is directly phosphorylated by ERK1/2, and once activated forms a ternary complex on serum response elements (SREs) that activate immediate early genes such as c-fos. In the putamen of heroin addicts, the protein expression of Elk-1 was significantly increased ($133.26 \pm 8.21\%$, $p < 0.01$; $F_{1,46} = 7.38$; Figure 42a). However, there is a significant reduction in the level of phosphorylated Elk-1 ($86.92 \pm 3.35\%$, $p < 0.05$; $F_{1,41} = 5.21$; Figure 42b) in the putamen of heroin addicts.

a) ELK1　　　　　　　　　　　　**b) pELK1**

Fig. 42. Protein levels of a) ELK1 b) pELK1 in the putamen of control subjects and heroin abusers. Results are expressed as mean ± SEM with respect to control subjects (100%). * P < 0.05, ** P < 0.01.
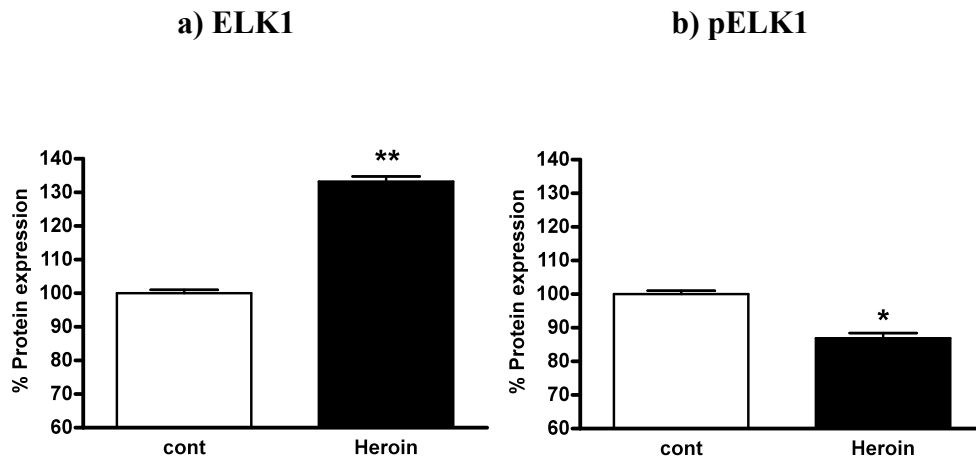
5. DISCUSSION

5.1 ALCOHOL

5.1.1 Gene expression

It is generally difficult to clearly understand whether changes in them RNA levels reflect a possible involvement of a specific gene in the execution of a behavior (i.e. alcohol intake) or the effect of alcohol by itself on this particular gene. Because prolonged exposure to EtOH by itself changes the expression of several genes (Worst *et al.,* 2005), the main goal of our study was to try to correlate the effects of different exposure to EtOH on opioid genes expression using a direct administration protocol.

The majority of studies describing the EtOH effects utilized different protocols of administrations such as forced drinking (Schulz *et al.,* 1980), liquid diet (Seizinger *et al.,* 1983), injection procedures (Lindholm *et al.,* 2000), placement in a vapor chamber (Zapata *and* Shippenberg, 2006) or using a genetically selected line for high alcohol drinking behavior (Arlinde *et al.,* 2004).

A clear distinction between the role of a specific gene in drinking behavior or in EtOH effect is still confounding; thus, we decide to use binge intragastric protocol to minimize the potential confounding factors induced by EtOH administration in rats.

The first main result of the present study is that the experimental conditions used were able to evoke tolerance to alcohol effects and dependence in rats, as measured by the progressive decrease of intoxication signs and the occurrence of clear withdrawal signs, respectively. Our results are thus consistent with very recent published data showing that this is a reliable methodology that effectively induces physical dependence upon EtOH over a short time period (Braconi *et al.,* 2010).

Changes in PDYN/KOP system gene expression

Many of the studies examining dynorphinergic system activity following exposure to alcohol (Przewlocka *et al.,* 1997; Rosin *et al.,* 1999; Lindholm *et al.,* 2000) reported inconsistent results probably arising from the various paradigm of administration used and experimental models, but also for the complex role of this system.

Here, we observed an up-regulation of PDYN mRNA following a single day of EtOH administration in both the brain region investigated, the AM and the PFCx. Acute EtOH has been already shown to stimulate, in rats and humans, the release of opioid peptides, such as b-endorphin and enkephalins (Dai *et al.,* 2005; Marinelli *et al.,* 2005), contributing to the reinforcing effects of EtOH, and also DYN (Marinelli *et al.,* 2006), this last neuropeptide exerting compensatory alterations.

In this respect, the aversive role of k-opioid agonists is well known (Land *et al.,* 2008). In the AM, the increased activity of the dynorphinergic system has also been observed in dependent animals (5D group), where KOP mRNA resulted up-regulated and PDYN gene expression returns to levels comparable with control. Moreover, PDYN gene expression resulted to be increased in the AM in the early withdrawal (EW) group. Our data are consistent with the hypothesis that the blockage of KOP induces a decrease in EtOH intake in rats that are physiologically dependent on EtOH, but not in nondependent rats (Walker *and* Koob, 2008).

DYN system changes during dependence and in EW are consistent with the previously suggested recruitment of this system during the different neurochemical and behavioural phases of alcohol intake history (Walker *et al.,* 2010).

Overall, these effects are here probably linked to the negative dysphoric state in withdrawal, that EtOH could relieve as previously proposed (Walker *and* Koob, 2008). This is a transient initial effect, because in the next withdrawal interval, up to Day 7, no alteration in the gene expression of the precursor was observed.

Changes in PNOC/NOP system gene expression

We did not observe any alteration in NOP mRNA levels in both the brain regions investigated. In contrast, the PNOC gene expression was increased by EtOH administration both after 1 day (1D) as well as in dependent (5D group) and in EW group animals in the AM, a key region in regulating alcohol consumption (Koob, 2003).

Intracerebro-ventricular injection of NOC as well as administration of NOC into the central AM significantly inhibited EtOH self-administration in the alcohol preferring animals (Economidou *et al.,* 2008).

In the central AM of EtOH-dependent rats, an enhanced sensitivity to NOC effects was found because the peptide blocked the EtOH-induced augmentation of inhibitory postsynaptic currents (Roberto *and* Siggins, 2006).

An important role of the NOC system in neuronal circuits involved in reinforcing or conditioning effects of EtOH was also proposed by behavioral data (Kuzmin *et al.,* 2007).

Thus, our findings, showing a constant upregulation of PNOC gene expression in the AM during EtOH intake and, to a lesser extent, in EW, appear to be consistent with a role of functional antagonism played by this system (Mogil *and* Pasternak, 2001) towards the positive reinforcing actions of EtOH associated with mu-acting opioids.

5.1.2 Epigenetic studies

We here studied the involvement of epigenetic mechanisms in the recently observed changes on PDYN and PNOC genes expression evoked by different EtOH administrations (D'Addario *et al.,* 2011b). We observed a close relationship between selective chromatin modifications and PDYN and PNOC genes expression, especially in rats treated for 1 day with alcohol (1D group).

Epigenetic mechanisms have already been proposed to be responsible for the cellular actions of EtOH (Shukla *and* Aroor, 2006; Kim *and* Shukla, 2006) and recently chromatin remodeling was suggested to be a plausible characteristic of alcoholism (Pandey *et al.,* 2008).

It has been observed that chronic EtOH exposure causes a more open structure of chromatin, consistent with a switch from heterochromatin to euchromatin (Mahadev *and* Vemuri, 1998).

In rat hepatocytes, EtOH induced a selective, post-translational acetylation of H3K9 in a dose-dependent and time-dependent manner (Park *et al.*, 2003), and distinct methylation patterns in histone H3K4 and H3K9 correlate with up- and down-regulation of genes (Pal-Bhadra *et al.*, 2007).

Our study revealed an inverse relationship of H3K27me3, a repressive mark, and H3K9Ac, activating mark, in both PDYN and PNOC promoters, in animals treated for 1 day with EtOH in the AM.

This may point to a covalently modified chromatin domain, already observed by us for the PDYN gene in SH-SY5Y cells exposed to alcohol and acetaldehyde (D'Addario *et al.*, 2011a) and characterized for many other genes (Azuara *et al.*, 2006; Roh *et al.*, 2006; Pan *et al.*, 2007; Mikkelsen *et al.*, 2007; Zhao *et al.*, 2007).

Alterations of H3K9Ac, but not of H3K27me3, were still present in the 5D group for the PNOC promoter, again in accordance with the gene expression increase.

None of the histone modifications under study were not directly associated with gene expression changes in the W-1D group.

This could be possibly explained by different hypothesis. First of all, it has to be taken under consideration the complexity of gene regulation by histone modifications (Barski *et al.*, 2007) and that multiple modifications may function cooperatively to prepare chromatin for transcriptional activation. Moreover, in addition to epigenetic mechanisms, gene expression is also regulated by many components of the complex transcriptional machinery.

One possibility for PNOC gene could be also that a long-term maintenance of epigenetic chromatin state (Boggs *et al.*, 2002; Peters *et al.*, 2002) could determine accessibility for transcription factors eventually inducing specific transcription even in absence of the modification not needed anymore.

Changes in DNA methylation in the promoter region of different genes such as the alpha-synuclein (Bönsch *et al.*, 2005), dopamine transporter (Hillemacher *et al.*, 2009), homocysteine-induced endoplasmic reticulum protein genes (Bleich *et al.*, 2006), as well as PDYN (Taqi *et al.*, 2011) have recently been observed in alcohol dependent patients.

However, in our study no significant alterations in DNA methylation following different alcohol exposures were present.

This data is anyhow in agreement with our previous in vitro observation showing no changes in DNA methylation of PDYN promoter in SH-SY5Y exposed to alcohol (D'Addario et al., 2011a). Moreover, we did not observe any alteration of H3K4me3 in all the experimental groups under study, and thus the latter findings suggest the selectivity of alcohol effects.

Overall our data indicate a linkage between gene expression alterations and epigenetic modulation in PDYN and PNOC promoters following 1 day and up to 5 days of alcohol treatment and we observed a cross-regulation of histone modifications in the 1D group.

In our experimental conditions even we did not observed any epigenetic alteration in the promoter regions of the genes investigated during withdrawal, we could confirm the possible chromatin remodeling mechanism already proposed for alcoholism (Pandey et al., 2008).

5.2 HEROIN

5.2.1 Proteins level studies

One of the key intracellular pathways activated by MOR is the MAPK pathway. To determine whether there are perturbations in protein levels of the MAPK pathway as a consequence of chronic heroin abuse, we focused on the core components of the pathway including MEK1/2 and ERK1/2.

In this context, chronic morphine exposure in rats resulted in structural changes of neurons compatible with the induction of synaptic plasticity (Sklair-Tavron et al., 1996; Robinson and Kolb, 1999; Spiga et al., 2003; Liao et al., 2005; Ballesteros-Yáñez et al., 2007).

Although some signaling pathways have been associated with morphine-induced changes in neuronal size, synaptic connectivity and behavioral plasticity, increasing

evidences indicate that proteins of the apoptotic pathways can also play relevant roles in promoting various forms of synaptic plasticity (Gilman *and* Mattson, 2002; Mattson *and* Gleichmann, 2005; Mattson, 2007).

In the putamen of heroin addicts, the protein expression of MEK1 was significantly reduced and on the two known targets of the MEK1/2, we observe an opposite effects: significant decrease in the protein level of ERK1 but, conversely, a significant increase in the protein levels of ERK2.

The activation of ERK1 is mediated by both MAPK1/2 and the decrease that we observed is clearly evoked by MEK1 level reduction. Conversely ERK2 activation is mediated only by MEK2, that didn't change in our experimental condition, and is also be under negative regulation by ERK1, which can inhibit its activation.

The reduction of the levels of ERK1 protein may lead to a reduction of the block that this exerts on the protein ERK2. In this way the effect of a prolonged exposure to heroin means, upstream, with a reduction of the activity of MEK1 which has the effect downstream to increase the levels of ERK2.

The control that ERK2 exerts on the protein ELK1 is positive, but the direct effect, observed in the putamen of heroin addicts, is a significantly increased in the global level of ELK1 but a significant reduction in the level of phosphorylated Elk-1 form.

Previous studies have shown that amphetamine activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) resulting in cAMP response element-binding protein (CREB) and Elk-1 phosphorylation in striatal neurons (Choe *and* Wang, 2002) and our data support the hypothesis that prolonged exposure to substance abuse causes a dysregulation of the MAPK pathway in the areas delegated to the reward.

In fact, the striatum is essential for motivation and drug reinforcement. Moreover, several basal ganglia dysfunction occurs in drug addiction and its alteration have been suggested to be responsible for enhanced liability to abuse drugs (Spreckelmeyer *et al.,* 2011).

The opposite direction in the levels of ELK1 and pELK1 remains controversial. Under normal conditions we can expect that the levels of total protein and its phosphorylated form going in the same direction after drugs exposure. Often, however, the opposite happens. Seems that the direct effect of ERK2 activation been the increase in ELK1 total level; the subsequent reduction observed in its phosphorylated form could be due to a compensatory effect induced by prolonged

activation of the transcription factor, or may simply result from the fact that, even if they are fresh-frozen tissue, the time is not sufficient to observe the up-regulation of the active form. This is possible if we consider that the exposure to drugs of abuse results in adaptation in the brain involving changes in gene expression and transcription factors (Martín *et al.,* 2011).

6. CONCLUSION AND PERSPECTIVES

Over the last decade, the distinct processes of addiction to drugs of abuse are being investigated as specific forms of drug-induced neural plasticity, and much attention has been focused on the genetic, cellular, molecular and behavioral mechanisms underlying the induced changes in brain function (Kalivas *and* O'Brien, 2008; Thomas *et al.,* 2008). Thus, opiate addiction in humans can be approached as a form of neuroplasticity, in which the lasting and aberrant adaptations in the brain would play major roles in the development of the principal features of this chronic medical disorder: opiate tolerance and dependence, behavioral sensitization, and compulsive drug use that underlines the long persistence of relapse risk in the addicted person (Hyman, 2005; Christie, 2008).

Conclusion I

The data about alcohol exposure confirm a role of the PDYN/KOP system in the negative hedonic state associated with alcohol addiction (Walker *and* Koob, 2008) and the hypothesis that the NOC system could function as a 'brake' to limit EtOH intake (Roberto *and* Siggins, 2006).

The linkage between gene expression alterations and epigenetic modulation in PDYN and PNOC promoters following alcohol treatment confirm the possible chromatin remodeling mechanism already proposed for alcoholism (Pandey *et al.,* 2008).

Our results could be important to partially fill the lack of knowledge of how EtOH by itself affects the opioid system in the brain, and suggest the possibility of using drugs acting on these systems for the treatment of withdrawal symptoms and alcohol dependence (Walker *and* Koob, 2008).

Conclusion II

The data, about heroin exposure, confirm the presences of perturbations in protein levels of the MAPK pathway as a consequence of chronic heroin abuse.

Moreover, we have shown that the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) resulting in Elk-1 phosphorylation in striatal neurons (Choe *and* Wang, 2002) supporting the hypothesis that prolonged exposure to substance abuse causes a dysregulation of MAPK pathway, in the areas delegated to the reward.

Our results could be important to clarify the role of ELK1 in the regulation of several gene expression, for changes in neuronal size, in the synaptic connectivity and in behavioral plasticity (Gilman *and* Mattson, 2002; Mattson *and* Gleichmann, 2005; Mattson, 2007).

Knowledge of the neuroanatomy, neurophysiology, neurochemistry and neuropharmacology of addictive drug action in the brain is currently producing a variety of strategies for pharmacotherapeutic treatment of drug addiction, some of which appear promising.

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