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Role of the endogenous opioid system in addiction to alcohol and cocaine

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
β-EP	β-endorphins
aAMP	Cyclic AMP
CaM	calmodulin
CaMKII	calcium-calmodulin kinase II
CNS	central nervous system
CS	conditioned stimulus
CTOP	[Cys^2-Tyr^3-Orn^5-Pen^7]-somatostatinamide
DA	dopamine
DAMGO	[D-Ala^2-*N*-Me-Phe^4,Gly-ol^5]-enkephalin
E-LTP	early-LTP
FR	fixed-ratio
HFS (2T)	high frequency stimulation two trains
HFS (4T)	high frequency stimulation four trains
ICSS	intracranial self-stimulation
IP	intra peritoneal
Iv	intravenous
L-LTP	late-LTP
LTP	long-term potentiation
MSN	medium spiny neurons
Nacc	nucleus accumbens
NMDAr	NMDA receptors
POMC	pro-opiomelanocortin
sc	Subcutaneous
SN	substantia nigra

STD	short-term depression
STP	short-term potentiation
US	unconditioned stimulus
VTA	ventral tegmental area
v/v	volume/volume
w/v	weight volume
10E	10% ethanol solution

1.1 Drug addiction

Long time ago, people discovered that many substances found in nature -primarily leaves, seeds, and roots of plants, but also some animal products- had medicinal qualities. They discovered herbs that prevented infections, that promoted healing, that reduced pain, which helped to provide a night's sleep. Together with this, our ancestors also discovered "recreational drugs", which produced pleasurable effects when eaten, drunk or smoked. The most universal recreational drug, and perhaps the first one discovered, is ethanol. In addition, Asians discovered the effects of the sap of the opium poppy and the beverage made from the leaves of the tea plant, and Indians the effects of the smoke of cannabis. South Americans discovered the effects of chewing coca leaves and making a drink from coffee beans, and North Americans discovered the effects of the smoke of the tobacco leaves. Together with their pleasant effects, some of these drugs may be associated with displeasing tastes and aftereffects; however people have learnt to tolerate them. The motivation for this is provided by an effect common to all recreational drugs: activation of the brain's reinforcement mechanisms. Some drugs can have very potent reinforcing effects, which can lead some people to abuse them or even to become addicted to them.

In the modern society drug addiction has become one of the most serious threats to public health, in terms of diffusion, lost lives and decrease in productivity. It is estimated that about 30 million individuals in the United States and Western Europe suffer from alcohol abuse and other drug addictions. Worldwide, at the beginning of 2000s, there were 29 million amphetamine abusers, 14 million cocaine abusers and 13 million abusers of heroin and other opiates (United Nations World Drug Report 2006). The human and economic costs of substance abuse to society are extremely high: it is estimated that it ranges between 1% to more than 3.5% of gross domestic product (GDP) in European countries and North America.

Drug addiction is defined by the World Health Organization as "a state psychic and sometimes also physical, resulting from the interaction between a living organism and a drug, characterized by behavioral and other responses that always include a compulsion to take a drug on a continuous or periodic basis in order to experience its psychic effects, and sometimes to avoid the discomfort of its abstinence" (WHO, 1969). Once established, addiction can be a life-long condition in which individuals show intense drug craving and an increased risk for relapse after years or even decades of abstinence.

1.2. The brain reward system

In order to induce addiction a drug has to be *rewarding* (interpreted by the brain as intrinsically positive) and *reinforcing* (behaviors associated with such drugs tend to be repeated). The neural substrate underlying reward and reinforcement consists of strictly interconnected forebrain structures referred to as the *brain reward system*. This circuitry is very old from an evolutionary point of view, and has evolved to mediate an individual's response to natural rewards, such as food, sex and social interaction. It consists of ascending and descending fibers that connect rostral basal forebrain and midbrain structures and includes noradrenergic, dopaminergic and serotoninergic fibers. In particular, positive reinforcement is determined by activation of the mesocorticolimbic dopamine system, which is involved in the mediation of both cognitive and emotional functions. This system can, in fact, be considered as an interface between emotional and motivational information, which are processed in the *limbic system*, and the regulation of motor functions that takes place in the *basal ganglia*, nuclei that contribute to the extra-pyramidal motor system.

1.2.1. Anatomy

The basal ganglia take up a wide area deeply in the cerebral hemispheres, in a position predominantly lateral to the thalamus. The principal components of this brain area are the caudate nucleus, the putamen (which together constitute the neostriatum) and the globus pallidus. The term *striatum* is often used to indicate

both the neostriatum, also defined as the *dorsal striatum*, and its ventral extension, whose principal component is the *nucleus accumbens* (NAcc). Two additional subcortical nuclei, functionally and anatomically interconnected with the neostriatum, are the subtalamic nucleus and the substantia nigra (SN), which can be divided into a pars compacta, principally made of dopaminergic fibers, and a pars reticulata, whose neurons are GABA-ergic. The basal ganglia constitute an accessory motor system, which operates in association with the cerebral cortex and the corticospinal system. Here the information carried by the corticostriatal projection is integrated with the afferents innervating the striatum and finally, once processed, it is conveyed to the exiting basal ganglia nuclei: the SN pars reticulata and the entopeduncolar nucleus. These afferents, through the mediolateral portion of the thalamus, are mainly directed to the prefrontal cortex, which includes areas involved in motor planning.

The corticostriatal terminals are excitatory, and employ glutamate as neurotransmitter: the fibers directed to the dorsal striatum originate in the neocortex, whereas those reaching the NAcc arise in the so-called limbic system. This system, also defined as limbic lobe, comprises subcortical structures such as the hippocampus, the amygdaloid complex and some areas in the prefrontal cortex, which are involved in emotional regulation. In addition to the glutamatergic innervations, the striatum receives dopaminergic afferents arising form the SN and the ventral tegmental area (VTA), which constitute the nigrostriatal and the mesocorticolimbic circuits respectively. The nigrostriatal circuit targets the dorsal striatum, while the mesocorticolimbic fibers innervate the NAcc, the olfactory tubercle, the most ventral portion of the caudate nucleus and the putamen and the amygdala.

1.2.2. The mesocorticolimbic system

This dopaminergic circuit is the major substrate in the mediation of positive reinforcement and reward, as it is activated by positive natural stimuli (and by drugs of abuse). This activation involves an increase in firing rate of the dopaminergic neurons in the VTA and a subsequent increase in the levels of

dopamine released in the NAcc, and in other limbic regions involved in the brain reward system. Within these brain areas, a key role is played by the NAcc, the main component of the limbic-motor interface, as it integrates the cortical glutamatergic information with the dopaminergic one arising in the midbrain.

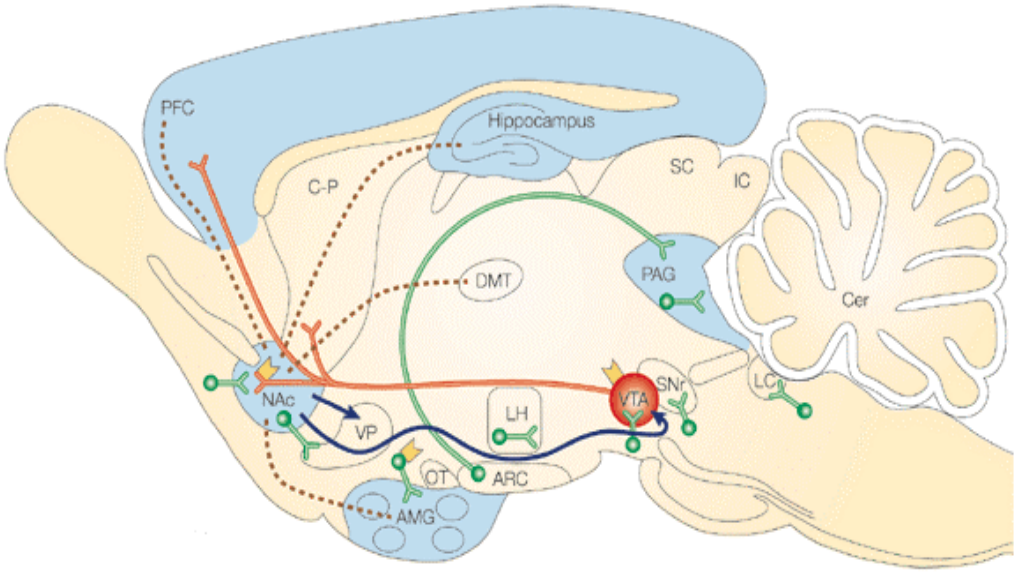


Figure 1. The brain reward system. Dootedlines indicate the glutamatergic afferents arising in the limbic system and directed to the NAcc. Red lines are the projections of the mesolimbic DA system involved in the mediation of reward and positive reinforcement. The DA neurons originate in the VTA and reach the NAcc and other limbic structures: the olfactory tubercle (OT), the most ventral portions of the caudate nucleus and the putamen (C-P), the amygdala (AMG) and the pre-frontal cortex (PFC). (Neslter, 2001)

1.2.3. The nucleus accumbens

Himmunocytochemical studies have revealed organizational complexity of the NAcc as a heterogeneous structure that can be divided into shell and core regions. The great majority of the cellular elements making up the NAcc are characterized by an elevated number of dendritic endings, thus being generally called “medium spiny neurons” (MSN). These neurons are GABA-ergic, and are normally

quiescent, as they are hyperpolarized: their membrane potential is about -85 mV. In this so-called "downstate", MSN neurons are not capable of generating an action potential. In order to do so, it is necessary that synchronous activation of excitatory glutamatergic inputs bring these neurons into an activated "up-state". In this condition, the membrane potential of these cells rises to –60 mV, a value closer to the activation threshold. Once the transition between down- and up-state has occurred, these neurons can maintain the activated state for a variable amount of time, which at times can be longer than a second. In this state, an additional weak depolarization is able to generate an action potential (Nicola et al. 2000).

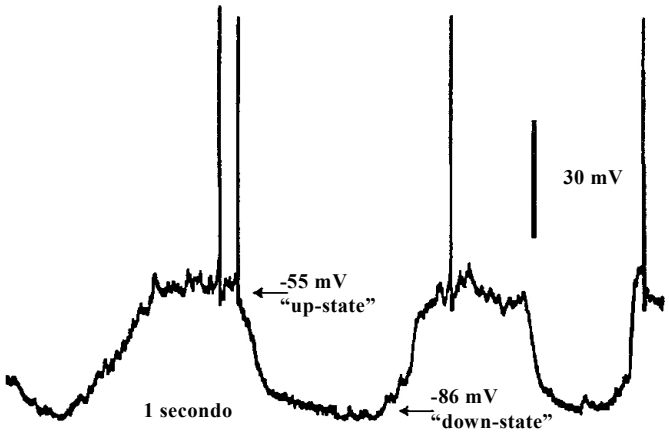


Figure 2. Transitino states of a medium spiny neuron's membrane potential. (Wilson e Kawaguchi, 1996)

1.3. Dopamine

The role played by the glutamatergic synapses on striatal neurons activity is supported by experimental evidences, which show that ablation of cortical afferents impairs MSN transition from the down- to the up-state (Wilson 1993). The effect of dopaminergic input on NAcc neurons activity, instead, has not been as yet fully understood. In fact, although extensively studied, dopamine actions on MSN are still debated, as the results obtained are controversial. Dopaminergic effects are mediated by dopamine receptors, which are divided into two major

subclasses, i.e. the D1-like (D_1 and D_5) and the D2-like (D_2 D_3 and D_4), based upon their pharmacological and protein sequence similarities. Both receptors are G-protein coupled controlling the activity of adenylate cyclase. Stimulation of D1-like receptors results in increased levels of cAMP, whereas stimulation of D2-like receptors causes the opposite effect (see Civelli et al., 1993; Missale et al., 1998). D1-like receptors appear mainly located postsynaptically outside the synaptic cleft (Gonon 1997), whereas D-2 like receptors are both located presynaptically, as autoreceptors, and postsynaptically. D2-like autoreceptors exert an inhibitory influence on the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxilase, and are therefore termed synthesis-modulating autoreceptors (Cooper et al., 1996). Presynaptic D2-like autoreceptors may also decrease the probability of dopamine release by indirectly modulating Ca^{2+} levels in the terminal (Cooper et al., 1996).

The NAcc MSN express both D1-like and D2-like receptors (Surmeier et al., 1996), though the D1-like receptors are largerly resctriced to a subpopulation of MSN expressing dynorphin and substance P and projecting to the pars retuculata of the SN, whereas the D2-like receptors are largely restricted to a subpopulation of MSN expressing enkephalin and projecting primarily to the pallidum (Gerfen, 1992). A third subpopulaton of MSN coexpresses substance P and enkephalin and similarly coexpresses D1-like and D2-like dopamine receptors (Surmeier et al., 1996); the projection of this subpopulation of neurons is not known. The net effect of dopamine on neuronal excitability is difficult to assess, as it has been shown to both inhibit and excite striatal neurons. Dopamine application in the NAcc depresses MSN firing evoked by stimulation of the amygdala (Yim and Mogenson, 1982) and the hippocampus (Yang and Mogenson, 1984); again, endogenous dopamine released as a consequence of VTA stimulation produces an inhibitory effect on MSN neurons activity, this effect has been attributed to D2-like receptors activation (Liang et al., 1991). On the other side, it has been reported that chemical stimulation of the VTA induces an increase in the firing rate of MSN mediated by D1-like receptors (Gonon, 1997). More recently it has been demonstrated that co-activation of both

dopamine receptors subtypes increases striatal neurons response to exogenous applied glutamate (Hu and White, 1997).

From what reported herein it is evident that dopamine actions at its receptors, D1-like and D2-like, are largely unknown, because the circumstances in which their activation results in a synergistic or an antagonistic effect have not been established yet. However, the involvement of dopamine in the mediation of reward and reinforcement has been extensively documented. Several microdialysis studies have demonstrated that dopamine is increased in response to natural rewards (Westerink 1995; Salamone 1996). In addition, studies of intra-cranial self-stimulation have shown that application of drugs such as neuroleptics, which are dopamine receptors antagonists, reduces operant responses in animals (Wise 1982).

Despite dopamine plays a pivotal role in reward, according to the current theory it is not implicated in the mediation of reward itself, but rather in the anticipation, intended as the prediction, of its effects. Probably the best explanation of the role played by dopamine in reward comes from *in vivo* electrophysiological studies performed in monkeys. These experiments have demonstrated that VTA dopaminergic neurons show phasic activations in response to unexpected rewards. When reward delivery is anticipated by an environmental cue (i.e. tones or lights), once this association is learnt, dopamine neurons respond to the stimulus rather than to the actual reward (Mirenovicz and Schultz 1994, 1996). These findings concurred to advance the hypothesis that dopamine is involved in a form of memory. According to this theory, phasic responses of dopamine neurons to primary rewards function as learning signals, which serve to establish the behaviors that elicited the rewards (Schultz 1998). This system can therefore be considered fundamental in the acknowledgement of the actions that promote the survival of the individual and the species.

1.4. Effects of addictive drugs on the mesocorticolimbic system

The mesocorticolimbic system has received much attention in research because of its involvement in the reinforcing properties of many dependence- producing drugs (Fibiger and Phillips, 1988). Similarly to natural stimuli, drugs of abuse are capable of activating the brain reward system with a strength, time course and reliability that exceeds almost any natural stimulus. Since every action associated with the activation of this system is recognized as intrinsically positive, the brain's inability to distinguish between natural (e.g. food, sex and social interaction), and "artificial" (abused drugs) rewards (Wise et al., 1998), clarifies the reinforcing effects elicited by these drugs, and consequently explains, at least in part, their ability to induce compulsive drug seeking. The assumption that abused drugs and natural rewards interact with the same cerebral circuitry is supported by experimental evidence. Experiments of intracranial self-stimulation have demonstrated that drugs such as cocaine, amphetamine, nicotine, opioids, ketamine, cannabinoids, barbiturates and alcohol, play a synergistic effect on the threshold for brain stimulation: in the presence of these drugs the threshold current intensity of the stimulation is reduced in a dose-dependent manner (Bespalov et al., 1999). This and other experimental evidence demonstrate that the mesocorticolimbic dopamine system is the convergent site for the rewarding effects of most, if not all, classes of abused drugs.

1.4.1 Ethanol

The mechanism through which ethanol influences the activity of the brain reward pathway has not been completely identified, but recent findings have demonstrated the involvement of several neurotransmitter systems. The effect of ethanol occurs at the soma of the dopaminergic cells in the VTA, rather than on their striatal terminals (Yim and Gonzales 2000); this is supported by experiments performed *in vivo* (Gessa et al., 1985), on brain slices (Brodie et al., 1990), and on isolated cells (Brodie et al., 1999). The fine mechanism through which ethanol increases the firing rate of the DA neurons in the VTA is still debated. One hypothesis states an indirect mechanism, through the GABA-ergic interneurons in

this brain area. These neurons exert a negative modulation on the activity of the DA cells. Therefore the inhibitory effects of ethanol on these interneurons cause a reduction of the tonic control on the DA neurons, resulting in activation. A second theory suggests a direct action of ethanol onto the DA neurons of the VTA. According to this hypothesis, this effect is mediated by the serotonin 5-HT_3 receptors on the DA terminals, whose activity is increased in the presence of alcohol (Campbell et al., 1996). This hypothesis is also supported by the evidence that administration of ondansetron, a selective 5-HT_3 inhibitor, blocks both the release of DA in the NAcc and ethanol consumption in experimental animals (Fadda et al., 1991).

1.4.2. Cocaine

The psychostimulant cocaine activates the brain reward system at both ends of the circuitry. Cocaine is able to induce an increase in DA levels in the NAcc by binding and deactivating the monoamine transporter proteins, thus blocking the reuptake of DA, serotonin and norepinephrine after these neurotransmitters are released by the terminal buttons in the synaptic cleft. Moreover, similarly to alcohol, cocaine has been shown to directly increase the activity of the DA cells in the VTA through the 5-HT_3 receptors. Evidence of this comes from studies in which systemically administered 5-HT_3 antagonists decrease cocaine-induced release of DA in the NAcc, and reduce cocaine-induced conditioned place preference as well as locomotor activity (Kankaanpaa et al., 2002).

A recent theory considers the increase in the firing rate of the DA neurons in the VTA as a consequence of the interaction between the endogenous opioid system and non-opioid drugs of abuse, such as cocaine and ethanol. There is in fact ample evidence that both ethanol and cocaine produce effects on the β−endorphin and enkephalin systems that may be responsible for their actions in the central nervous system (CNS). In preclinical studies opioid antagonists have been shown to reduce the reinforcing efficacy of cocaine as measured by the threshold for brain stimulation (Bain and Kornestky, 1986), and the rate of cocaine self-administration during acquisition (De Vry et al., 1989) and its

maintenance (Corrigal and Coen, 1991). A recent clinical study has demonstrated an increased μ opioid receptor binding in cocaine-dependent men, detected by PET (Zubieta et al. 1996). Moreover opiate antagonists have proven to inhibit the reinforcing effects of ethanol in a variety of animal models, reducing home cage voluntary ethanol intake (Davidson and Amit 1997) as well as operant ethanol self-administration (Altshuler et al., 1980).

1.5. The endogenous opioid system

The endogenous opioid system is constituted by three distinct families of endogenous peptides -β–endorphin, enkephalin and dynorphin- that are defined by their precursor molecules (Khachaturian et al., 1985). Pro-opioimelanocortin (POMC) gives rise to β–endorphin, which is synthesized in the brain in the arcuate nucleus and in a small group of neurons in the nucleus tractus solitarii (Crine et al., 1978). β–Endorphin neurons of the arcuate nucleus project to various brain regions including the VTA, NAcc, septum, amygdala, hippocampus, frontal cortex and periaqueductal gray (Khachaturian et al., 1985). Proenkephalin gives rise to 4 methionine (met)-enkephalin molecules and 1 of each met-enkephalin-Arg^6-Phe^7, met-enkephalin-Arg^6-Gly^7-Leu^8 and leucine (leu)-enkephalin (Bloom 1983). Prodynorphin gives rise to dynorphins, α-neoendorphins and leu-enkephalin (Kakidani et al., 1982). Neurons synthesizing enkephalins and dynorphins are widely distributed throughout the brain (Khachaturian et al., 1985). This system is involved in three major physiological functions: modulation of the response to painful stimuli and stressors; reward and reinforcement; and homeostatic adaptive functions, such as regulating body temperature and food and water intake (Olson et al., 1990).

The opioid peptides interact with three major classes of opioid receptors - μ, δ and κ- that have been identified and characterized; and each peptide presents different affinities for each of the opioid receptors. β–Endorphin binds with about equal affinity to μ and δ opioid receptors, met- and leu-enkephalins bind with 10- to 25-fold greater affinity to δ than μ opioid receptors, and dynorphins bind

selectively to κ opioid receptors (Charnes 1989). Interactions of endorphins and enkephalins with μ and δ opioid receptors have been shown to increase DA release in the nucleus accumbens and contributes to the processes associated with reward and reinforcement (Koob 1992), whereas dynorphins binding to κ opioid receptors, which has been shown to produce aversive states and decrease DA release, may prevent reinforcement (Koob 1992). Opposing effects of these agents have also been observed at the neurochemical level within the mesolimbic dopamine system: μ agonists increase, whereas κ agonists decrease, DA release in the NAcc. In particular, microdialysis studies demonstrated that VTA microinjection of [D-Ala^2-*N*-Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO), a μ selective agonist, enhances, whereas [Cys^2-Tyr^3-Orn^5-Pen^7]-somatostatinamide (CTOP), a μ selective antagonist, decreases DA release in the NAcc (Di Chiara and Imperato, 1988; Spanagel et al., 1990). Administration of both substances in the NAcc, instead, did not elicit any effect (Di Chiara and Imperato, 1988; Spanagel et al., 1990). On the contrary, κ-selective agonists inhibit DA release when administered in the NAcc, without eliciting any effect when applied in the VTA (Di Chiara and Imperato, 1988; Spanagel et al., 1990).

These results suggest that the maintenance of basal release of DA in the NAcc depends on a balance between the stimulatory μ-opioid systems and the inhibitory κ-opioid system (Spanagel et al., 1992). In agreement with this observation, it has been shown that a repeatedly administered μ agonist (morphine), elicits a compensatory increase in the activity of the dynorphinergic system, functionally opposed to the endorphinergic one in the NAcc, and/or a hypoactivity of the μ-opioid system.

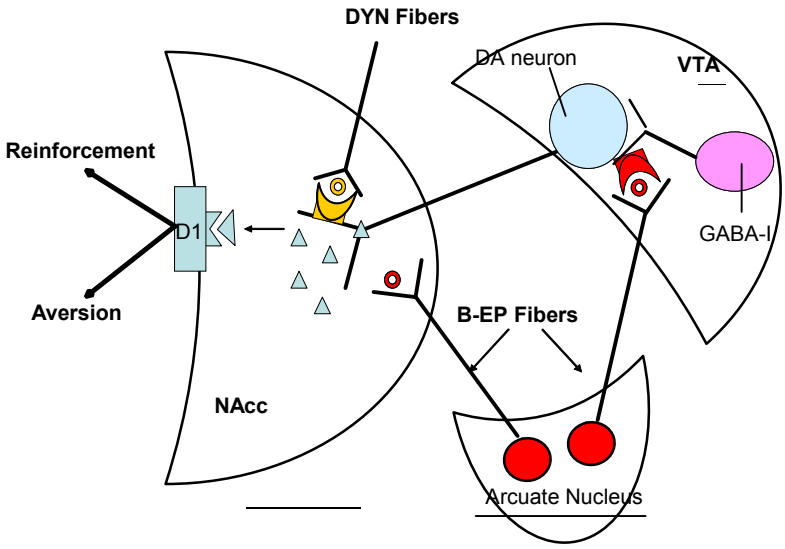


Figure 3. Hypothetical model of the interaction between endogenous opioids and DA release in the Nacc. VTA DA neurons are under tonic inhibition of the GABA-ergic interneurons. Stimulation of pre-synaptic μ-opioid receptors on the GABA-ergic interneurons stimulates DA release from the DA neurons of the Nacc. Activation of κ-opioid receptors, on the contrary, inhibits DA release in the Nacc. Beta-endorphins released by the ascending fibers of the hypothlamic arcuate nucleus activate μ-opioid receptors in the VTA.

The increase in the levels of DA in the NAcc after cocaine or alcohol exposure results probably by the concurrence of the above mentioned neurochemical systems and represents the neural basis for their rewarding and reinforcing effects. During chronic drug exposure, however, this continuous and sustained activation of the mesocorticolimbic system induces adaptive defensive changes, aimed at counterbalancing the drug-induced stimulation of this system. These changes, which can be considered an example of synaptic plasticity (which will be discussed later on in this text), consist of molecular and cellular changes, such as regulation of receptors and ion channels expression, of signal transduction mechanisms and of gene expression. The consequence of these changes is a hypo functionality of the mesocorticolimbic system, which is a significant condition toward drug addiction. In fact, the reduced ability of the abused drug to increase DA levels in this brain areas, promotes additional drug consumptions, which in turn may cause aversive effects. This process of habituation of the DA transmission is a form of pharmacodynamic tolerance, resulting from homeostatic responses to excessive drug stimulation, which act to reinstate the activity of the

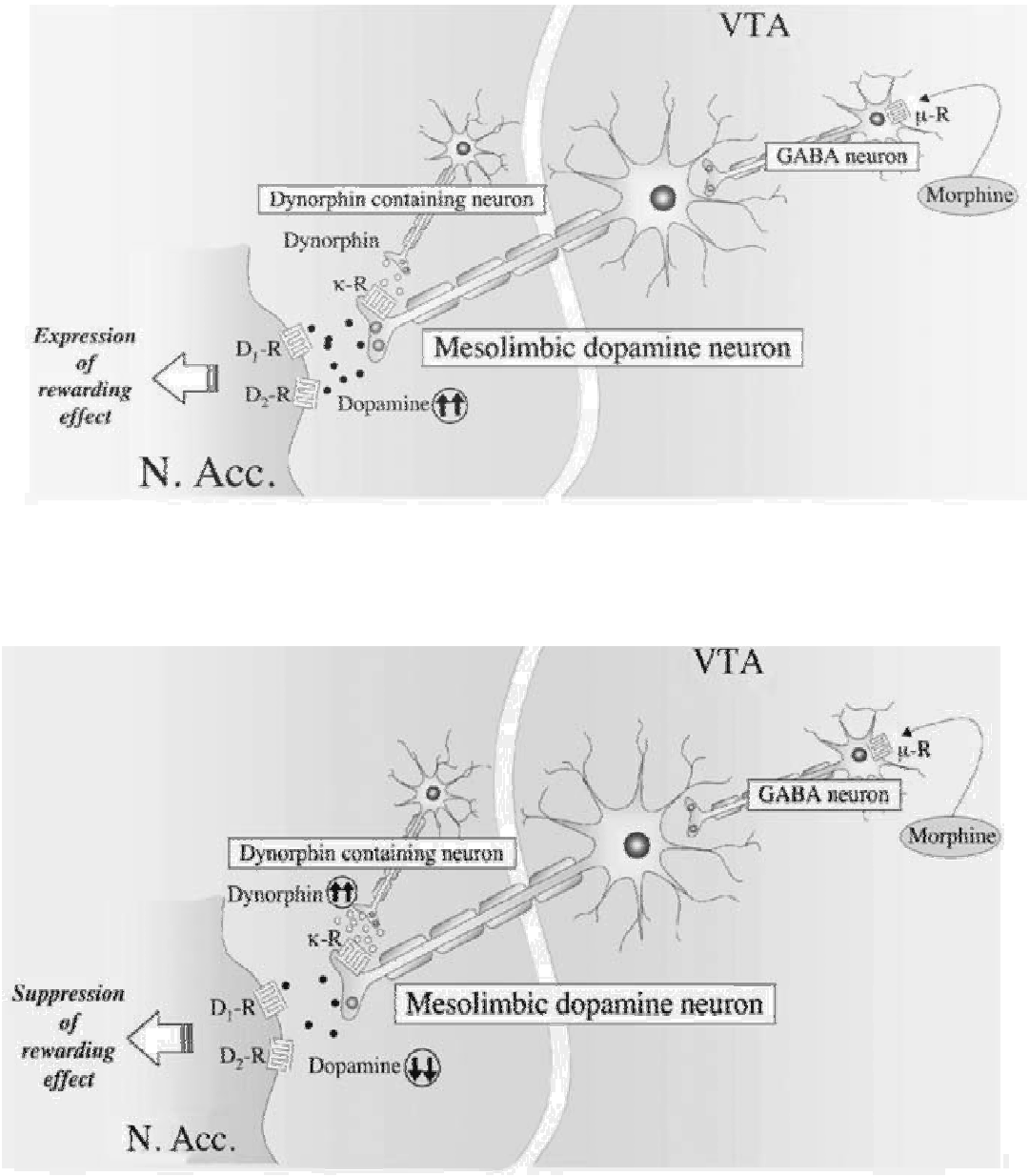


Figure 4 . Schematic representation of the modulation of the mesocorticolimbic system by morphine (A) and hypothetical compensatory action of the dynorphinergic system (B), at the DA fibers in the NAcc. Morphine induces an in increase in the release of DA in the NAcc through a dishinibiton of the GABA-ergic system in the VTA, which translates in reward (positive reinforcement). This effects is prevented by activation of the
k-oppioid receptors on the DA terminals, which arise in the VTA, thus decreseing DA release in the NAcc.

mesocorticolimbic DA system to its physiological levels. Once drug consumption is suspended, the lack of its effect on this compensated system, further lowers the efficiency of DA transmission. It is in fact demonstrated that abstinence induces a significant decrease in the activity of the DA neurons in the VTA (Diana et al., 1992; Shen and Chiodo, 1993), and a subsequent decrease in the levels of DA released in the NAcc (Rossetti et al., 1992; Weiss et al., 1996).

1.6. Relapse

It has been hypothesized that DA depletion in this brain areas can elicit "craving" for the abused drug. Craving, which can be defined as an intense desire for a psychoactive drug, motivates compulsive drug seeking, even after a long period of abstinence. At clinical level this translates into relapse, the most critical feature of drug dependence. Relapse can, in fact, occur after years or decades of abstinence and represents the main obstacle in the long-term treatment of drug abuse. Clinical reports and laboratory animals have shown that a number of factors contribute to relapse: re-exposure to the drug itself, or "drug priming" (Jaffe et al., 1989; de Wit, 1996; Chutuape et al., 1994), exposure to stressors (Sinha et al., 1999; Brown et al., 1993) and to environmental stimuli previously associated with drug taking (Childress et al., 1988, 1993; Monti et al., 1993). In particular, environmental stimuli repeatedly associated with the pharmacological effects of the abused drug are thought to be the most important factors contributing to increase an individual's vulnerability to relapse.

1.6.1. Animal model of relapse: the reinstatement paradigm

Relapse has been studied in animals using the "reinstatement procedure". This model results from a modification of an animal model of learning, the so-called *classic conditioning*, introduced by Ivan Pavlov at the beginning of the past century. The essence of classic conditioning consists of an association between *conditioned* and *unconditioned* stimuli. The conditioned stimulus (CS) comes from the environment, (i.e. a tone or a light), and is a neutral stimulus, in that it does not elicit any response in the animal. The unconditioned stimulus (US),

instead, is chosen because it is always able to elicit an innate response in the animal, i.e. food, which increases salivation in the animal. If the CS is repeatedly followed by the US, the animal will learn the association between the two, and therefore, presentation of the CS will evoke a *conditioned* response itself. Therefore, after repeated CS-US associations, the CS becomes a signal anticipating US availability, and the animal will respond to the CS, as if this primed the US. Classic conditioning represents a mean through which the animal learns to predict a relationship between an event and the environment. For example, if a light is repeatedly followed by food presentation, after a series of training sessions the animal will respond to the light as if it predicted the taste of food: the light alone will be sufficient to induce salivation. Another important principle of conditioning is that a stabilized conditioned response decreases in intensity when the CS is repeatedly presented in the absence of the US. Therefore the light that had been previously associated with food (US), will gradually cease to evoke salivation in the animal, if it is not followed by food presentation. This procedure is called *extinction*.

In the animal model of relapse, classic conditioning is modified into an *operant conditioning* paradigm, in which the experimenter introduces a contingency between the CS, which comes from the environment, and the US, also called reinforcer, which is the drug. In particular, in the reinstatement model animals are trained to self-administer a drug of abuse (US) by pressing a lever, in response to various types of environmental stimuli. After this phase, called "conditioning phase", the association between the stimulus and the reward is disrupted by the absence of both the drug and stimulus. In these conditions, the behavior associated with drug seeking, i.e. lever pressing, progressively extinguishes. After extinction, re-exposure to the stimuli previously associated with drug availability can reinstate operant responding, even in the absence of the reward. This model of relapse consists therefore of three phases: the first one called conditioning, in which the animal learns an association between stimulus, lever and drug; the second one called extinction, during which this association is eliminated; the

third, called reinstatement, during which lever pressing resumes in response to the environmental stimulus.

This experimental procedure varies in the type of environmental stimulus chosen as CS. Those that have been employed in alcohol and cocaine preclinical research includes a re-exposure to the drug, or drug priming, in which the first few lever presses are followed by drug availability (Chiamulera et al., 1995), environmental stimuli, such as tones or lights (Katner and Weiss, 1999) and, limitedly to cocaine addiction, the environmental context. Little information is available, in fact, about the role played by the environmental context in the resumption of ethanol- seeking behavior. This is an important aspect that is lacking in current research, as demonstrated for example by the high rates of relapse that occur when abstinent alcoholics return to their home environments after successful treatment (Childress et al., 1993).

The reinstatement model has proven to be a very useful tool in the elucidation of the neural mechanisms underlying relapse to drugs of abuse. In recent years, findings from several studies have strongly implicated the endogenous opioid system in relapse to alcohol and cocaine addiction. Notably, opiate antagonists appear to have clinical utility in reducing relapse in recovering alcoholics (O'Malley et al., 1992, Volpicelli et al., 1992, Kranzler and Van Kirk, 2001;), as well as craving and relapse in human cocaine addicts (Kosten et al. 1989). An acute cocaine administration (Gerrits et al., 1999), as well as ethanol injection (Olive et al., 2001) and its consumption (Gianoulakis et al., 1996) increases opioid peptide release in the brain. In animal studies, opiate antagonists are reported to attenuate reinstatement induced by alcohol (Bienkowski et al., 1999, Le et al., 1999) and cocaine (Gerrits et al., 2005) priming, or by exposure to ethanol-paired discriminative stimuli (Ciccocioppo et al., 2002, 2003; Katner et al., 1999).

1.7. Synaptic plasticity and drug addiction

The observation that drug addiction can be a life-long condition suggests that it is responsible for very stable functional changes in the CNS. Evidence has

accumulated over the past 15 years to support the hypothesis that the adaptations leading to addiction involve the same glutamate-dependent mechanisms that enable learning and memory. In other words, addiction can be viewed as a form of neuronal plasticity. Different lines of evidence support this hypothesis. First, imaging studies revealed that drug-related stimuli activate neuronal circuits implicated in learning and memory (Wilson et al., 2004). Other studies showed that that drugs of abuse and learning mechanisms activate common signal transduction pathways (Berke and Hyman, 2000; Hyman and Malenka 2001; Nestler, 2001). In addition, Ungless et al. (2001) found that a single exposure to cocaine induces a form of synaptic plasticity in the VTA.

Synaptic plasticity is defined as the ability of synapses to develop and reorganize in response to experience, and results as a change in the potency of synaptic transmission. The leading candidate for mediating neuronal plasticity is the so-called long-term potentiation (LTP), which was first found by Bliss and Lomo in 1973. LTP is induced by brief, high frequency stimulation (HFS), which leads to an increase in synaptic response after a single stimulus. As a result, a test stimulus applied after the HFS produces a larger response than the same stimulus applied prior to the tetanus. This effect can persist for many hours in brain slices and even for weeks in intact animals (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1972; Malenka and Nicoll, 1999). Potentiation can be defined as short-term (STP) when it lasts between 5 and 30 minutes after the HFS, and long-term (LTP) when it lasts more than 20 minutes. LTP can be further characterized as "early" (E-LTP), when it lasts up to 3 hours, and "late" (L-LTP) when it lasts 3-8 hours. These differences in time imply differences at the molecular level: only L-LTP involves, in fact, new protein synthesis, suggesting the involvement of nuclear functions (Soldering and Derkach, 2000). Another form of synaptic plasticity is synaptic depression, which can be induced by prolonged low frequency stimulation. As in the case of synaptic potentiation, also synaptic depression can be distinguished in long-term (LTD), and short-term (STD) depression (Castro-Alamancos et al., 1995).

1.7.1. Cellular mechanisms of synaptic plasticity

Synaptic plasticity is a fundamental property of the great majority of glutamatergic excitatory synapses in the mammalian CNS. Glutamate receptors are classified as iontropic, when directly associated to ion channels, and metabotropic, which can be coupled to ion channels or other cellular functions via a second messenger. Several studies have demonstrated that both types of glutamate receptors can be involved in the cellular mechanisms of synaptic plasticity. Iontropic glutamate receptors are classified in two subpopulations: NMDA receptors, which bind the analogous of glutamate N-metil-D-aspartate (NMDA), and non-NMDA. Non-NMDA receptors are further divided into AMPA, which can be activated by the α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA), and those that bind to kainic acid.

Glutamate receptors are constituted by several subunits: to date 7 subunits have been identified for non-NMDA receptors (called GluR1 to GluR7), and 5 subunits for NMDA receptors (called NR1, NR2a, NR2b, NR2c and NR2d). Glutamate binding to non-NMDA receptors activates non-selective ion channels permeable to monovalent cations (Na^+ and K^+), whereas activation of NMDA receptors opens non-selective cation channels, permeable to Na^+ and Ca^{2+}. In addition to the glutamate binding site, NMDA receptors show other binding loci: particularly relevant to synaptic plasticity is the site binding Mg^{2+} ions.

During basal synaptic transmission, glutamate is released from its pre-synaptic terminals, and binds to both AMPA and NMDA receptors; however, in this phase, NMDA receptors are blocked by extracellular magnesium molecules, and therefore sodium influx occurs only through the channels associated to the AMPA receptors. Only when the post-synaptic cell is depolarized by multiple convergent pre-synaptic neurons for a sufficient amount of time, Mg^{2+} dissociates from its binding site on the NMDA receptors, and Na^+ and Ca^{2+} can enter into the post-synaptic cell. The subsequent increase in the intracellular levels of calcium is the pivotal event for the development of the various types of synaptic plasticity. In particular, a synaptic response is increased after robust activation of the NMDA receptors (induced by HFS), which causes a remarkable calcium influx; the

efficiency of a synaptic response is instead decreased after a moderate NMDA receptor activation (induced by low frequency stimulations), with a subsequent moderate increase of calcium ions.

The increase in intracellular levels of calcium ions induces in turn a prolonged activation of the protein kinases, which persists even in the absence of elevated calcium levels. Calcium ions bind to the calmodulin (CaM) forming the complex calcium-calmodulin (Ca^{2+}-CaM), which in turn binds to the oligomeric protein calcium-calmodulin kinase II (CaMKII), activating it after phosphorylation. CaMKII can phosphorylate several proteic substrates, including the GluR1 subunit of the AMPA receptor (Lisman et al., 2002); in particular, LTP induction requires phosphorylation of GluR1 at Ser831, increasing channel conductance (Song and Huganir, 2002). CaMKII activation is also necessary for synaptic insertion of AMPA receptors, although the substrate is not GluR1 itself but rather some yet unidentified protein (Hayashi et al., 2000). In addition to CaMKII, other two kinases are involved in synaptic plasticity: protein kinase C (PKC) that, similarly to CaMKII phosphorylates Ser831, and c-AMP-dependent protein kinase (PKA), acts at Ser845. It seems that this site is already phosphorilated during basal synaptic transmission, and that it is dephosphorylated by PKA during LTD (Soderling and Derkach, 2000). GluR1 AMPA receptor phosphorylation and dephosphorylation appear to contribute to the mechanisms of expression of LTP and LTD, respectively (Lee et al., 1998). PKA activity, similarly to CaMKII, depends upon initial calcium influx into the post-synaptic neuron: Ca^{2+}-CaM, in fact, activates adenylate cyclase, with a subsequent increase in the intracellular levels of cAMP, necessary to activate PKA itself. PKC, together with PKA, plays a role at the pre-synaptic level, promoting neurotransmitter release (Lu and Gean, 1999). Protein kinases action starts about thirty minutes after potentiation induction, and can be maintained for up to three hours; it is therefore involved in early LTP; late LTP involves instead changes in gene expression and BDNF secretion (Aicardi et al., 2004; Santi et al., 2006), whereas its initial phase depends exclusively upon NMDA receptor and/or voltage-gated Ca^{2+} channels activation (Sweatt 1999).

In addition to the work by Ungless et al. (2001), several other studies demonstrated synaptic plasticity in the brain areas relevant to drug addiction. In particular, it has been shown that both LTP and LTD can be elicited at the excitatory synapses in the NAcc (for example see Artola and Singer 1993; Pennartz et al., 1993; Kombian and Malenka, 1994; Hyman et al. 2006; Nestler, 2005 Thomas and Malenka, 2003). Like other forms of plasticity studied in the hippocampus, both LTP and LTD in the hippocampus require NMDA receptors activation and elevation in postsynaptic calcium levels. Instead, LTP in this brain area has the unique feature that the enhancement of the AMPA receptor-mediated component is accompanied by a decrease in the NMDA receptor-mediated component (Kombian and Malenka, 1994).

Considered the above-mentioned evidences of functional interactions between the endogenous opioid system and addiction to non-opioid drugs of abuse, it has recently been proposed that synaptic plasticity in the nucleus accumbens -the major reward area of the brain– might be modulated by endogenous opioid peptides. In support of this hypothesis it has been shown that κ-receptor activation inhibits glutamatergic transmission in the NAcc through an action at the presynaptic terminal (Hjelmstad and Fields, 2001). In addition, Martin et al. (1997), demonstrated that μ opioid receptors regulates NMDA receptor-mediated processes by reducing glutamate release presynaptically, while augmenting responses of NMDA receptors postsynaptically.

These observations, combined with the fact that endogenous opioids are physiologically involved in reward, suggest a more general common role for the endogenous opioid system in the mediation of the addictive properties of non-opioid drugs of abuse. Therefore, the present project was aimed at studying whether endogenous opioids are involved in relapse to alcohol and cocaine, and to verify if opioid antagonists may interfere with synaptic plasticity in the NAcc.

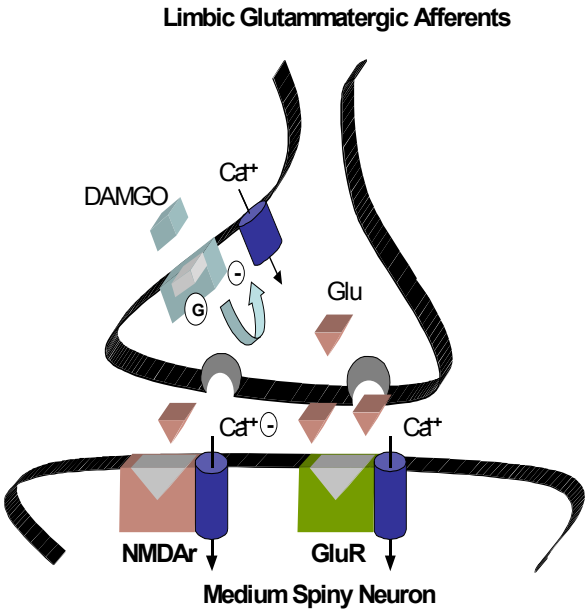


Figure 5. Schematic representation of the hypothetical model of μ-opioid receptor regulation on the glutamatergic synapses, as proposed by Martin et al. (1997). The μ-selective agonist DAMGO, acts at the μ-opioid receptor (MOR) at both pre- and post-synaptic level. Pre-synaptic activation inhibits glutamate release, probably by inhibiting calcium currents.

1.8. **SPECIFIC AIMS OF THIS STUDY**

1) To delineate the involvement of the environmental context on alcohol-seeking behavior after long-term abstinence, using the reinstatement model.
2) To determine whether the endogenous opioid system is involved in the mediation of context-induced relapse to alcohol addiction.
3) To verify whether opioid antagonists influence cocaine-seeking behavior in response to cocaine-associated cues.
4) To study the effect of blockade of endogenous opioids receptors on LTP in the NAcc.

2.1. Behavioral study

2.1.1. Alcohol self-administration

The part of the project regarding the aclohol self-administration training was entirely carried out in the laboratory of Prof. Patricia H. Janak, Ernest Gallo Clinic and Research Center, Neurology Department, UCSF, San Francisco, CA, USA.

Animals. Male Long-Evans rats (Harlan, Indianapolis, Ind., USA), weighing 150-180 g at the beginning of the experiments, were used. Rats were housed individually in a climate-controlled facility with a 12-h light/dark cycle (lights on at 6:00 a.m., off at 6:00 p.m.). Standard laboratory rat chow and water were available *ad libitum* in the home cage, except as noted below. All training and experimental sessions were conducted during the light cycle between 11 a.m. and 2 p.m. Attempts to minimize animal discomfort and to limit their numbers were made. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Ernest Gallo Clinic and Research Center at the University of California, San Francisco, and are in accordance with "PHS Policy on Humane Care and Use of Laboratory Animals," Office of Laboratory Animal Welfare, National Institutes of Health, revised 2002.

Drugs. Ethanol solutions were prepared in tap water from 95% (v/v) ethanol. Naltrexone hydrochloride (Sigma, St. Louis, Mo., USA) was dissolved in saline and was injected subcutaneously (SC) at a dose of 0.30 mg/kg 20 min prior to the reinstatement test. This dose was chosen because similar doses have been shown to reduce both ethanol-primed and cue-induced reinstatement of ethanol-seeking in rats (Katner et al., 1999; Le et al., 1999). The injection volume was 1 ml/kg.

Apparatus. Operant conditioning chambers (Med Associates Inc., Georgia, Vt., USA) were enclosed in sound attenuating, ventilated environmental cubicles. Each chamber was equipped with a drinking reservoir (3 cm in diameter) located 3 cm above the grid floor in the center of the right wall of the chamber, and photocells detected entries to the reservoir. Syringe pumps delivered ethanol solution into a fluid receptacle (ethanol port). Two retractable levers (one active and one inactive, on the right and left of the receptacle, respectively) were located 7 cm above the grid floor and 1 cm from the right or left edge of the side walls. A computer with Med-Associates software controlled fluid delivery and recorded operant responses and entries into the ethanol port.

Two-bottle choice procedure. At least one week after arrival from the vendor, subjects were trained to consume alcohol using a variation of the sucrose fading procedure (Samson, 1986), wherein alcohol and water solutions were available 23.5 hr/day, on the home cage. First, rats were given access to a solution containing 10% v/v ethanol + 10% w/v sucrose for 1 week. This process was repeated with a gradual decrease in the sucrose concentration (i.e. 5%, 2%, and finally 0%) every week. During this procedure water was always available. To avoid a side preference, the position (left or right) of each solution on the cage was alternated daily. At the end of this procedure the oral ethanol operant self-administration training began. Subjects trained to self-administer sucrose received no treatment during this time.

Data analysis. The total number and time of occurrence of lever presses, and the number of photocell breaks detected at the ethanol port were measured. These measures were analyzed using a paired t-tests and ANOVA as appropriate. Significant main effects and interactions were followed by planned comparison's using the Students Newman-Keul's test, or the post hoc LSD test (Experiment 1). The latencies to the first active lever press and to the first port entry were

compared using the nonparametric Mann Whitney rank sum test. SigmaStat (SPSS, Inc., Chicago, IL) was used for all data analysis.

Experiment 1: Context-induced reinstatement of ethanol- and sucrose-seeking behavior in water-restricted subjects.

Animals underwent 24 hours of water restriction before the first operant conditioning session. From this day onward, subjects received 1-hour free access to water in the home cage. When the delay between sessions was longer than 3 days, water restriction started 24 hours before the experimental session. Subjects in the ethanol (n=12) and sucrose groups (n=10) were trained to self-administer a solution of either ethanol (10% v/v) or sucrose (10% g/v) on a continuous fixed-ratio-1 schedule of reinforcement (FR-1), in response to lever press. The experiment consisted of three phases: acquisition in one context (referred to as Context A) which contained a steel-rod floor, transparent walls and celing, and a strawberry air deodorizer taped to the inside of the front of the sound-attenuating chamber, extinction in a different context (referred to as Context B), which had black walls and ceiling, and a 10% acetic acid solution sprayed in the bedding underneath the smooth Plexiglas floor, and reinstatement in the acquisition context (Context A). Assignment to the contexts was random and counterbalanced. At the beginning of all sessions the house light was switched on and the retractable lever appeared in the box. Both groups were initially trained to lever press in an over-night session in Context A. Operant responses were reinforced with the delivery of 0.1 ml of either ethanol or sucrose, available in the reward cup for 3 sec. Rats next underwent 15-20 daily 60-min acquisition sessions in Context A. At the end of this phase, operant responses were extinguished in Context B by eliminating reward delivery (4-6 daily sessions). Following extinction, eight and six randomly chosen animals from the ethanol and sucrose groups, respectively, were tested for operant response reinstatement with no reward availability in the acquisition context (Context A). Reinstatement sessions occured the day after the last extinction session, 2 weeks later, and 3 weeks later. Animals were also tested in the extinction context (Context B) 15 days after the

last extinction session. The remaining four animals from each group (ethanol and sucrose) were tested 24 hrs after the last extinction session, for novelty effects on operant response reinstatement by exposing them to Context C, which had black and white diagonally striped walls (ceiling, front door and back wall), a rough semitransparent sheet of Plexiglas covering the grid floor, and a green pine-scented air freshener taped on the back wall.

Experiment 2: Reinstatement of ethanol-seeking behavior after placement in the context previously paired with ethanol availability in non water-restricted subjects

To facilitate the learning of the lever press response, 13 subjects were water-deprived for 24 hours and were then placed overnight (12-14 h) in the operant chambers. The following day, daily 1-hour sessions began. During the overnight session and for the first 5 days of training only the active lever extruded from the wall, and each lever press resulted in the delivery of 0.1 ml of 10% ethanol (10E) (fixed-ratio-1 (FR-1) schedule). At this time of training water availability was limited to 1 hour a day, at the end of each session. From day 6 and on, a second, inactive, lever was introduced, the pressing of which produced no further consequence. In addition, the program was set so that pressing the active lever resulted in reward delivery only after the animal had poked its head into the ethanol port. On the same day and for the next two days, access to water in the home cage was gradually increased; starting on day 8 and during all subsequent training and testing, water was available ad libitum in the home cage. During the self-administration training, subjects underwent 38 daily sessions that lasted one hour each, in either context A or B, counter-balanced across subjects. At the end of the self-administration phase, rats were subjected to daily 1-hour extinction sessions for a total of 15 days. This phase took place in the context that a subject had not been trained within, namely context B for the animals that were trained to self-administer ethanol in context A and vice versa. During these sessions, responses at the previously active lever activated the syringe pump, but no ethanol was delivered. The ability of the environmental context to reinstate responding for

ethanol was tested one day after the final extinction session. It consisted of a single 1-hour session in which subjects were placed back into the self-administration context. During this session, responses at the previously active lever activated the syringe pump, but no ethanol was delivered.

Experiment 3: Effect of the opioid antagonist naltrexone on the context-induced reinstatement of ethanol-seeking behavior

There were twenty-two subjects in Experiment 3, and they were trained exactly as subjects in Experiment 2. During the extinction sessions, these subjects were habituated to the s.c. injection procedure by administering saline prior to the last three extinction sessions. On the reinstatement day, one-half of the subjects were treated with naltrexone (0.3 mg/kg) 20 minutes prior to the behavioral session. The second half of subjects received a saline injection under the same conditions. All subjects were placed back into the ethanol self-administration context for the 1-hour reinstatement test. During this test, responses at the previously active lever activated the syringe pump, but no ethanol was delivered.

Experiment 4: Test of the potency of the odor stimulus to control context-induced reinstatement of ethanol-seeking

The eight subjects in Experiment 4 were trained in the following manner: following 24 hours of water restriction, subjects were placed overnight (12-14 h) in the operant chambers; this was repeated for 2 more nights. The following day, daily 1-hour sessions began, and water restriction was terminated. For all sessions, both an active and inactive lever was present. Responding on the active lever was reinforced by 0.1 ml of 10% ethanol (10E) on an FR-1 schedule for the first 5 days, followed by 4 days on an FR-2 schedule. The following session, all subjects were moved to an FR-3 response requirement to determine if a higher response requirement would increase responding during test. During the self-administration training, subjects underwent 27 daily sessions that lasted one hour each in a specific context, context A or B, as described for Experiment 1. To determine whether context-dependent reinstatement is observed after a longer

extinction training, rats were subjected to daily 1-hour extinction sessions for a total of 30 sessions in the context (A or B) in which they had not been trained. At this time, two of the subjects still were responding more than 10 times on the ethanol lever, and so we adopted an extinction criterion: subjects were run under extinction conditions until they reached a criterion of < 6 presses over 3 sessions. This criterion was maintained during the series of extinction sessions conducted between the four reinstatement tests. Dependence of context-induced reinstatement on the odor stimulus was tested by comparing the reinstating ability of the ethanol-associated context to the ethanol-associated context without the odor and to the odor presented in a novel context (white walls, green plastic floor). The effect of placement into the novel context (white walls, green plastic floor, no odor) was also tested. The order of the tests was counterbalanced across subjects to control for order effects.

Experiment 5: Test of the effect of naltrexone on responding in extinction

The same eight subjects from Experiment 4 were allowed 12 reminder sessions of ethanol self-administration in the self-administration context and were then placed on extinction in the extinction context for 15 sessions, with the assignment of context A and B consistent with the assignments for Experiment 4. The effects of naltrexone administered prior to an extinction session was compared with its effects during reinstatement. Specifically, ethanol-seeking was extinguished in the extinction context for 10 sessions, eight of which were preceded by saline injections; prior to the 11^{th} extinction session, subjects received an injection of 0.3 mg/kg naltrexone. Following an additional three extinction sessions coupled with saline injections, all subjects were tested in the ethanol context with half of the subjects receiving a saline injection and the other half receiving an injection of 0.3 mg/kg naltrexone. After an additional 4 extinction sessions coupled with saline injections, a second reinstatement test was conducted with the saline and naltrexone treatment assignments reversed.

2.1.2. Cocaine self-administration

The part of the project regarding the cocaine self-administration training was entirely carried out in the laboratory of Behavioral Pharmacology, directed by Dr. L. Cervo at the Istituto di Ricerche Farmacologiche Mario Negri, Milan.

Animals. Twenty-four male Sprague-Dawley CD®IGS rats (Charles River, Italy), weighing 225-275 g at the beginning of the experiments were housed individually in a climate-controlled facility with a 12-h light/dark cycle (lights on at 7:30 a.m., off at 7:30 p.m.) with food and water ad libitum. All training and experimental sessions were conducted between 9 a.m. and 6 p.m. All experimental procedures were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358,1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Drugs. Cocaine hydrochloride (MacFarlan-Smith, Edinburgh, UK) was dissolved in sterile 0.9% saline. Naltrexone hydrochloride (Sigma-Aldrich, Milan, Italy) was dissolved in saline immediately before use and injected s.c. in a volume of 1 mL/kg and at doses of 0.25, 1 and 2.5 mg/kg 20 min before the reinstatement tests. Doses and pretreatment times were chosen on the basis of previous findings (Gerrits et al., 2005; Burattini et al., 2006).

Apparatus. The self-administration stations consisted of standard rodent operant conditioning chambers (ENV 007, Med Associates, St Albans, VT, USA) enclosed in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with two retractable levers (one active and one inactive, 12 cm apart, 7 cm above the grid floor) and three lights (2.8 W, 24 V): the house light was positioned in the center of the ceiling, and the others on the front panel, 4 cm above each lever. Auditory stimuli were presented through a loud-speaker on the back panel. Intravenous infusions were administered by a syringe pump (Med

Associates, St Albans, VT) located inside the sound-attenuating cubicles and connected to a chronic jugular catheter on each rat's back by a connector (C313G, 5UP; Plastic One, VT, USA). The infusion tubing for cocaine was enclosed in a wire coil and screwed into the external mount of the guide cannula. This coil was connected through a swivel apparatus (Med Associates, St Albans, VT, USA) to the syringe infusion pump. A computer with dedicated software (Med Associates, St Albans, VT, USA) controlled the input and output to operant cages.

Intravenous Catheters. Catheters were made in-house according to Cervo et al. (2003). Each catheter consisted of 13 cm of silastic tubing (Hoechst Marrion Russel, Milan) fitted to a guide cannula (C313G, 5UP; Plastic One) bent at a right angle. The cannula was encased in dental cement (Heraeus Kuler GmbH, Wahrheim, Germany) and anchored to a 1,5-cm diameter circular nylon mesh (Small Parts Inc., Miami Lakes, Fla. USA). Rats were then anaesthetized with equithensin (9,7 mg sodium pentobarbital in saline, 42,6 mg/ml chloral hydrate in propylene glycol, 21,2 mg/ml Mg_2SO_4 in ethanol) at the dose of 3 ml/kg i.p. and implanted with the chronic silastic catheter in the right jugular vein. The catheters, sterilized in 70% alcohol, were implanted with the proximal end reaching the heart through the right jugular vein, continuing subcutaneously over the right shoulder, and exiting dorsally between the scapulae. During the 5-day post-surgery recovery rats received daily injections of ampicillin (Amplital®, Pharmacia Italia S.p.A., Nerviano, Milan, Italy). Catheter patency was maintained using daily IV infusions of heparinized (30 UI/ml) sterile 0,9% saline before and after each self-administration session. Subjects trained to self-administer sucrose received no treatment during this time of training.

Data analysis. The data are presented as the mean ± S.E.M. of active and inactive lever-presses during self-administration, extinction and reinstatement. In each experiment the number of cocaine infusions or sucrose pellets earned in the two separate daily sessions, the last three days of saline or no-reward self-administration and the last three days of extinction before and between the

different reinstatement sessions were analysed separately by one-way analysis of variance (ANOVA) for repeated measurements (cocaine experiment) or mixed factorial ANOVA (sucrose experiment) with sessions as the main factor. Since there was no difference in responding, these values were averaged for further analyses. Thus, the effects of naltrexone on reinstatement induced by cocaine- or sucrose-associated cues were analysed by two-way ANOVA for repeated measurement or mixed factorial ANOVA, respectively, with test session as the main factor. Post-hoc comparisons were done with the Newman-Keuls test.

Experiment 1: Reinstatement of cocaine-seeking behavior after exposure to cocaine-associated stimuli.

Eight animals were trained to self-administer cocaine hydrocloride or saline in daily sessions on a FR-1, in which an active lever press resulted in the delivery of a cocaine (0.25 mg/0.1 ml), or a saline injection, infused over a period of 6 seconds. Each reinforced response was followed by a 20 sec time-out (TO) period, during which the lever remained inactive to prevent accidental overdosing.

To facilitate the acquisition of operant responding subjects were food-restricted for 24 hours and allowed to lever press for 45-mg food pellets. During this time of training only the active lever extruded from the wall, and each lever press resulted in the delivery of one 45-mg food pellet. The first session consisted of 30 min of non-contingent delivery of one food pellet every 30 sec. From the second 30-min session food pellets were made available only after the animal had pressed the active lever. Food availability was limited to 20 g a day, at the end of each session. Subjects received a minimum of three 30-min food training sessions, and once they had earned 100 pellets they returned to *ad libitum* feeding and were surgically prepared with catheters implants, as reported above.

After five days of recovery from surgery, rats were trained to lever press for cocaine reinforcement in daily 2-hour sessions. Cocaine availability was signaled by a white noise 20dB above the background (S^{D+}), and each lever press activated simultaneously the infusion pump and the stimulus light above the active lever (CS+). The lever light remained activated during the subsequent TO period. From

day 3 and on, a second inactive lever was introduced, the pressing of which produced no further consequence. Once animals had demonstrated stable cocaine self-administration, the 2-hour sessions were divided into two one-hour sessions separated by 60 minutes, during which animals returned to their home cage. After a 2-day adaptation to this new schedule an additonal one-hour saline self-administration session was introduced. Saline availability (i.e., nonreward) was signaled by the illumination of the chamber's house light (S^{D-}) and each lever press, resulting in the delivery of a saline injection, was paired with a 20-s intermittent tone (7KHz, 70dB) (CS-). During the remainder of the discrimination training animals underwent two cocaine self-administration sessions and one saline self-administration session daily for 5 days a week. Drug and saline sessions were presented in random order. After establishing a stable pattern of responding for cocaine (i.e., the number of drug infusions per session stabilized to within ±10% on 3 consecutive days) and saline (i.e., less than 5 lever presses for three consecutive days) rats were placed on extinction conditions. During this phase rats were subjected to daily hourly extinction sessions until they reached the criterion of less than 5 lever presses for three consecutive days. During extinction both the active and the inactive lever extruded from the wall, but no discriminative stimuli were presented; responses at the previously active lever activated the syringe pump but neither cocaine or saline, nor the response-contingent cues were delivered. The day after individual animals met the extinction criterion, they were tested for operant response reinstatement. Reinstatement sessions lasted one hour each and consisted in exposing the animals to the same conditons as in the conditioning phase of the experiment, except that neither cocaine nor saline were available. Sessions started with the extension of both levers and presentation of the discriminative stimuli (either S+ or S-) which remained active throughout the entire session. Responses at the previously active lever activated the syringe pump and the conditioned stimulus: the lever light as CS+ in the reward-associated (S+) condition, or the white noise, as CS- in the non-reward associated (S-) condition. Each subject was tested once under the S- conditions and 4 times under the S+ conditions. Distinct

reinstatement tests were separated by extinction sessions; before being tested for reinstatement each animal had to meet the extinction criterion (Cervo et al., 2003, 2006). Reinstatement was measured counting the number of reinforced lever presses emitted during the test session.

Experiment 2: Effect of naltrexone on seeking behavior induced by cocaine-associated cues.

There were eight subjects in Experiment 2, and they were trained exactly as subjects in Experiment 1. During the extinction sessions, these subjects were habituated to the s.c. injection procedure by administering saline prior to the last three extinction sessions. In a within-subject design, each rat was tested once with the saline-associated cues and once with each dose of naltrexone or vehicle, and the cocaine-associated stimuli. The five reinstatement sessions were presented according to a Latin square design. Reinstatement sessions were separated by extinction sessions; before being tested for reinstatement each animal had to meet the extinction criterion (Cervo et al., 2003, 2006).

Experiment 3: Effect of naltrexone on seeking behavior induced by sucrose pellet-associated cues

To verify whether the effect of naltrexone was specific for cocaine-seeking behavior, we tested its effect on seeking-behavior induced by re-introduction of sucrose-associated stimuli. Since these cues induce gradually less reinstatement over time (Baptista et al., 2004; Cervo et al., 2006), we used a mixed between-within-subjects experimental design. This experiment was conducted under the same conditions as the previous ones, except that rats were not catheterised and the reinforcer was a 45-mg sucrose pellet (Noyes formula F, Sandown Scientific, Esher, Surrey, UK).

Two groups of eight rats were trained to press a lever for sucrose pellets with simultaneous presentation of S^Ds predictive of sucrose availability - a white noise 20dB above the background (S^{D+}) - or non-availability, i.e. the house light on (S^{D-}). Sucrose pellets were available under an FR1 schedule of reinforcement, and

active lever-presses were followed by 20-s TO, signalled by the light on above the active lever for sucrose (CS+), and a 20-s tone for no-reward (CS-). To avoid satiety each session ended after 30 min or after 30 sucrose pellets had been earned. Extinction and reinstatement test sessions were identical to those for cocaine except that they lasted only 30 min.

After rats reached the extinction criterion (i.e less than five lever presses for three consecutive sessions), they were tested for reinstatement, after naltrexone (2.5 mg/kg SC, 20-min pretreatment) or vehicle pre-treatment. Each animal underwent two test sessions, one with sucrose pellet-associated cues, and one after re-introduction of no-reward-associated stimuli. Reinstatement sessions were separated by three sessions at the extinction criterion. Rats receiving naltrexone during the reinstatement session with the sucrose pellet-associated cues received vehicle with the no-reward-associated stimuli. In the second group of rats the pairing between stimuli and treatment was reversed.

2.2 Electrophysiological study

The part of the project regarding the electrophysiological study was entirely carried out in the laboratory of Prof. Aicardi, Department of Human and General Physiology, University of Bologna, Italy.

Slice preparation. The experiments were carried out in parasagittal brain slices including NAc, obtained from young (21- to 38-day-old) male Sprague-Dawley CD®IGS rats (Charles River, Italy). Slices were prepared as previously described by Li and Kauer (2004). Briefly, rats were decapitated in deep halothane (Sigma-Aldrich, Milan, Italy) anaesthesia, their brains were rapidly removed and immerged in ice-cold artificial cerebrospinal fluid (aCSF), containing (in mM): NaCl, 119; KCl, 2,5; NaH_2PO_4, 1; $NaHCO_3$; $MgSO_4$, 1,3; $CaCl_2$ 2,5; glucose, 10; saturated with 95% O_2, 5% CO_2 (pH 7,4). The cerebellum and medial aspects of

both hemispheres were removed and parasagittal slices (400 μm) were cut from the blocked brain using a vibratome.

Electrophysiology. After recovering for at least 60 min, a single slice was transferred to a submersion recording chamber and perfused at the rate of 2 ml/min with aCSF maintained at 34°C ± 0.2°C, saturated with 95% O_2 and 5%.CO_2, and added with 20 μM bicuculline metochloride (Tocris). Recordings started 60 min after the slice was placed into the chamber. Excitatory postsynaptic field potentials (fEPSP) evoked by glutamatergic afferent pathway stimulation were recorded in the NAc with an extracellular microelectrode (glass micropipette filled with 2 M NaCl, 1-3 MΩ) connected to a dc amplifier by an Ag/AgCl electrode. Constant-current square pulses (0.2 ms, 30-300 μA, 0.033 Hz) were applied by using a stimulus generator connected through a stimulus isolation unit to a concentric bipolar electrode (70-80 KΩ) positioned at the rostral edge of the NAc, bordering the cortex, just below the anterior commisure (at ≈400-600 μm from the recording electrode in rostral direction). Stimulus intensity was adjusted to induce ≈ 50% of the maximal synaptic response. After stable baseline recording, LTP was induced by a 100-Hz tetanic stimulation, consisting of four trains [HFS(4T)] of stimulations delivered 60 s apart, each one lasting 800 ms, at test stimulus intensity. A weaker stimulation protocol was also used, consisting of two trains of stimulations, instead of four, at 100 Hz [HFS(2T)]. In two additional sets of experiments, naltrexone (30 μM) or CTOP (10 μM) (Sigma-Aldrich, Milan, Italy) were added to the slice perfusing medium during the recording period. For all experiments, the values given are the mean ± SEM.

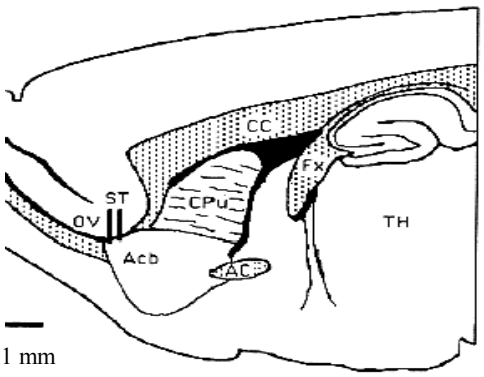


Fig 1. Schematic representation of a brain slice used in these experiments. Vertical bars indicate the stimulation electrode (ST). The abbreviation Acb (nucleus accumbens) is centered in the recording area. AC, anterior commissure; OC, corpus callosum; Cpu, caudate-putamen; Fx, fornix; OV, olfactory ventricle; TH, thalamus. Based on Paxinos and Watson (1986). Calibration: 1 mm.

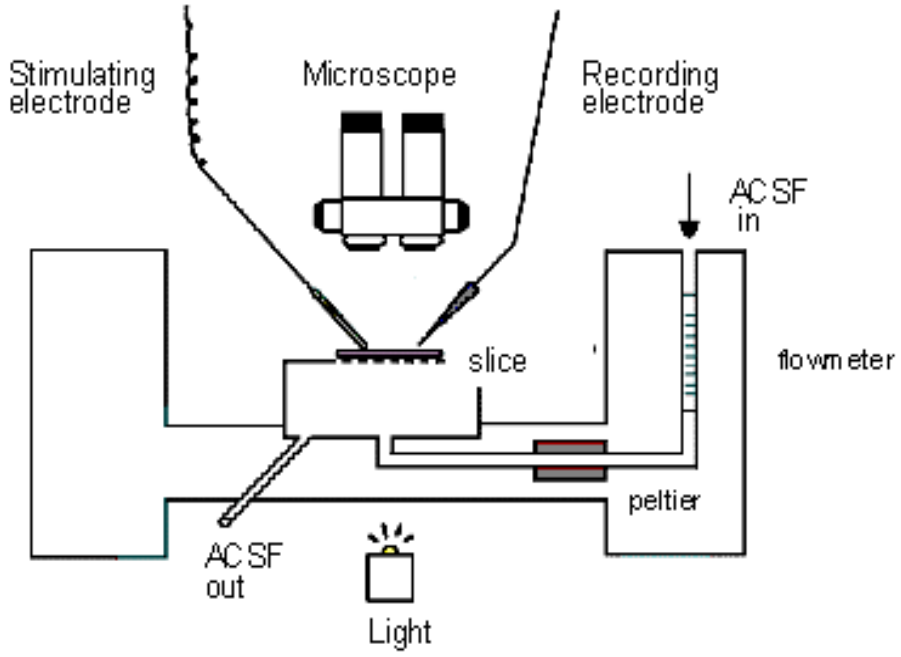


Fig. 2: Schematic representation of the recording setup.

Data Analysis. The amplitude of the synaptic peak of the fEPSP (hereinafter defined fEPSP amplitude) recorded after the tetanic stimulation were normalized with respect to the mean value of those recorded during the 10 minutes preceding the HFS, which was considered 100%. For statistical evaluations fEPSP amplitudes were recorded in five-minute intervals after the HFS. These values were compared with those recorded during five minutes preceding the HFS. Potentiation was considered significant when the mean fEPSP amplitudes were ≥10% as compared to the baseline (Ziakopoulos et al., 1999). Comparison of potentiations obtained in control conditions and in the presence of naltrexone and CTOP were done using a two-tailed Student's T-test ($p<0.005$).

3.1. Behavioral study

3.1.1. Alcohol self-administration

Experiment 1: Context-induced reinstatement of ethanol- and sucrose-seeking behavior in water-restricted subjects.

Fig. 1 shows the mean number of lever presses and rewards given during each session of acquisition/maintenance (in context A) and extinction (in context B) phases, for all the ethanol ($n = 12$) and sucrose ($n = 10$) trained subjects. The ethanol group animals consumed an average of 1.31 ± 0.07 g/kg per 1 h acquisition/maintenance session (range, 0.94–1.61). During extinction in context B, there was a gradual decrease in the number of operant responses. Compared to the final day of the acquisition/maintenance phase, mean responding in the ethanol group was significantly ($P < 0.01$, Student's *t*-test) lower by the second extinction session, while in the sucrose group the decrease was already significant ($P < 0.05$, Student's *t*-test) by the first session.

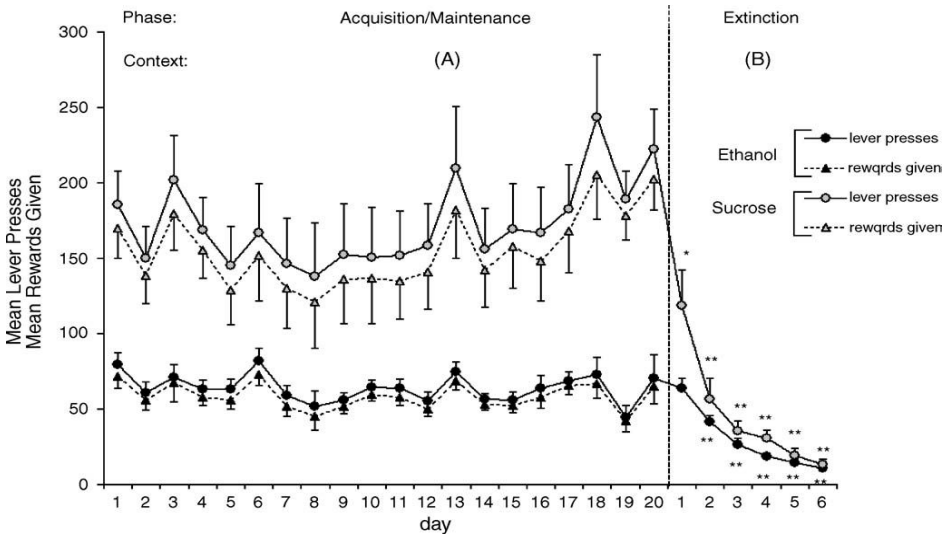


Fig. 1. Mean number of lever presses and rewards given during each session of acquisition/maintenance (context A) and extinction (context B) phases, for ethanol- trained($n = 12$) and sucrose- trained ($n = 10$) subjects. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the last acquisition/maintenance session (Student's *t*-test).

Twenty-four hours after the last extinction session, two groups of animals (ethanol, $n = 8$; sucrose, $n = 6$) underwent the first reinstatement test in context A with no reward delivery (reinstatement test, day 1, Fig. 2). Two and 3 weeks later, both groups were tested again for reinstatement in context A (reinstatement test, days 14 and 21, Fig. 2). Both groups were also tested in context B 15 days after the first reinstatement test (reinstatement test, day 15, Fig. 2). Placement in context A increased the mean number of lever presses for both the ethanol and the sucrose group, while responding in context B remained at extinction levels (Fig. 2). ANOVA of lever-press responding during the last extinction session and all four reinstatement sessions revealed significant context-dependent response reinstatement effects for both ethanol and sucrose groups ($F_{4,28} = 8.55$, $P < 0.0001$ and $F_{4,20} = 12.16$, $P < 0.00001$, respectively). Post hoc tests revealed that both groups significantly increased the number of operant responses when tested in the acquisition context (context A) 1 day after the extinction in context B (extinction versus reinstatement test, day 1, $P < 0.005$ for ethanol and $P < 0.0001$ for sucrose), as well as 2 weeks after the first reinstatement (extinction versus reinstatement test, day 14, $P < 0.0005$ for ethanol and $P < 0.01$ for sucrose). Placement back into context A 3 weeks later did not result in significantly increased responding in the sucrose group relative to the first extinction ($P = 0.19$), but did result in significantly increased responding in the ethanol group ($P < 0.005$). For both groups, responding in context B, tested 15 days after the first reinstatement was not different from the final extinction day in context B (all P's > 0.05). In addition, responding on all reinstatement days was significantly greater than that in the second context B exposure 15 days after extinction (reinstatement test, day 15 versus reinstatement test, days 1, 14 or 21, all P's < 0.05). The stability of the response reinstatement in context A was also tested. Analysis of the three reinstatement sessions in context A revealed a time-dependent effect on responding for the sucrose group ($F_{2,10} = 10.10$, $P < 0.004$). Specifically, responding was higher during the first reinstatement session as

compared to both the second (reinstatement test, day 14) and third (reinstatement test, day 21) session for the sucrose group ($P < 0.05$ and $P < 0.005$, respectively). In contrast, there was no effect of time for the ethanol group ($F_{2,14} = 0.17$ ns), indicating that responding was not different across the three reinstatement test.

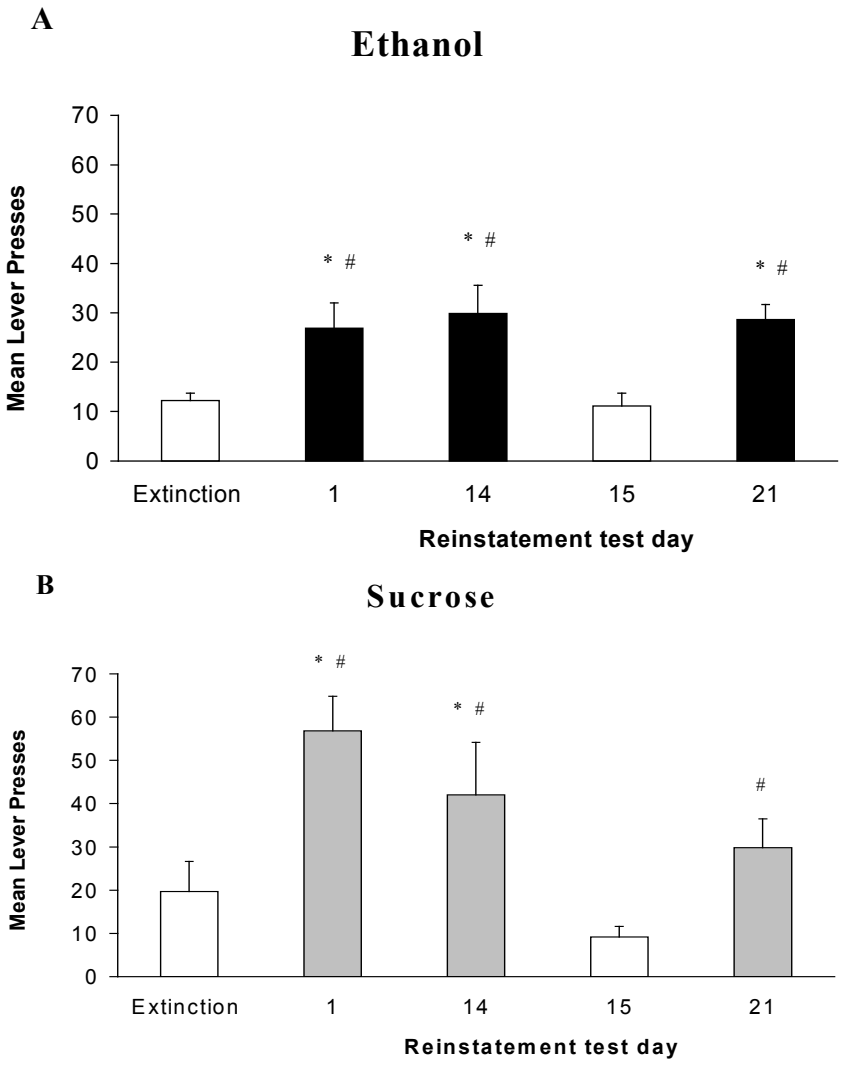


Fig 2. Mean number of lever presses emitted by (A) the ethanol-trained subjects ($n = 8$) and (B) the sucrose-trained subjects ($n = 6$) in the extinction context B (white columns) and in the acquisition/maintenance context A (black and gray filled columns for ethanol and sucrose group, respectively). Twenty-four hours after the last extinction session (extinction), animals underwent the first reinstatement test in context A (reinstatement test, day 1). Two and 3 weeks later, both groups were tested again for reinstatement of the operant response in context A (reinstatement test, days 14 and 21), and in context B 15 days after the first reinstatement test (day 15). No reward was delivered for any of the tests. $^{*}P < 0.01$ as compared to extinction and $^{\#}P < 0.01$ as compared to reinstatement test, day 15 (post hoc LSD test).

Twenty-four hours after the last extinction session, subjects trained to self-administer ethanol ($n = 4$, Fig. 3A) and sucrose ($n = 4$, Fig. 3B) were tested in context C, a novel environment, to verify whether exposure to a context different from the extinction context is sufficient to increase operant responding. As shown in Fig. 3, exposure to the novel environment increased the mean number of lever presses for both ethanol and sucrose groups. However, a comparison of responding during exposure to the novel environment to that on the last extinction session revealed a significant difference only for the sucrose group (Student's *t*-test, $P < 0.05$).

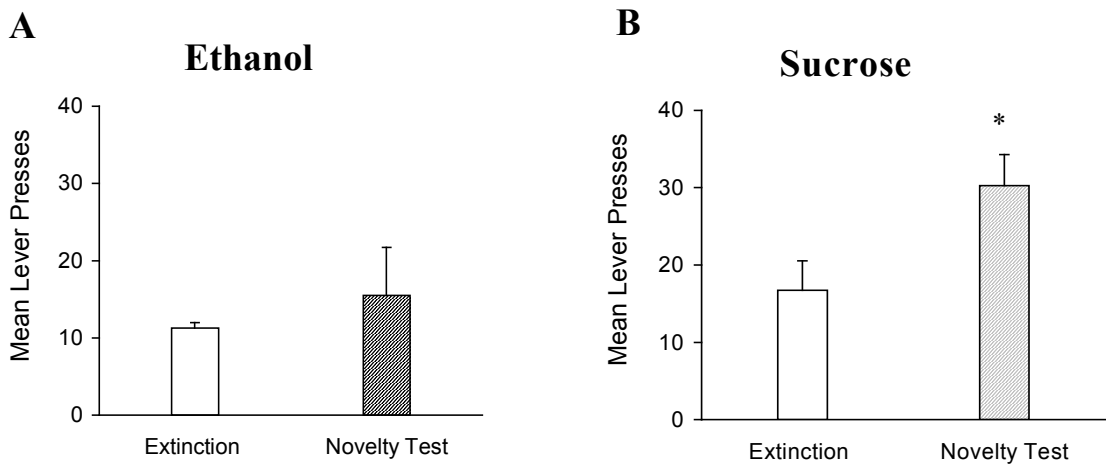


Figure 3. Mean number of lever presses emitted by (A) the ethanol-trained subjects ($n = 4$) and (B) the sucrose-trained subjects ($n = 4$) in the extinction context B (white columns) and in the novel context C (black and gray columns for the ethanol and the sucrose group, respectively). Twenty-four hours after the last extinction session (extinction), animals underwent a reinstatement test in context C (novelty test) with no reward delivery. $^{*}P < 0.05$ (Student's *t*-test).

Experiment 2: Reinstatement of ethanol-seeking behavior after placement in the context previously paired with ethanol availability in non water-restricted subjects

Following sucrose fading in the home cage, subjects in Experiment 2 (N=13) were trained to lever-press for 10% ethanol in the self-administration context. The mean number of ethanol lever presses during the last 5 days of ethanol self-administration was 44.38 + 5.69. These values correspond to an estimated ethanol consumption of 0.57 + 0.07 g/kg. The total number of port entries made by subjects during the behavioral session was treated as an additional measure of ethanol-seeking behavior, and during the last 5 days of self-administration the mean number of entries was 67.71 + 6.14. Extinction was conducted in the alternative context; behavioral responding decreased across 2 weeks of extinction. A comparison of the average number of responses the final 5 days of self-administration with the last day of extinction indicated that all three measures significantly decreased [(Paired t-tests, ethanol-associated lever presses: $t(12) = 7.15$, $P < 0.001$, inactive lever presses: $t(12) = 3.83$, $P < 0.003$, port entries: $t(12) = 10.34$, $P < 0.001$)] (Figure 4 A and B).

Rats were tested for reinstatement following the return to the self-administration context. Exposure to the ethanol-paired environment significantly reinstated ethanol-seeking behavior (Figure 4). A comparison of responding on the last day of extinction with responding during the reinstatement test session found a significant increase in the number of responses at the previously active ethanol lever (paired t-test, $t(12) = -4.39$, $P < 0.001$; Fig. 4A), as well as an increase in the number of port entries ($t(12) = -3.39$, $P < 0.005$; Fig. 4B) during the reinstatement test. Inactive lever responding did not change significantly ($t(12) = 1.23$, $P = 0.24$; Fig. 4A).

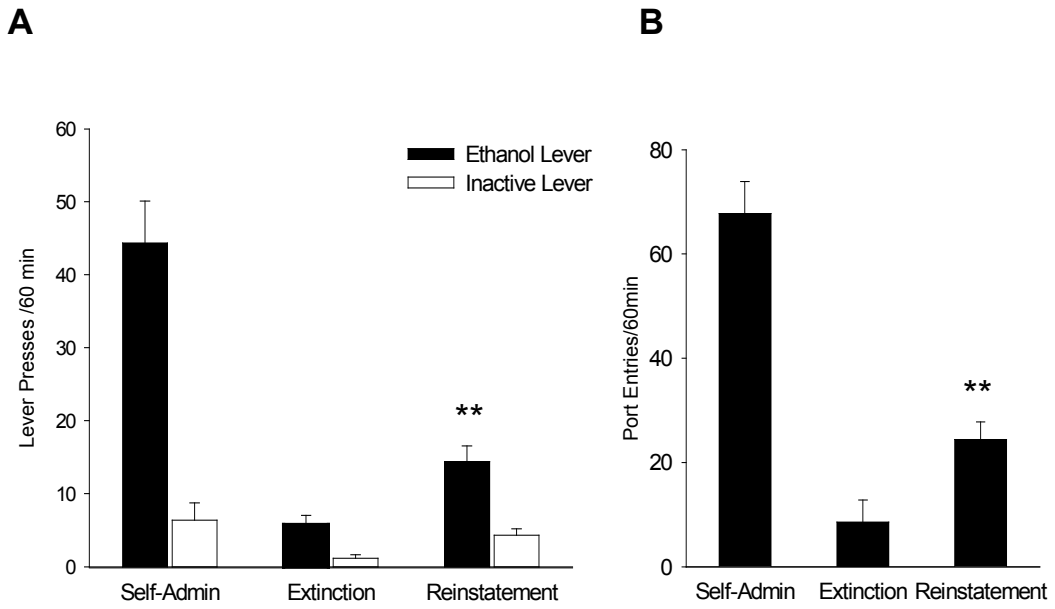


Figure 4. Return to the self-administration context after extinction increases ethanol-seeking. A. Mean (+/- S.E.M.) ethanol lever and inactive lever presses during the final 5 days of self-administration (Self-Admin), the final day of extinction, and during the reinstatement test. Self-administration and the reinstatement test occurred in the same context. Extinction occurred in a different context. No ethanol was delivered during the reinstatement test. N=13. **P<0.001, as compared to extinction responding. B. Mean (+/- S.E.M.) entries into the ethanol port by the same subjects as in Fig. 4A. **P<0.01, as compared to extinction responding

Experiment 3: Effect of the opioid antagonist naltrexone on the context-induced reinstatement of ethanol-seeking behavior

During the last 5 days of the ethanol self-administration phase, rats (N=22) were performing the operant response task with a mean number 54.33 + 5.40 ethanol lever presses. This value corresponds to an estimated g/kg of 0.62 + 0.06. The mean number of entries into the ethanol port during the last 5 self-administration sessions was 76.88 + 6.98. Ethanol-seeking was extinguished in the extinction

context across 15 daily sessions during which ethanol was not available. A comparison of the average number of responses the final week of self-administration with the last day of extinction indicated that all three measures significantly decreased (Paired t-tests, active lever presses: $t(21) = 9.25$, $P<.001$, inactive lever presses: $t(21) = 5.26$, $P<.001$, port entries: $t(12) = 10.89$, $P<.001$) (Figure 2).

Following administration of either saline (N=11) or 0.3 mg/kg naltrexone (N=11), subjects were placed back into the self-administration context. A two-factor ANOVA found a main effect of Session ($F(1,20)=18.98$, $P<0.001$) and a significant Session x Treatment interaction ($F(1,43)=5.05$, $P<0.037$), but no main effect of Treatment ($F(1,20) = 3.11$, $P = 0.09$). As can be seen in Figure 5A, saline-treated subjects showed significant increases in ethanol lever responding during reinstatement relative to extinction (Student-Newman-Keuls Test, $P < 0.001$), whereas naltrexone-treated subjects did not ($P=0.15$). In addition, the number of ethanol lever presses made by subjects that received naltrexone was significantly less than that made by saline-treated subjects ($P<0.008$). The analysis of port entries indicated a significant main effect of Session ($F(1,20)=29.86$, $P<.001$), accounted for by significant increases in port entries during reinstatement by subjects treated with both saline- ($P<0.001$) and naltrexone ($P<.015$) as compared to their extinction responding (Figure 5B). Figure 5B shows that the number of port entries during reinstatement is lower in naltrexone-treated subjects, but the main effect of Treatment and the Session x Treatment interaction were not significant. There were no effects of reinstatement or naltrexone treatment on mean inactive lever responding (main effect of Session: $F(1,20)=0.11$, $P=0.75$; main effect of Treatment: $F(1,20)=0.49$, $P=0.49$; Session x Treatment interaction: $F(1,43)=0.95$, $P=0.34$).

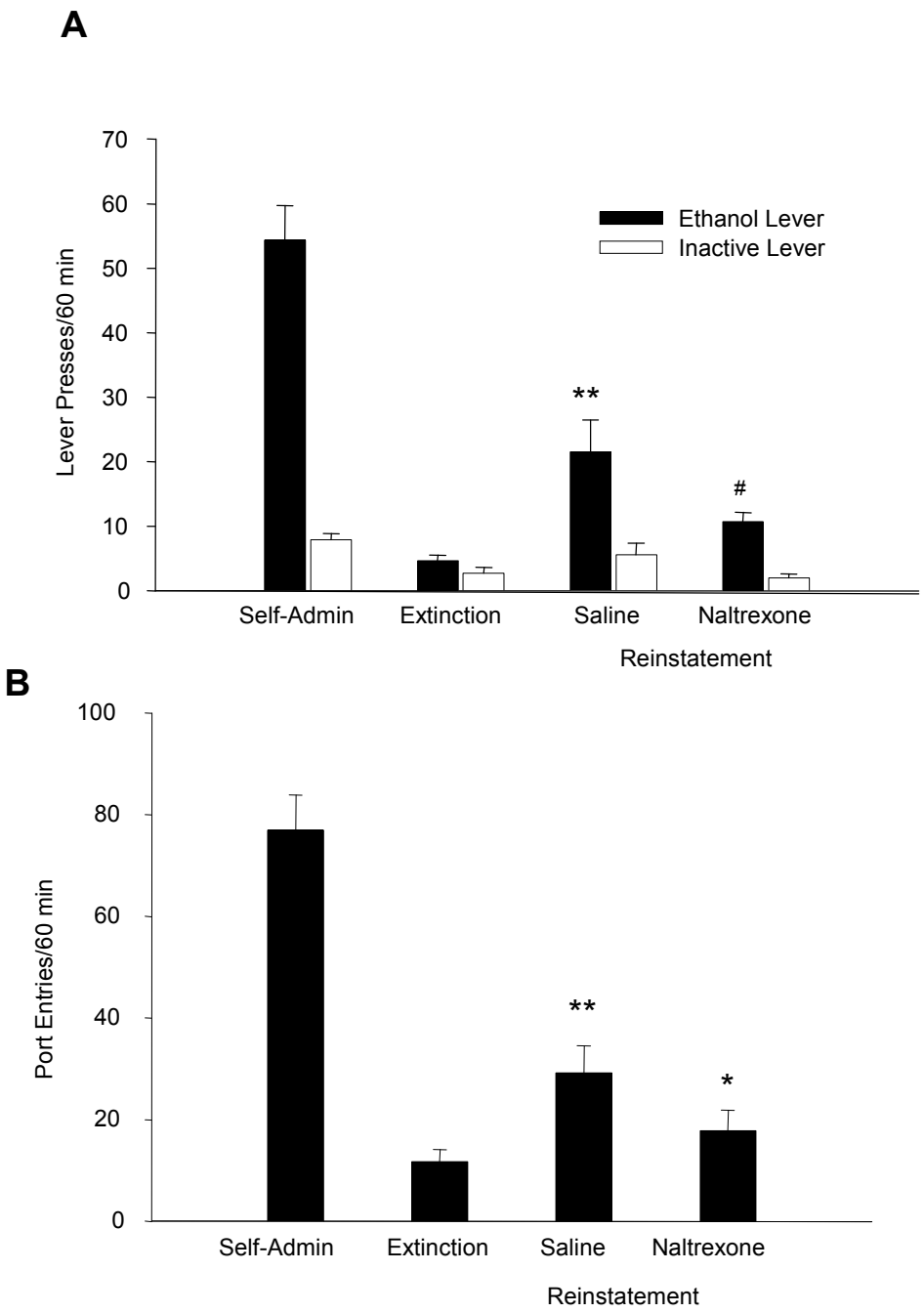


Figure 5. Naltrexone reduces the context-induced increases in responding at the ethanol lever when subjects are returned to the self-administration context.
A. Mean (+/- S.E.M.) ethanol lever and inactive lever presses during the final 5 days of self-administration (Self-Admin), the final day of extinction, and during the reinstatement test. Self-administration and the reinstatement test occurred in the same context. Extinction occurred in a different context. Subjects received a s.c. injection of saline (N=11) or naltrexone (0.3 mg/kg) (N=11) 20 minutes before the reinstatement test. No ethanol was delivered during the reinstatement test. **P<0.001, as compared to extinction responding; $^{\#}$P<0.01 as compared to saline reinstatement. B. Mean (+/-) S.E.M. entries into the ethanol port by the same subjects as in Fig. 2A. *P<0.05, **P<0.001, as compared to extinction responding.

The effect of naltrexone treatment on the latency to initiate the first lever press at the ethanol-associated lever and the latency to enter the port upon placement into the ethanol self-administration context was examined. These data are given in Table 1. No significant difference between naltrexone and saline-treated subjects was found for any of these measures, using the non-parametric Mann Whitney rank sum test (all P's>0.05).

Because naltrexone attenuated responding but not the latency to the first press, the pattern of responding during the reinstatement test was examined to define the effect of naltrexone on response reinstatement. An examination of Figure 6 reveals that the number of responses at the ethanol-associated lever made by saline-treated subjects and naltrexone-treated subjects was the same for the first 5 minutes, and then cumulative responding diverged subsequent to this time. An ANOVA test conducted on the cumulative number of ethanol lever presses in each 5-min time bin revealed a main effect of Session Time ($F(11,220)=12.43$, $P<0.001$) and a significant Treatment x Session Time interaction ($F(11,220)=3.75$, $P<0.001$), but not a main effect of Treatment ($F(1,20)=2.71$, $P=0.12$). The significant interaction indicates that the pattern of responding over the course of the session was significantly different between the two groups. Student-Newman-Keuls tests revealed that responding in the first 5 minutes by saline-treated subjects was significantly lower than cumulative responding during each of the remaining 11 bins, indicating that context continued to elicit responding throughout the session. In contrast, the first 5 minute time bin was not significantly different from the final 55-60 minute time bin for naltrexone-treated subjects ($P=0.17$). Examination of each individual time point revealed that naltrexone-treated subjects' cumulative responding was significantly less than that of saline-treated subjects during minutes 40-60 (the final 4 time bins; $P<0.05$ - $P<0.01$; see Figure 6).

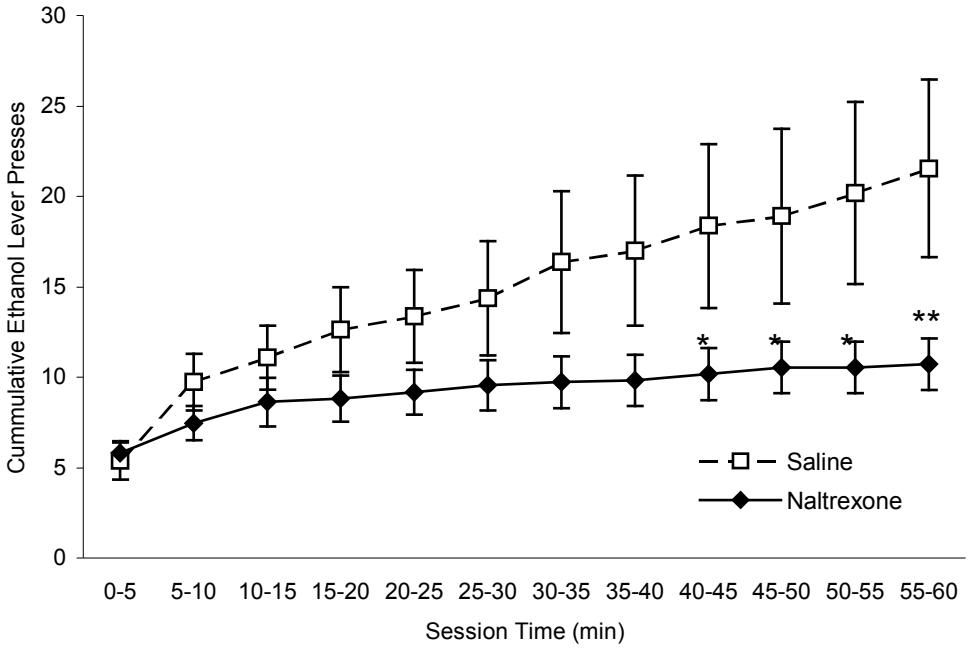


Figure 6. Time course of the effects of naltrexone on responding during the reinstatement test. Cumulative responding at the ethanol-associated lever depicted in 5 minute intervals for the entire 60-minute reinstatement test session following saline or naltrexone (0.3 mg/kg) treatment. Naltrexone did not alter the number of responses in the first 5 minutes. Cumulative responding during minutes 40-60 was significantly less following naltrexone treatment as compared to saline treatment. *P<0.05, **P<0.01. Same subjects as in Fig. 5.

Experiment 4: Test of the potency of the odor stimulus to control context-induced reinstatement of ethanol-seekin

During the last 5 days of the ethanol self-administration phase, rats (N=8) were performing the operant response task with a mean number 67.3 +/- 19.5 ethanol lever presses (FR-3). This value corresponds to an estimated g/kg of 0.35 + 0.09. The mean number of entries into the ethanol port during the last 5 self-administration sessions was 85.07 + 18.86. Unlike in Experiments 2 and 3, ethanol-seeking was extinguished in the extinction context to criterion. The mean days to criterion (average total lever presses over 3 days less than 6) was 35.7 +/-

3.8 (range, 30-53The ability of the ethanol context to reinstate responding for ethanol was compared with the reinstatement obtained from the ethanol context without the odor, the odor presented in a novel context, and a novel context alone, with the order of the tests counterbalanced across subjects. Subjects received extinction sessions in the extinction context between each reinstatement test. Figure 7 depicts the mean responding during self-administration, extinction, and the reinstatement tests. The data from two subjects was lost due to procedural error, leaving a group size of six. A 2-factor repeated measures ANOVA with factors of Session (extinction session vs. test session) and Reinstatement Type found a main effect of Session [$F(1,5) = 19.72$, $P<0.008$] and a significant Session x Reinstatement Type interaction [$F(3,15) = 3.72$, $P<0.036$]. Further comparisons indicated that these effects were due to an increase in responding during reinstatement with the full ethanol context ($P<0.001$); no other groups showed significant reinstatement, although the responding during exposure to the context without the odor approached significance ($P=0.08$) (Figure 7A). In addition, responding during the reinstatement test to the full ethanol context was significantly greater than responding during exposure to the context without the odor ($P<0.03$), exposure to the odor alone ($P<0.004$), and exposure to a novel context ($P<0.001$), while responding during these later three tests was statistically equivalent. Responding during extinction was not different for any of the four reinstatement conditions.

Analysis of the port entries during extinction and the four different reinstatement conditions found a significant effect of Session [$F(1,5) = 7.33$, $P<0.043$], carried primarily by a significant increase in port entries following placement into the full ethanol context ($P<0.005$), but not any of the other tests (Figure 7B).

In contrast to Experiments 2 and 3, there was a significant effect of Session [$F(1,5) = 8.27$, $P<0.05$] and a significant Session x Reinstatement Type interaction [$F(3,15) = 3.8$, $P<0.04$] for inactive lever responding, accounted for by an increase in inactive responding during the reinstatement test for the full ethanol context ($P<0.002$) and the novel context ($P<0.03$).

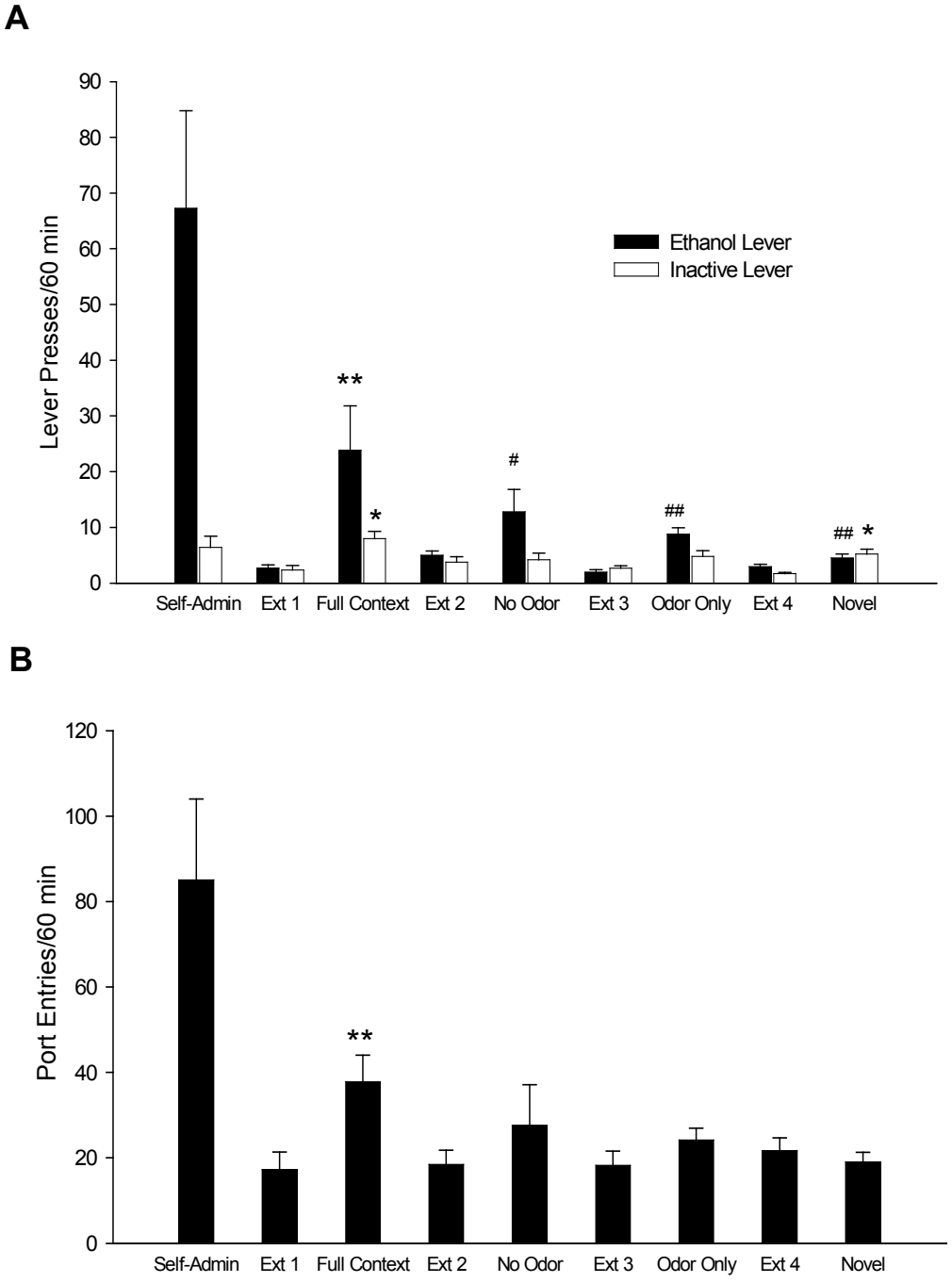


Figure 7. The olfactory stimulus alone does not reinstate ethanol seeking.
A. Mean (+/- S.E.M.) ethanol lever and inactive lever presses during the final 5 days of self-administration (Self-Admin) and during four different reinstatement tests including the full configuration of stimuli of the ethanol context (Full Context), the ethanol context without the olfactory stimulus (No Odor), the olfactory stimulus presented in a novel context (Odor Only), and a Novel context. The mean responding in the extinction context the last day of extinction before each test is also depicted. . **P<0.001, *P<0.05 as compared to Extinction responding; $^{\#\#}$P<0.005, $^{\#}$P<0.05 as compared to reinstatement in the full context. B. Mean (+/-) S.E.M. entries into the ethanol port by the same subjects as in Fig. 7A. **P<0.005, as compared to extinction responding

Experiment 5: Test of the effect of naltrexone on responding in extinction

The subjects from Experiment 3 were given 12 reminder ethanol self-administration sessions at FR-3. For the last five days of retraining, they responded at the ethanol-associated lever with a mean of 92.43 +/- 22.11 responses, corresponding to an estimated g/kg of 0.42 +/-0.09. These means do not include the data from one subject who was removed from the study at this point, after responding less than 5 presses per session for the last 5 self-administration sessions, leaving a group size of 7. The mean number of entries into the ethanol port during the last 5 self-administration sessions was 122.17 +/- 21.74. Unlike their first experience with extinction, these subjects extinguished rapidly on this occasion. A comparison of the average number of responses the final week of self-administration with the last day of extinction indicated that all the number of ethanol lever and port entry responses decreased (Paired t-tests, active lever presses: $t(6) = 3.95$, $P<0.009$, port entries: $t(6) = 3.93$, $P<0.009$). The mean number of inactive responses did not change ($p=0.24$), as they were already quite low during self-administration.

Placement back into the ethanol context reinstated ethanol lever responding, and this increase was attenuated by naltrexone; in contrast, naltrexone administered prior to placement into the extinction context did not alter responding (Figure 8). When responding following saline and naltrexone injection were compared with responding during the prior extinction session we found a main effect of Test Context [$F(1,6) = 30.44$, $P<0.001$], a main effect of Session [$F(2,12) = 11.31$, $P<0.002$], and a significant Test Context x Session interaction [$F(2,12) = 7.81$, $P<0.007$]. Planned comparisons revealed that treatment with saline prior to placement into the ethanol context significantly increased active lever responding ($P<0.001$) relative to their extinction baseline, whereas after naltrexone treatment, significant reinstatement was not observed ($P=0.07$) (Figure 8A). In addition, active lever responding after saline was significantly greater than after naltrexone in the ethanol context ($P<0.001$). Treatment with saline or naltrexone prior to placement into the extinction context did not significantly alter active lever responding (all P's > 0.05).

To better compare the effect of naltrexone injection in the extinction and ethanol self-administration environments, a 2 x 2 ANOVA comparing Treatment (saline vs. naltrexone) and Context (extinction vs. ethanol self-administration) was conducted. This analysis found a main effect of Treatment [$F(1,6) = 11.66$, $P<0.015$], Context [$F(1,6) = 16.36$, $P<0.007$], and a Treatment x Context interaction [$F(1,6) = 20.93$, $P<0.004$] when active lever responding was considered. There was a significant difference between responding under saline and naltrexone in the ethanol self-administration context ($P<0.001$), but not in the extinction context ($P=0.19$), suggesting that naltrexone did not affect responding when tested under extinction conditions.

There were no significant effects on inactive lever responding (Figure 8A) or on port entries (Figure 8B) in this experiment. In addition, the latency to first press and to first port entry following placement in the extinction or reinstatement context did not differ after saline or naltrexone treatment (Mann Whitney rank sum test, all P's>0.05; Table 1).

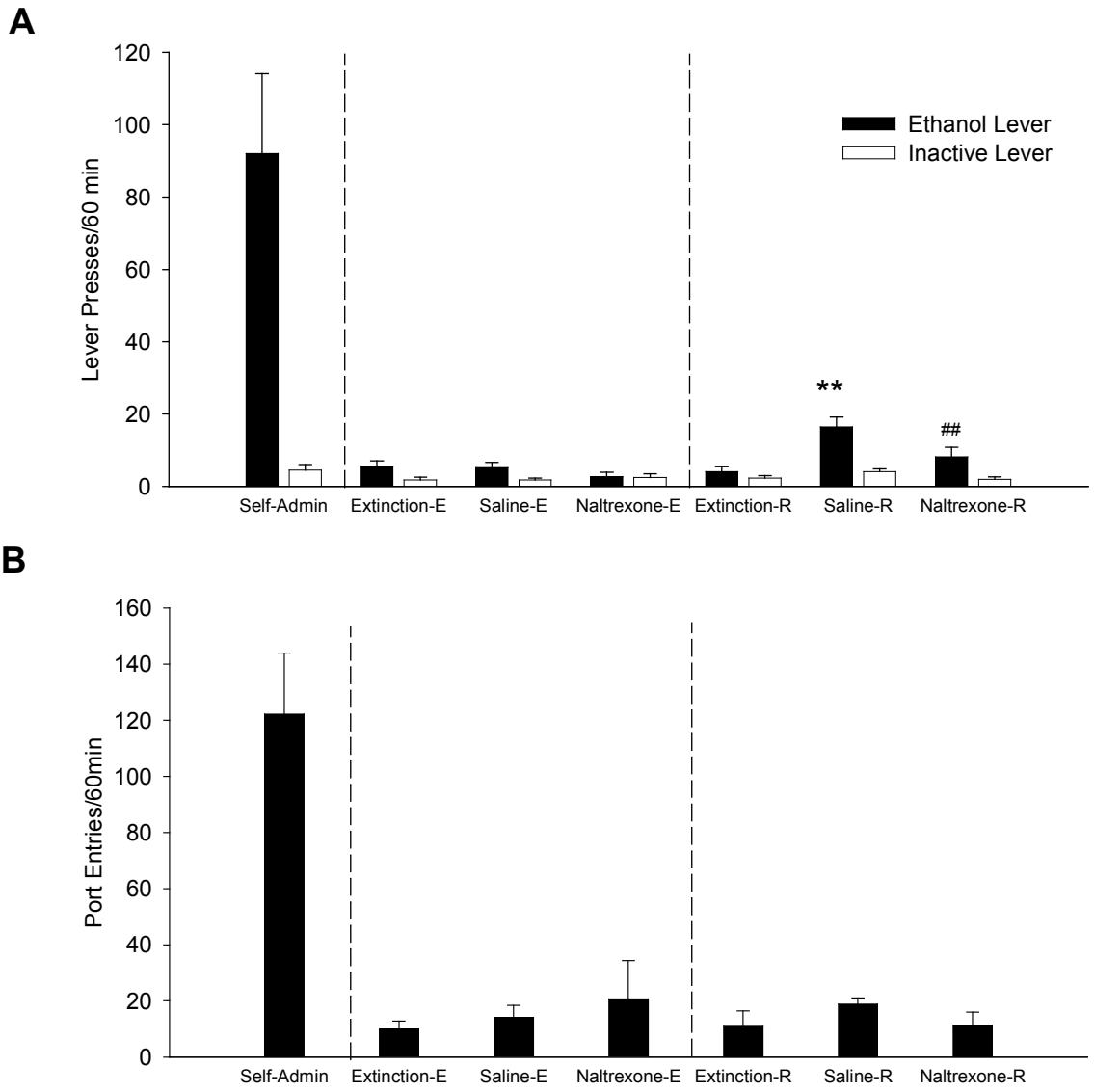


Figure 8. Naltrexone reduces the context-induced increases in responding at the ethanol lever when subjects are returned to the self-administration context, but has no effect in the extinction context.

A. Mean (+/- S.E.M.) ethanol lever and inactive lever presses during the final 5 days of self-administration (Self-Admin), during saline or naltrexone injection in either the extinction context (Saline-E, Naltrexone-E) or the ethanol self-administration reinstatement context (Saline-R, Naltrexone-R). Responding in the extinction context on the last day of extinction before treatment is shown for each series of injections (Extinction-E, Extinction-R). Saline or naltrexone (0.3 mg/kg) was administered 20 minutes before the reinstatement test. No ethanol was delivered during the reinstatement tests. **$P<0.001$, as compared to extinction (Extinction-R) responding; $^{\#\#}P<0.001$ as compared to saline reinstatement (Saline-R).

B. Mean (+/-) S.E.M. entries into the ethanol port by the same subjects as in Fig. 8A.

Table 1. Mean and median latencies to first responses during the reinstatement test following saline or naltrexone.

Treatment	Ethanol Lever Press	Port Entry
Experiment 2		
Saline		
Mean (SEM)	84.1 (24.4)	44.1 (10.4)
Median (min, max)	60.7 (14.7, 255.6)	26.7 (15.5, 127.2)
Naltrexone		
Mean (SEM)	50.8 (16.8)	45.0 (8.1)
Median (min, max)	35.4 (8.1, 207.5)	50.6 (7.0, 83.8)
Experiment 4		
Extinction		
Saline		
Mean (SEM)	1061.71 (483.8)	676.20 (228.4)
Median (min, max)	610.85 (14.0, 3600.0)	455.55 (6.3, 1405.2)
Naltrexone		
Mean (SEM)	1842.49 (586.8)	1105.90 (645.1)
Median (min, max)	1911.63 (19.5, 3600.0)	99.73 (20.6, 3600.0)
Reinstatement		
Saline		
Mean (SEM)	207.65 (168.0)	47.51 (13.7)
Median (min, max)	21.95 (8.6, 1208.2)	40.85 (1.0, 107.9)
Naltrexone		
Mean (SEM)	47.69 (27.9)	49.16 (19.3)
Median (min, max)	13.5 (1.71, 207.9)	35.5 (0.5, 135.9)

All data are in second

3.1.2. Cocaine self-administration

Experiment 1: Reinstatement of cocaine-seeking behavior after exposure to cocaine-associated stimuli.

All animals developed stable cocaine self-administration in 16.3±1.7, while the number of lever presses for saline gradually decreased. There were no differences in responding during the final 3 days of training or between the first and second daily hours of self-administration. During extinction the number of active lever presses gradually decreased and rats met the extinction criterion after an average of 16.9±2.7 sessions. Responding on the inactive lever was minimal during both conditioning and extinction. Following extinction, reintroduction of cocaine-associated cues led to strong recovery of responding on the active lever, whereas exposure to the saline-associated cues did not result in enhanced lever-pressing. When cocaine-associated stimuli were repeatedly presented they modified the number of presses on the active lever ($F(29,173)=12.9$, $P<0.01$, one-way ANOVA for repeated measurement) but not on the inactive one. *Post hoc* comparison by the Newman–Keuls test indicated that the presentation of the cocaine-associated cues revived responding during the first four tests, with increasingly more lever presses than with the saline-associated stimuli ($P<0.05$) and more than during the three extinction sessions preceding each cocaine-associated stimuli presentation (data not shown, $P<0.01$). These data are depicted in figure 9.

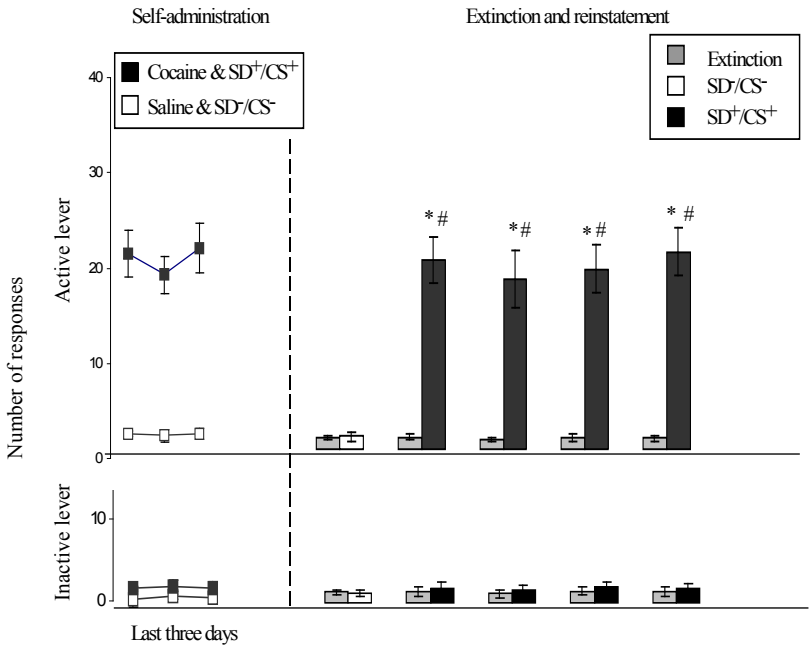


Figure 9. Reinstatement of cocaine-seeking behavior after exposure to cocaine-associated stimuli Responses on the active and inactive lever (mean ± SEM of eight rats) during reinstatement sessions under saline- and repeated cocaine-associated stimuli presentation (see Materials and methods for further details). Data were analyzed by one-way ANOVA for repeated measures followed by NewmanKeuls *post hoc* comparison. *P*<0.05, different from the no-rewarding stimuli presentation and the three preceding extinction sessions, NewmanKeuls test

Experiment 2: Effect of naltrexone on seeking behavior induced by cocaine-associated cues.

All animals rapidly acquired cocaine self-administration, reaching the training criterion in 14.5 ± 0.5 days of the discriminative regimen. In parallel, the number of lever presses during the saline self-administration sessions gradually decreased. Responding at the inactive lever remained low throughout training. During extinction sessions, active lever pressing gradually decreased and animals reached the extinction criterion in 18 ± 1.2 sessions. Two-way ANOVA for repeated measures found no significant difference in the number of lever presses during the last three days of self-administration, no-reward and extinction on either active or inactive levers. Figure 10 shows the response on the active and inactive levers during self-administration training (mean of the last three sessions), extinction (mean of the last three sessions before reinstatement tests) and reinstatement phase. For comparison, the Figure also shows the mean number of lever presses during the last three days of self-administration. Two-way ANOVA for repeated measurement found a significant effect of test sessions ($F(9,140) = 13.6$, $p<0.01$) and of levers ($F(1,140) = 74.0$, $p<0.01$).

Moreover a significant interaction between test sessions and levers was also found ($F(9,140) = 9.4$, $p<0.01$). Post-hoc comparisons by Newman-Keuls test revealed that re-introduction of cocaine-associated stimuli in vehicle-pretreated rats significantly increased the number of pressings on the active lever ($p<0.01$ vs. saline-associated cues presentation and vs. the three previous extinction days, Newman-Keuls test) but not on the inactive one ($p>0.05$ vs. saline-associated cues presentation and vs. the three previous extinction days, Newman-Keuls test).

Naltrexone pre-treatment significantly modified rats' behaviour after presentation of cocaine-associated stimuli on the active but not on the inactive lever. In fact, post-hoc comparisons by the Newman-Keuls test showed that 1 and 2.5 mg/kg naltrexone significantly reduced the effect of the presentation of cocaine-associated cues ($p<0.05$ vs. vehicle treated group), which at the highest dose of naltrexone was no longer different from those emitted during the three preceding extinction days ($p>0.05$).

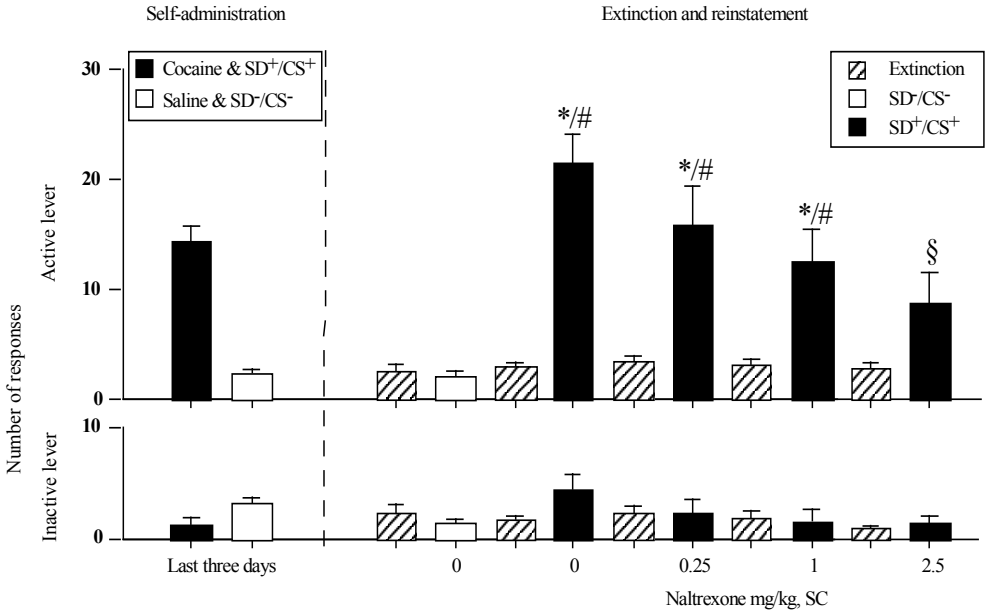


Figure 10. **Effects of naltrexone on reinstatement induced by cocaine-associated cues**.Histograms represent the mean (n=8) ± S.E.M. number of presses on the active and inactive levers. The number of presses during self-administration training (mean of last three days), extinction (mean of last three days before reinstatement sessions), and in the presence of stimuli associated with no-reward during reinstatement sessions is also shown. Data were analyzed by two-way ANOVA for repeated measurements (with test session as main factor) followed by Newman-Keuls post-hoc comparison. $^{*}P<0.01$ different from the no-reward stimuli and $^{\#}P<0.01$ different from the three previous extinction sessions. $^{\S}P<0.05$ different from the vehicle + cocaine-associated stimuli group, Newman-Keuls test.

Experiment 3: Effect of naltrexone on seeking behavior induced by sucrose pellet-associated cues

All rats in both groups developed stable sucrose self-administration, while responding during the no-reward sessions gradually decreased, and the training criterion was reached in respectively 25 ± 3.4 and 23.2 ± 1.7 discriminative sessions. No differences in responding by the two groups of animals were observed during the two daily sessions of sucrose pellet self-administration on either active or inactive levers ($p<0.05$, mixed one-way ANOVA). Likewise, no differences were found in the number of lever-presses during the last three days of self-administration (mean ± S.E.M were 25.5 ± 3.8 and 27.8 ± 3.1, and 1.7 ± 1.4 and 2.9 ± 1.8, for the active and inactive levers, respectively), no-reward (mean ± S.E.M were 3.3 ± 2.1 and 2.9 ± 1.8, and 2.0 ± 1.4 and 2.5 ± 1.8) in the two groups of animals ($p>0.05$, mixed one-way ANOVA).

During extinction sessions, active lever pressing gradually decreased and the two groups of rats reached the extinction criterion in 15.7 ± 4.3 and 17.5 ± 1.6 sessions. No differences were observed in the mean of the three days preceding the reinstatements test.

Mixed factorial ANOVA found a significant effect of sessions ($F(3,28)=23.6$, $p<0.01$) and of levers ($F(3,9)=93.8$, $p<0.01$) as well as an interaction between session x levers ($F(9,84)=p<0.01$). Post-hoc comparisons by Newman-Keuls test revealed that in vehicle treated animals sucrose-associated cues re-introduction significantly revived active ($p<0.01$) but not inactive ($p>0.05$) lever pressing. Naltrexone 2.5 mg/kg did not modify the number of lever presses induced by sucrose-associated cues ($p>0.05$ compared to vehicle treated rats and $p<0.01$ compared to extinction or no-sucrose-associated cues reintroduction). Thus, independently of the treatment, sucrose-associated stimuli revived a significant seeking behaviour ($p<0.01$ vs. respective extinction and no-sucrose associated stimuli presentation, Newman-Keuls test). These data are reported in figure 10.

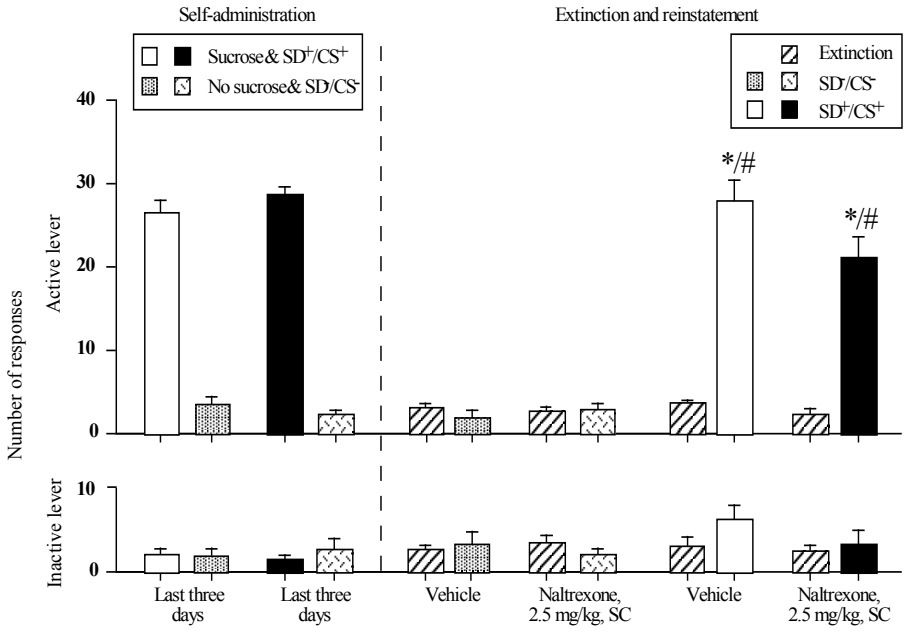


Figure 10. Effects of naltrexone on reinstatement induced by sucrose-associated cues. Results are the mean (n=8+8) ± S.E.M. number of presses on the active and inactive levers. Reinstatement data were analyzed by mixed factorial ANOVA for repeated measurements followed by Newman-Keuls post-hoc comparison (with test session as main factor). *P<0.01 compared with respective no-sucrose-associated stimuli group. # P<0.01 compared with respective extinction, Newman-Keuls test.

3.2. Electrophysiological study

Field potentials evoked by glutamatergic fibers stimulation are characterized by two negative peaks, as previously reported (Pennartz et al., 1990). The first peak (N1) is non-synaptic, has a latency of 2-4 ms, and its amplitude does not increase significantly in response to HFS. The second one (N2), instead, is the synaptic response, has a latency between 8 and 10 ms and a mean amplitude of 0.3 mV in basal conditions. As opposed to N1, N2 amplitude significantly increases after effective HFS (Fig. 11).

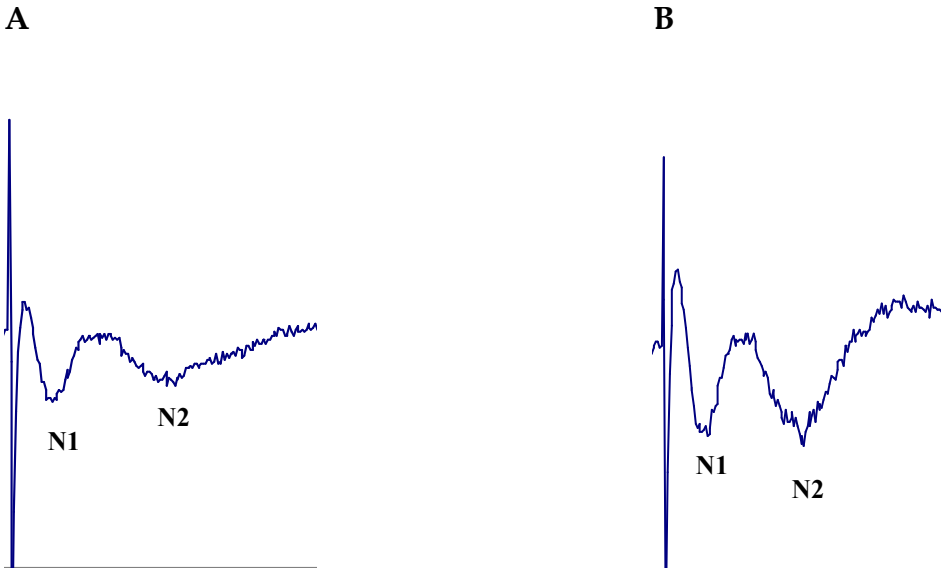


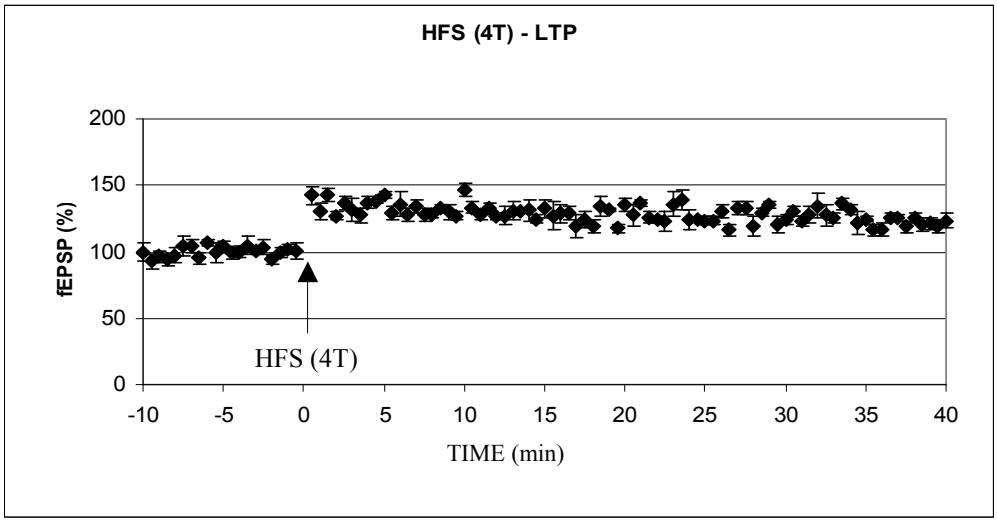
Figure 11. Example of an excitatory postsynaptic field potential recorded in the nucleus accumbens in a rat brain slice before (A) and after (B) HFS.

Synaptic potentiation has been evaluated by comparing N2 amplitude before and after HFS (4T) or HFS (2T). Using both stimulation protocols three sets of experiments have been carried out: the first set of slices was perfused with normal aCSF solution, whereas in the second and in the third one the solution was added with naltrexone (30 μM) or CTOP (10 μM), respectively. The results obtained have been classified as: LTP, when the increase in N2 amplitude was significant for more than 30 minutes after HFS; STP, when the increase in N2 amplitude was significant between 5 and 30 minutes after HFS, but not after 30 minutes.

Experiment 1: HFS (4T)

Control experiments. Control experiments have been performed in the experimental conditions reported by Li and Kauer (2004), in which the HFS (4T) stimulation protocol induced LTP. LTP was obtained in four experiments: fEPSP amplitude increased significantly ($p<0.005$), from 100.59±4.09% to 133.59±5.8%, during the first 5 minutes after HFS (fig. 12A); the increase was still significant after 30 minutes. In two cases the recording was interrupted after 40 minutes: the increase in amplitude was significant ($p<0.005$) between 36 and 40 minutes after HFS (128.33±1.77%). In one case the recording was interrupted after 185 minutes: fEPSP amplitude was still significantly ($p<0.005$) higher as compared to baseline (112.38±3.03%) between 181 and 185 minutes after HFS. In six experiments, instead, HFS (4T) induced STP: fEPSP amplitude increased significantly ($p<0.005$), from 99.42±2.6% to 123.26±4.93%, during the first 5 minutes and decreased to 112.39±4.8% (value sill significantly different as compared to baseline, $p<0.005$) between 16 and 20 minutes after HFS; then the fEPSP amplitude further decreased to values not significantly different from baseline (fig 12B).

A



B

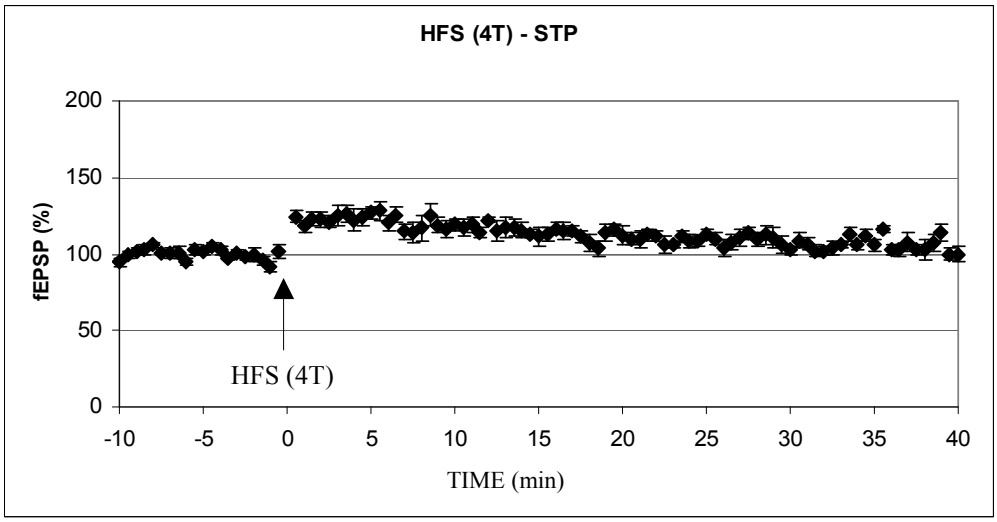
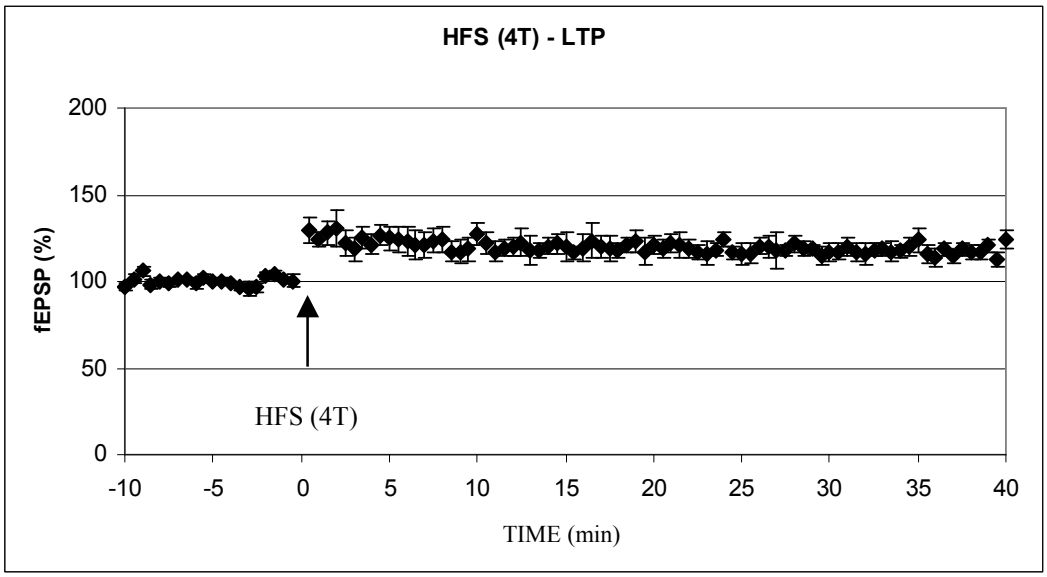


Figure 12. Amplitude of extracellular field potentials recorded in the nucleus accumbens in rat brain slices before and after HFS (4T). A: HFS (4T) induces LTP (n=4). B: HFS (4T) induces STP (n=6). Data are reported as mean% (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM.

Naltrexone experiments. Naltrexone (a non-selective opioid receptor antagonists) was used to verify the involvement of endogenous opioids in LTP. Data obtained are shown in figure 13. LTP was obtained in four experiments: fEPSP amplitude increased significantly ($p<0.005$), from 99.59±2.3% to 125.15±6.78%, during the first 5 minutes after HFS; this increase was still significant between 36 and 40 minutes after HFS (118.5±1.46%) (Fig 13A); recordings were interrupted after 40 minutes. In four experiments, instead, HFS (4T) induced STP: fEPSP% amplitude increased significantly ($p<0.005$) from 99.03±2.79% to 118.26±4.04%, during the first 5 minutes and decreased to 110.03±5.6% (value not significantly different from the baseline) between 11 and 15 minutes after HFS (fig 13B).

CTOP experiments. The selective μ-opioid receptor antagonist CTOP was used to verify the involvement of the endorphinergic system in the cellular mechanism of LTP. LTP was obtained in the 2 experiments performed: fEPSP amplitude increased significantly ($p<0.005$), from 100.63±3.23% to 150.41±5.46%, during the first 5 minutes after HFS; this increase was still significant between 36 and 40 minutes after HFS (126.54±1.47%) (Fig 14); the recording was interrupted after 40 minutes.

A



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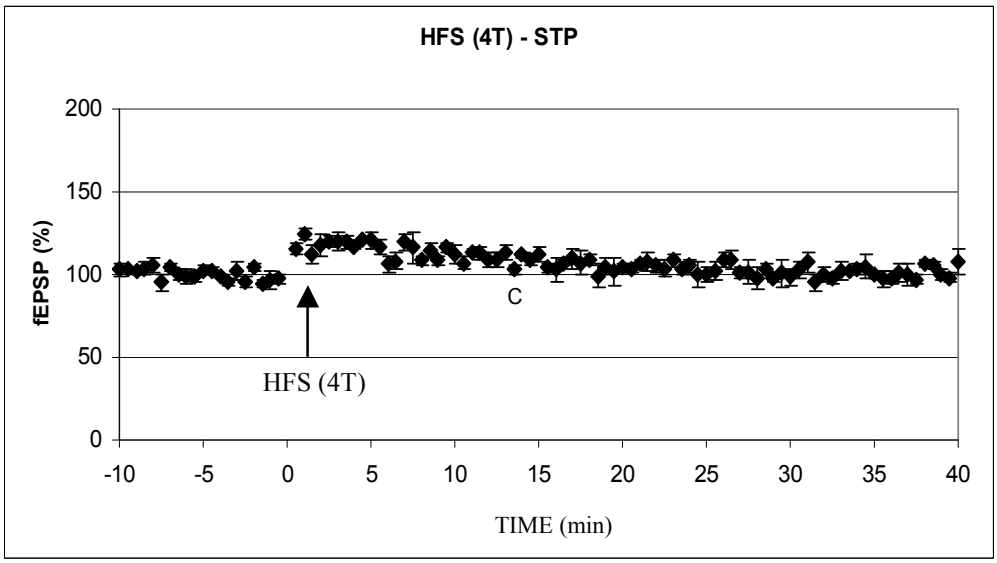


Figure 13. Amplitude of extracellular field potentials recorded in the nucleus accumbens in rat brain slices before and after HFS (4T) in the presence of naltrexone (30 μM). A: HFS (4T) induces LTP (n=4). B: HFS (4T) induces STP (n=4). Data are reported as mean% (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM.

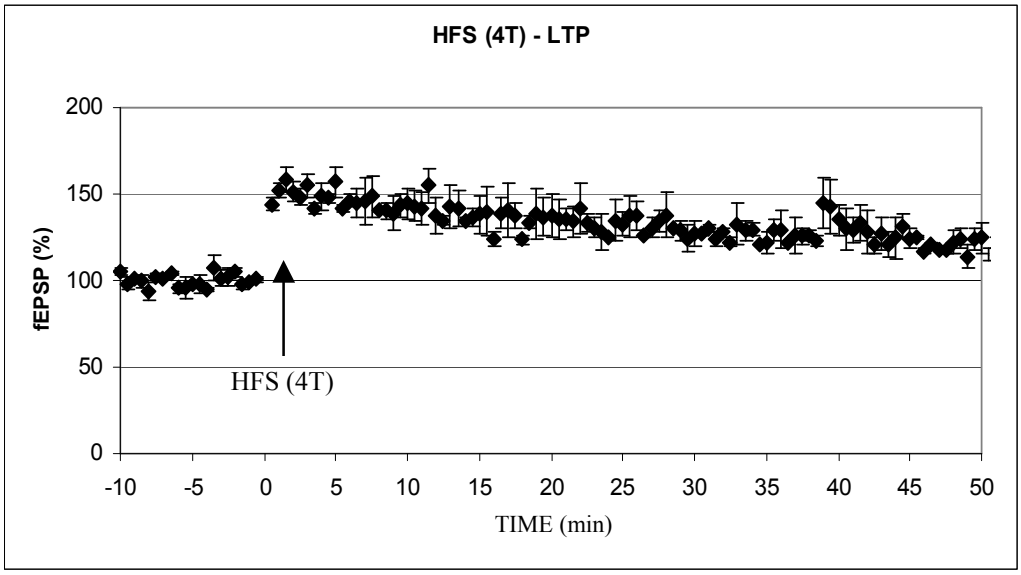
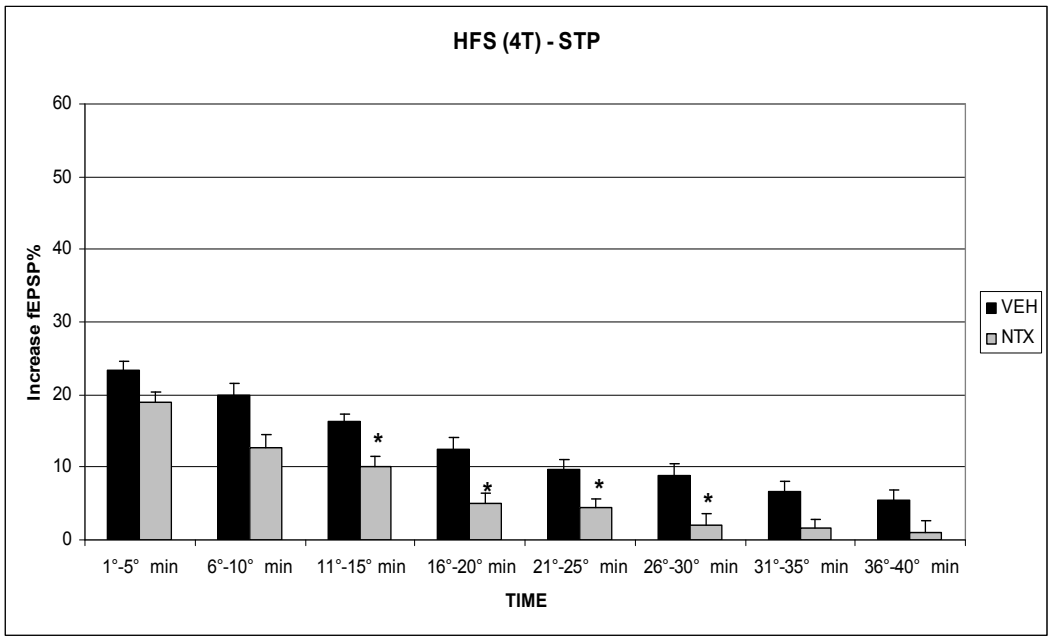


Figure 14. Amplitude of extracellular field potentials recorded in the nucleus accumbens in rat brain slices before and after HFS (4T) in the presence of CTOP (10 μM). HFS (4T) induces LTP (n=2). Data are reported as mean% (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM.

Comparison between control and pharmacological treatment after HFS (4T). fEPSP increase after HFS (4T) is greater in control conditions as compared to that obtained in the presence of naltrexone, both in LTP and STP. A comparison of fEPSP values at 5-minute time intervals during STP reveals a significant ($p<0.005$) difference between control (n=6) and naltrexone (n=4) during minutes 11-30 after HFS (111.75±0.75% and 105.36±0.76%, respectively) (Fig. 15A). During LTP, instead, a significant ($p<0.005$) difference between control (n=4) and naltrexone (n=4) is observed during minutes 1-5 (135.55±1.75% and 125.15±2%, respectively), 11-15 (129.85±1.48% and 119.91±1.84%, respectively), 21-25 (128.45±1.96% and 118.92±1.56% respectively) and 31-35 (128.33±1.77% and 118.5±1.46%, respectively) after HFS. In the experiments performed in the presence of CTOP (n=2), synaptic potentiation in LTP was greater with respect to both control and naltrexone. The difference in amplitude was significantly ($p< 0.005$) different from control during minutes 1-15 (144.86±1.29% and 132.48%±0.96%, respectively) and 36-40 (130.43±2.71% and 121.27±1.34%, respectively) after HFS (Fig. 15B).

A



B

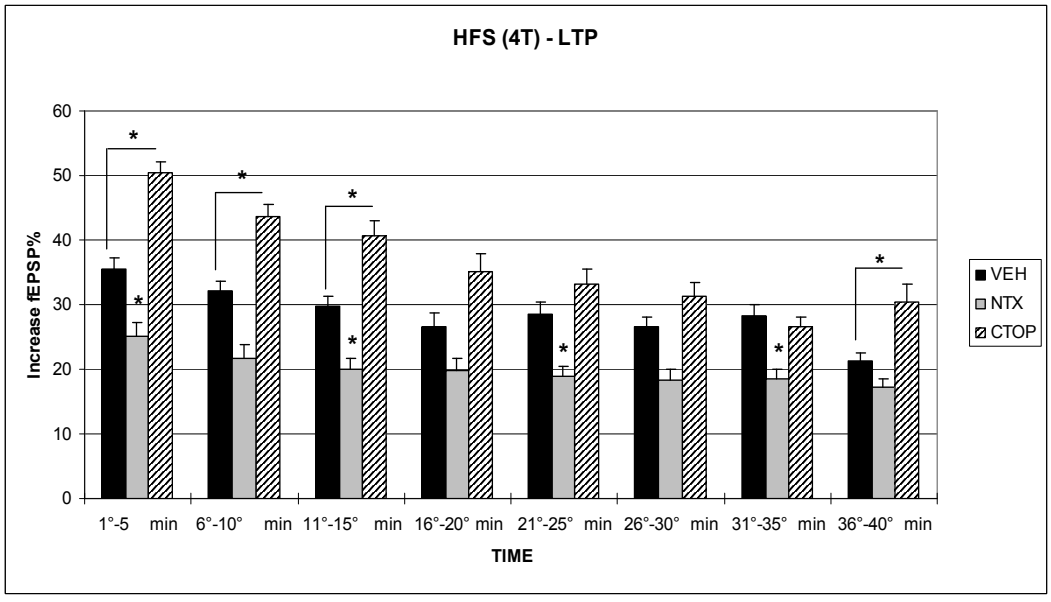


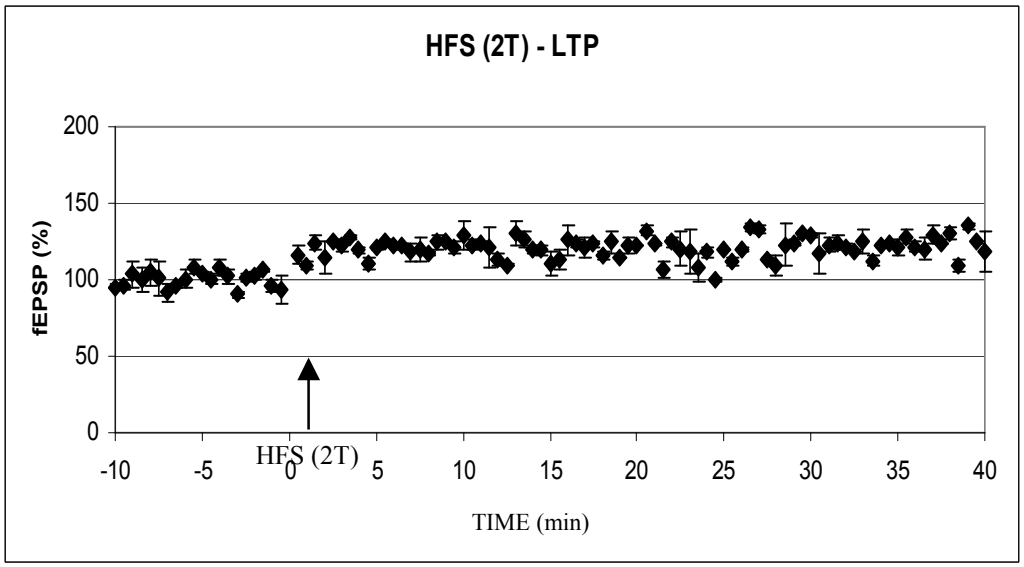
Figure 15. Comparison between the increases in fEPSPS% recorded in the nucleus accumbens in rat brain slices before and after HFS (4T), in control conditions and in the presence of naltrexone (30 μM) or CTOP (10 μM). Histograms represent the mean of the data recorded in five-minute time intervals after HFS, and are expressed as increase in % as compared to baseline values (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM. A: Comparison between data obtained in STP experiments in control conditions (n=6) and in the presence of naltrexone (n=4). B: Comparison among data obtained in LTP experiments in control conditions (n=6), in the presence of naltrexone (n=4) and CTOP (n=2).

Experiment 2: HFS (2T)

Control experiments. LTP was obtained in two experiments: fEPSP amplitude increased significantly ($p<0.005$), from 100.26±3.55% to 118.93±3.76% (n=2) during the first 5 minutes after HFS (fig. 16A); the increase was still significant after 30 minutes. In one case the recording was interrupted after 40 minutes: the increase in amplitude was significant ($p<0.005$) between 36 and 40 minutes after HFS (120.82±1.55%). In the other one the recording was interrupted after 185 minutes: fEPSP amplitude was still significantly ($p<0.005$) greater as compared to baseline values (120.43±2.48%) between 181 and 185 minutes after HFS. In four experiments, instead, HFS (2T) induced STP: fEPSP% amplitude increased significantly ($p<0.005$) from 99.41±3.57% to 114.78±3.61%, during the first 5 minutes and decreased to 114.69±3.88% (value sill significantly different as compared to baseline, $p<0.005$) between 6 and 10 minutes after HFS; then the fEPSP% further decreased to values non significantly different from baseline (fig 16B).

Naltrexone experiments. The data obtained are shown in figure 17. LTP was obtained in four experiments: fEPSP amplitude increased significantly ($p<0.005$), from 102.47±8.66% to 131.66±5.22%, during the first 5 minutes after HFS; this increase was still significant between 36 and 40 minutes after HFS (127.14±2.62%) (Fig 17A); the recordings were interrupted after 40 minutes. In four experiments, instead, HFS (2T) induced STP: fEPSP% amplitude increased significantly ($p<0.005$) from 102.95±3.58% to 118.77±3.05%, during the first 5 minutes and decreased to 111.78±5.21% (value not significantly different as compared to baseline) between 11 and 15 minutes after HFS (fig 17B).

A



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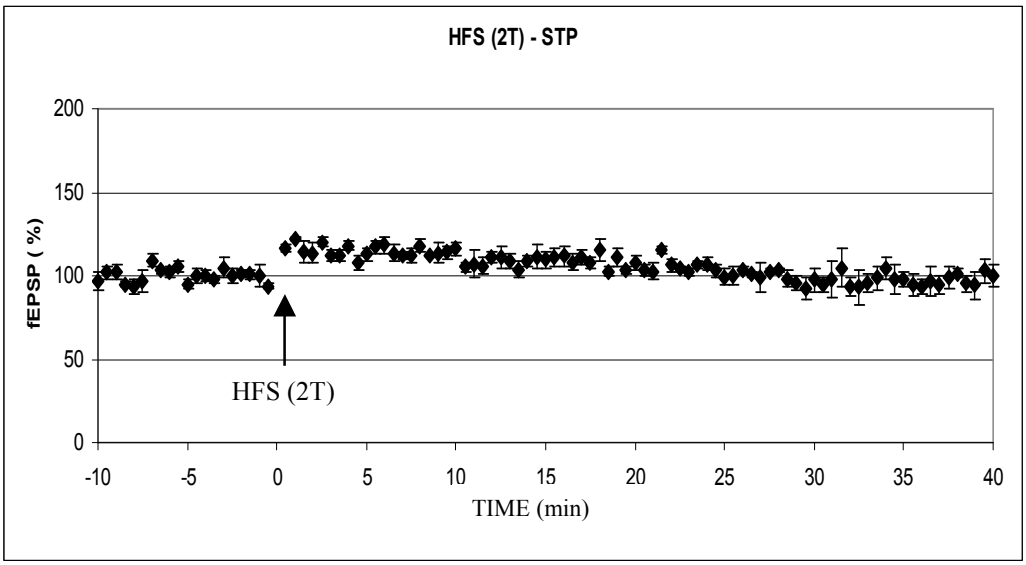
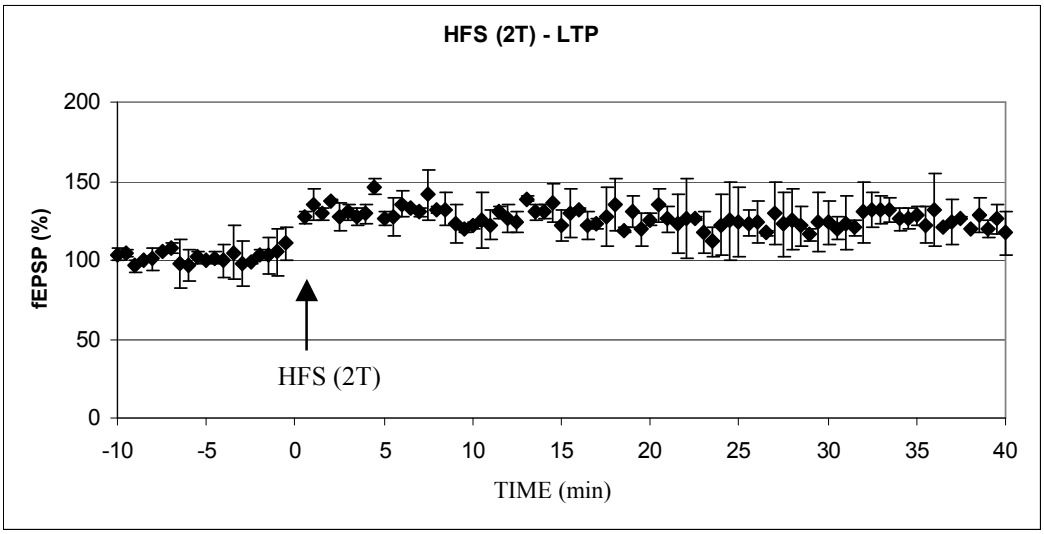


Figure 16. Amplitude of extracellular field potentials recorded in the nucleus accumbens in rat brain slices before and after HFS (2T). A: HFS (4T) induces LTP (n=2). B: HFS (4T) induces STP (n=4). Data are reported as mean% (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM.

A



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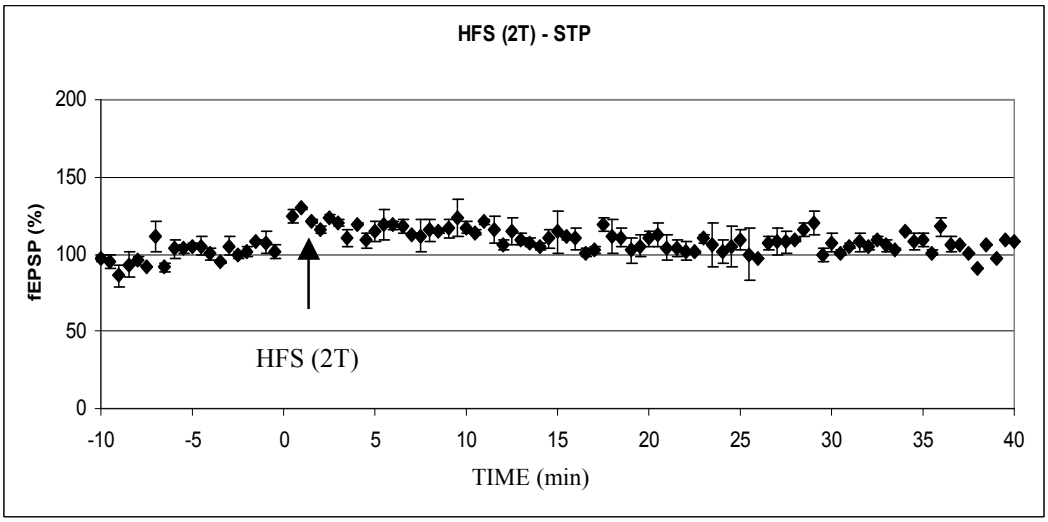


Figure 17. Amplitude of extracellular field potentials recorded in the nucleus accumbens in rat brain slices before and after HFS (2T) in the presence of naltrexone (30 μM). A: HFS (4T) induces LTP (n=4). B: HFS (4T) induces STP (n=4). Data are reported as mean% (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM.

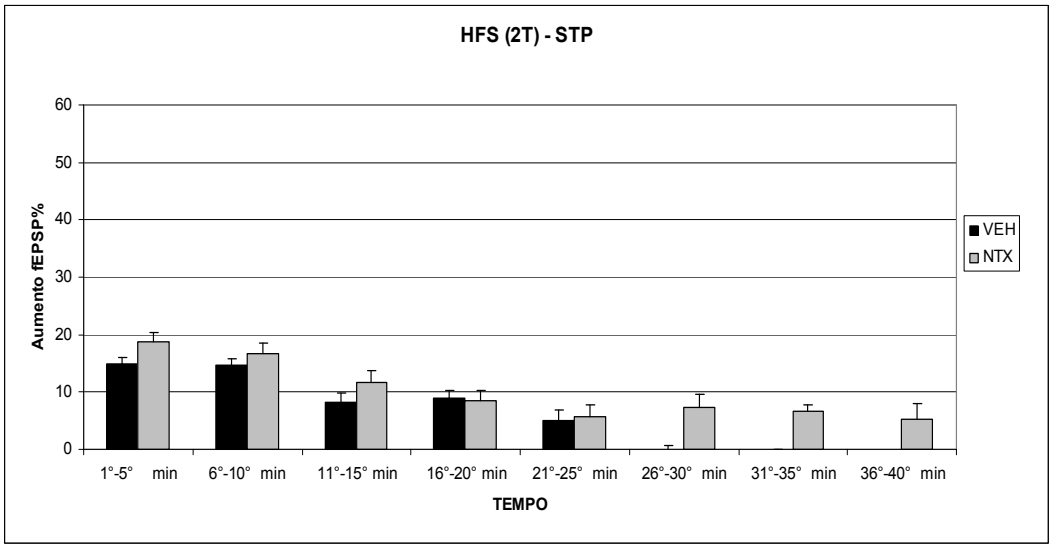
Comparison between control and pharmacological treatment after HFS (4T). fEPSP increase after HFS (2T) application is smaller in control conditions than in the presence of naltrexone, in contrast to what observed with HFS (4T). A comparison of fEPSP values at 5-minute time intervals during STP reveals a non-significant ($p>0.005$) difference between control (n=4) and naltrexone (n=2) (Fig. 18A). During LTP, instead, a significant ($p<0.005$) difference between control (n=2) and naltrexone (n=2) is observed during minutes 1-5 (118,93±3,76% and 131,66±5,22%, respectively) and 31-35 (120,82±1,55% and 127,14±2,62%, respectively) after HFS (Fig. 18B).

Comparison between the two stimulation protocols in control conditions and in the presence of naltrexone.

Control. In control conditions, fEPSP increase after HFS (2T) is significantly ($p<0,005$) smaller as compared to that obtained after HFS (4T). During STP a significant ($p<0,005$) difference between the data obtained with the two stimulation protocols, HFS (4T) (n=6) and HFS (2T) (n=4), is observed during minutes 1-5 (123.26±1.52% and 114.78±1.26%, respectively), 11-15 (116.22±1.53% and 108.28±1.54%, respectively), 26-40 (106.95±0.83% and 98.06±1.1%, respectively) after HFS (Fig. 19A). During LTP, instead, a significant ($p<0.005$) difference between the two protocols, HFS (4T) (n=3) and HFS (2T) (n=2), is observed during minutes 1-5 (133.59±2.03% and 118.93±1.74% respectively), and 31-35 (128,48±1,78% and 120,82±1,55%, respectively) after HFS (Fig 19B).

Naltrexone. In the presence of naltrexone, fEPSP increase after HFS (2T) is greater than after HFS (4T), but a significant ($p<0,005$) difference between the two protocols, HFS (4T) (n=4) and HFS (2T) (n=2), is observed only in LTP, during minutes 31-35 after HFS (118,5±1,46% and 127,14±1,72,624%, respectively) (Fig 20).

A



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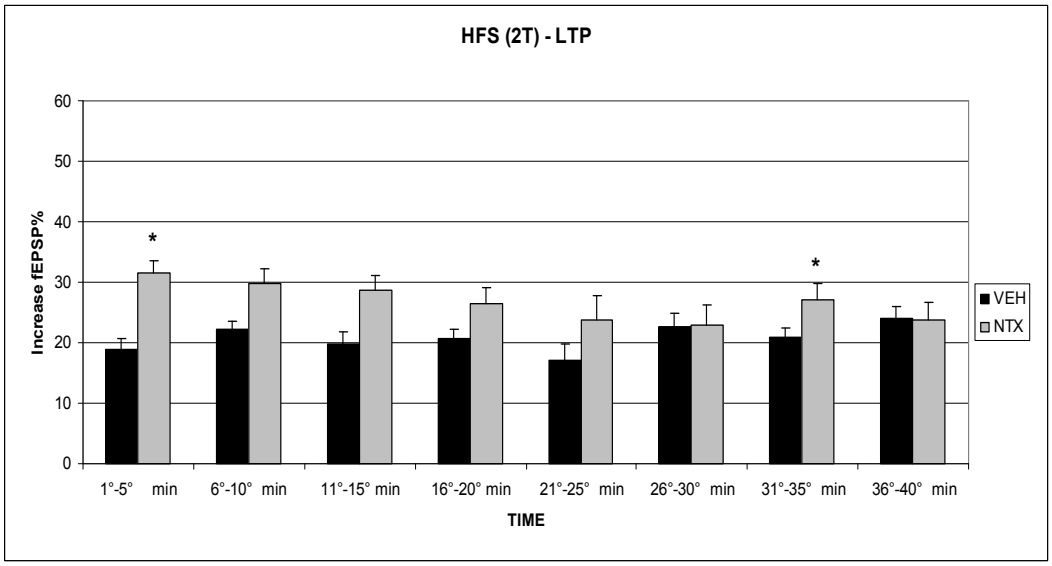
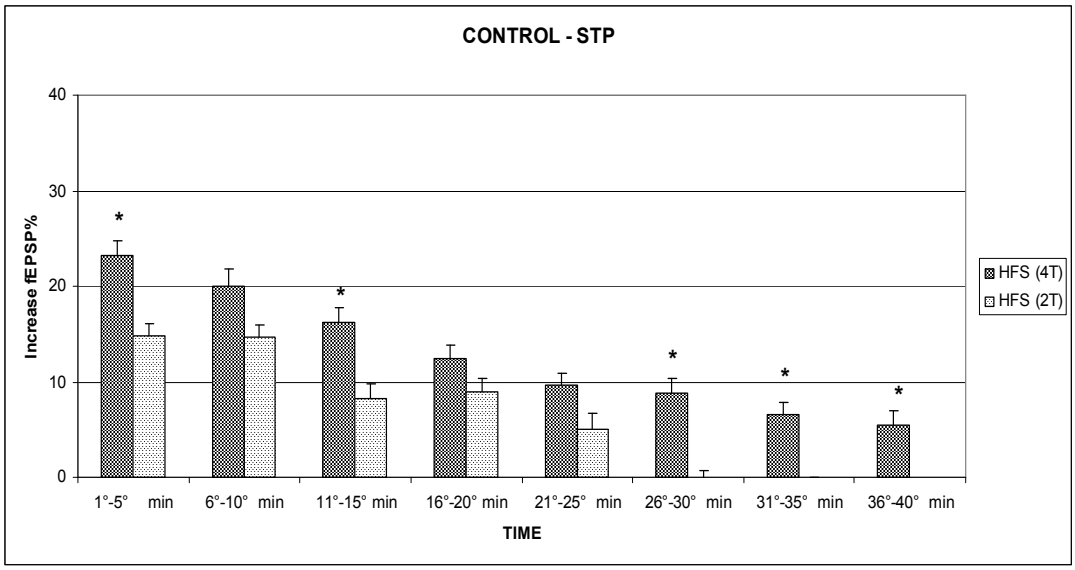


Figure 18. Comparison between the increases in fEPSPS% recorded in the nucleus accumbens in rat brain slices before and after HFS (2T), in control conditions and in the presence of naltrexone (30 μM). Histograms represent the mean of the data recorded in five-minute time intervals after HFS, and are expressed as increase in % as compared to baseline values (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM. A: Comparison between the STP experiments, in control conditions (n=4) and in the presence of naltrexone (n=2). B: Comparison between the LTP experiments, in control conditions (n=2) and in the presence of naltrexone (n=2).

A



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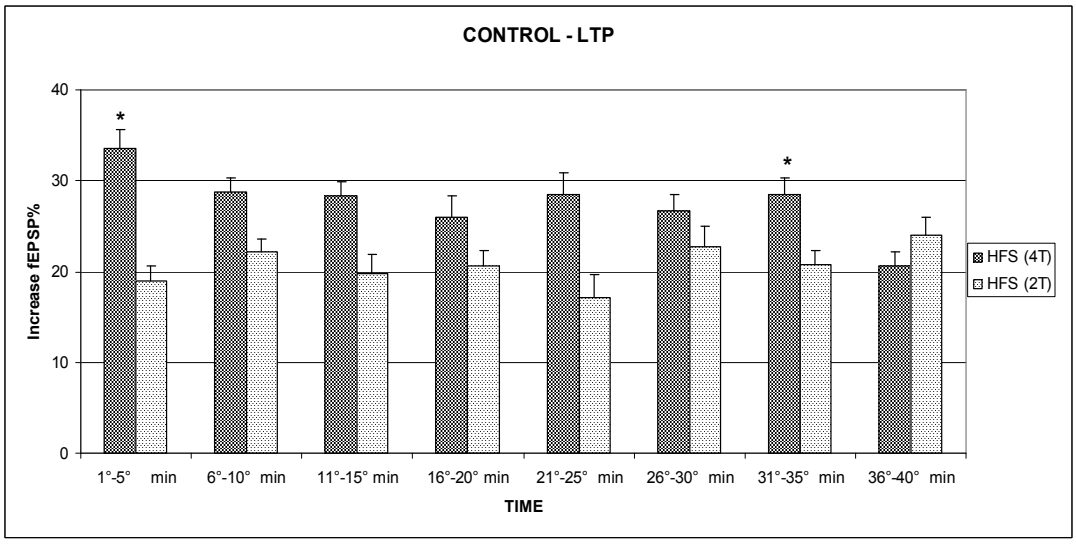
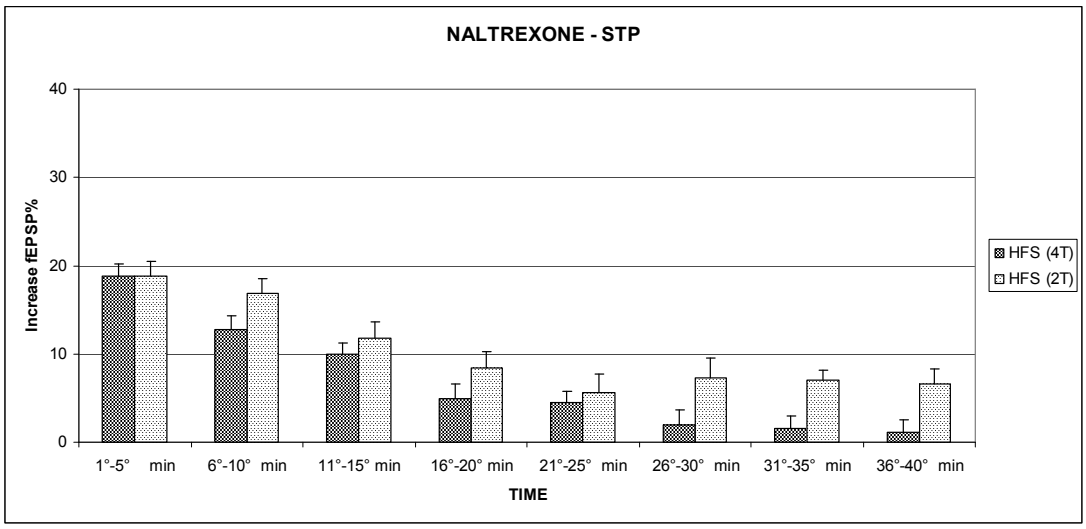


Figure 19. Comparison between the increases in fEPSPS% recorded in the nucleus accumbens in rat brain slices before and after HFS (4T) or HFS (2T) in control conditions. Histograms represent the mean of the data recorded in five-minute time intervals after HFS, and are expressed as increase in % as compared to baseline values (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM. A: Comparison between data obtained in STP experiments, after HFS (4T) (n=6) and HFS (2T) (n=4). B: Comparison between data obtained in LTP experiments, after HFS (4T) (n=3) and HFS (2T) (n=2).

A



B

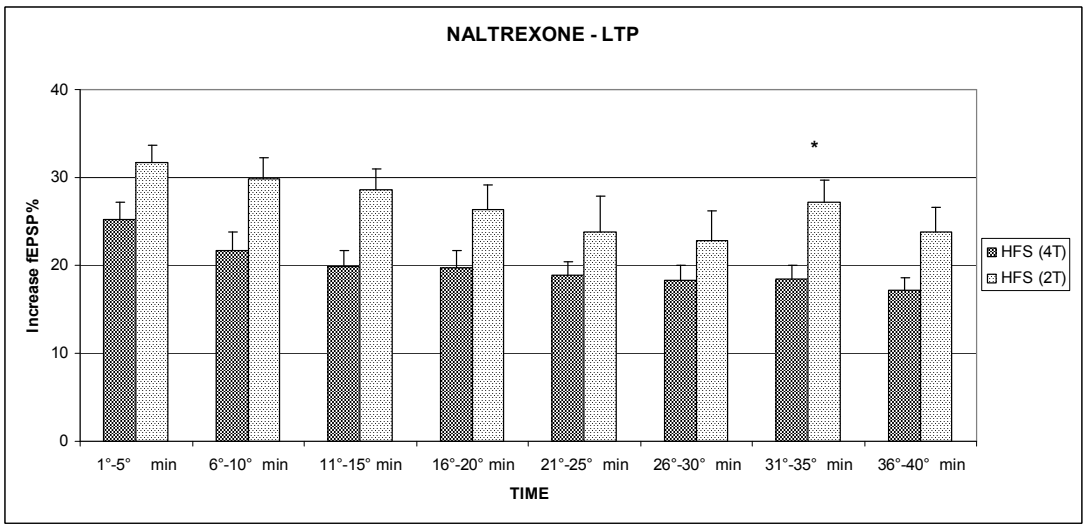


Figure 20. Comparison between the increases in fEPSPS% recorded in the nucleus accumbens in rat brain slices before and after HFS (4T) or HFS (2T) in the presence of naltrexone (30 μM). Histograms represent the mean of the data recorded in five-minute time intervals after HFS, and are expressed as increase in % as compared to baseline values (in each experiment, 100% is the mean of the values recorded during 10 minutes before HFS) ± SEM. A: Comparison between data obtained in STP experiments, after HFS (4T) (n=4) and HFS (2T) (n=2). B: Comparison between data obtained in LTP experiments, after HFS (4T) (n=4) and HFS (2T) (n=2).

4.1. Behavioral study

4.1.1. Alcohol self-administration

The results of the first experiment indicate that the environmental context can induce relapse to ethanol seeking behavior after operant response extinction and in the absence of ethanol availability. Memory for the ethanol-associated context was specific, as placement into the non-ethanol extinction context did not induce ethanol seeking. In addition, memory for the ethanol-associated context was persistent, lasting up to three weeks, the final time point tested. This result suggests that pharmacological effects of self-administered ethanol are sufficient to produce long-lasting modification of animals' behavior, leading to relapse phenomena even after prolonged abstinence.

The second group of subjects trained to self-administer sucrose provided an interesting control for the effects of ethanol. In particular, we found that subjects trained to lever press for sucrose in a distinctive context, and then extinguished in a different context, reinstate lever press responding when placed back into the sucrose-associated context**.** However, the degree of reinstatement decreased over repeated tests, returning to extinction values after three weeks. These findings indicate that reward-associated contexts can induce seeking behavior for both drug and natural rewards, but this behavior is long lasting only for drug rewards. Similarly, the ability of cocaine-associated cues to reinstate responding lasts far longer than cues associated with a non-drug reinforcer (Ciccocioppo et al., 2004; Drummond et al., 1990).

It might be proposed that, after a prolonged time of no reward availability in either context, animals are no longer able to remember which environment was

associated with reward, and that the responding at test reflects spontaneous recovery of the extinguished responding. We therefore tested the subjects in the extinction context two weeks after the first extinction session. The non-significant effect of the extinction context on operant response reinstatement for both the ethanol- and sucrose-trained groups confirmed that animals are able to recall the specific association of one context with previous reward availability over a considerable time. We also saw a difference in the effect of a third novel context between reward substances. Specifically, the novel environment induced significant reinstatement in sucrose-trained subjects, but not in ethanol-trained subjects. This finding indicates that the reward-paired context may be particularly critical for reinstating ethanol-seeking behavior, although the small sample size for this manipulation precludes firm conclusions. Interestingly, this finding agrees with Bossert et al. (2004) who found that a novel context did not reinstate responding for heroin. On the other hand, studies of contextual control of responding for appetitive reinforcers after extinction have found that conditioned-responding is increased by a novel context (for review see Bouton, 2004). Hence, contextual control over responding for drug and alcohol unconditioned stimuli may be stronger than for natural rewards, like sucrose.

We restricted the water intake of the experimental subjects to ensure high ethanol intakes. Ethanol doses such as those consumed herein (1.31 ± 0.07 g/kg) are reported to alter hippocampal spatial function. For instance, Melia *et al*. (1996) found that 1.0-1.5 g/kg of ethanol administered before training disrupts contextual fear conditioning. In our experiments, subjects showed significant and long-lasting reinstatement to the context, indicating that regular consumption of ethanol at these doses does not impact the formation of the context-ethanol association. These findings are in agreement with Boulouard *et al*. (2002) who found that chronic exposure to ethanol produced tolerance to the spatial memory impairing effects of acute ethanol.

Previously, we (Nie & Janak, 2003) and others (Katner *et al*., 1999; Ciccocioppo *et al*., 2003; Backstrom et al., 2004) found that conditioned and/or discriminative cues can reinstate ethanol-seeking behavior after extinction. Hence both discrete

cues and environmental contexts that are associated with ethanol availability can induce ethanol-seeking behavior after a period of abstinence. In the current study as well as in our previous study (Nie & Janak, 2003), we found that animals trained to self-administer sucrose demonstrated a similar effect of context or discrete cues, respectively, on reinstatement of sucrose-seeking, indicating that the conditioning processes engaged during ethanol self-administration are general processes involved in conditioned associations with reward. However, here we found that the ability of ethanol to reinstate the seeking behavior was more persistent over time compared to sucrose, supporting the view of a peculiar effect of ethanol on memory processes involved in addictive behavior.

In the second experiment we found that exposure to the specific context in which subjects had previously self-administered ethanol reinstates ethanol-seeking behavior also in non water-restricted subjects. The ability for the multi-modal context to increase responding does not depend on the presence of the odor stimulus alone, but requires the presence of the complete configuration. In addition, acute treatment with the opioid antagonist, naltrexone, attenuates the increase in lever-press responding induced by the return to the self-administration context. The training parameters used in the second experiment are notable in two respects. First, sucrose fading, in which ethanol is introduced to subjects in a sweetened solution to overcome rats' initial aversion to the taste of ethanol, occurred in the home cage. This allowed subjects to become accustomed to the taste, smell, and pharmacological effects of ethanol prior to the initiation of operant training. Hence, subjects learned to lever-press for a 10E solution, the only reinforcer the subjects experienced in the ethanol self-administration chambers. Importantly, this training procedure produced responding comparable to our previous work in which the sucrose fading occurred during lever-press training within the operant chamber (Janak and Gill, 2003, Nie and Janak, 2003). The second notable aspect of training for these experiments is that the home cage availability of water was restricted only for a few days to facilitate the initial acquisition of lever-press responding; for the remainder of the operant training,

subjects were not food or water restricted, in contrast to the first experiment. Taking into account both of these training parameters, it is likely that responses emitted by subjects during the reinstatement tests in these experiments are motivated by the memory of the reinforcing attributes of 10E, rather than by associations of the environment with sucrose and/or thirst. An additional alteration in the training parameters from an FR-1 to an FR-3 response requirement did not appear to increase the behavioral output observed during the reinstatement tests.

Upon placement back into the original self-administration context in the absence of ethanol, the number of responses at the ethanol-paired lever significantly increased, suggesting that animals recalled the previously-learned relationship between the self-administration context and ethanol availability. The finding that placement into a novel context did not alter responding further supports a specific recollection of the ethanol self-administration environment, in agreement with our previous findings (Zironi et al., 2006) and with findings with other drugs of abuse (Bossert et al., 2005b; Crombag and Shaham, 2002). The increase in responding in the ethanol context could indicate a direct context-ethanol association, or it could reflect the role of the ethanol context as an occasion setter that triggers the recall of the stimulus-response and response-reinforcer associations that control instrumental responding for ethanol. Previous research on the effects of context on extinguished conditioned responses appears to favor the latter interpretation (Bouton and Peck, 1989).

In two separate experiments, pretreatment with naltrexone significantly attenuated response reinstatement induced by placement within the self-administration context, suggesting that endogenous opioid release contributes to this responding. This finding is in agreement with previous studies showing that opioid antagonists block reinstatement triggered by a discriminative stimulus (Ciccocioppo et al., 2002, 2003; Katner et al., 1999) or ethanol priming (Bienkowski et al., 1999, Le et al., 1999). Collectively, these findings indicate that naltrexone blocks ethanol seeking, in addition to its well-known decreases in ethanol consumption (Bienkowski et al, 1999; Hyytia and Kiianmaa, 2001; June et al., 1999; Le et al.,

1993; Sharpe and Samson, 2001; Ulm et al., 1995; Williams et al., 1998). The present results add to the previous findings by providing an examination of the time course of the naltrexone effect during the reinstatement test. Naltrexone did not alter the latency to first lever press or port entry, and did not alter the number of responses emitted in the first 5 minutes of the test session. These findings suggest that the reduction in responding after naltrexone is not related to motor or memory (see below) impairments. In addition, we demonstrated that naltrexone does not affect responding in the extinction context, suggesting that this effect is specific for responding during reinstatement, although the low levels of responding during extinction make this type of conclusion tentative. One interpretation of the ability of naltrexone to decrease reinstatement triggered by the self-administration context is that it blocks the ability of the ethanol context to retrieve the appropriate memory(ies) of ethanol self-administration. A recent study has shown that inhibition of μ-opioid receptors in the hippocampus impairs spatial memory retrieval (Meilandt et al., 2004). Thus, naltrexone-treated subject might have lost the capability to discriminate between extinction and self-administration contexts. If this were true, then one would expect initial responding after naltrexone treatment in the ethanol context to resemble that seen in the extinction context. In addition, initial responding in the ethanol context would be different in saline- vs. naltrexone-treated subjects. However, neither of these was the case, as can be seen in an examination of the latency to the first lever-press response and the number of ethanol lever responses during the first 5 minutes of the session. After placement into the ethanol context, these parameters were very similar following saline and naltrexone treatment. Naltrexone reduced responding only after a number of non-reinforced responses had been emitted. Thus the 0.3-mg/kg dose of naltrexone does not seem to reduce responding reinstated by the ethanol context by impairing memory; rather, it appears to shorten the amount of time subjects are willing to respond in the absence of the ethanol reinforcer. Similarly, Cunningham et al. (1998) found that naloxone reduced the expression of ethanol-conditioned place preference, but only in the final 40 minutes of a 60-minute test session, an observation similar to that reported here (i.e., the initial

response to the ethanol-paired context was not altered by opioid receptor antagonism). Thus, the present results support the hypothesis that naltrexone enhances extinction of the conditioned rewarding effects of ethanol (Cunningham et al., 1998). This view is in agreement with the findings of Bienkowski and coworkers (1999) who observed that naltrexone reduces responding during extinction (enhances extinction learning) in subjects trained to lever-press for oral ethanol. In addition, Cunningham et al. (1998) also found that naloxone enhanced extinction of ethanol-conditioned place preference. Interestingly, in alcoholics, naltrexone reduces the subjective effects of alcohol-associated cues but only after repeated exposure to those cues over multiple 3-minute intervals (Rohsenow et al., 2000).

In the present experiments, we included the secondary measure of entries into the ethanol port to explore the influence of reinstatement procedures on this conditioned measure of ethanol seeking. We found in general that increases and decreases in port entries paralleled the increases and decreases in lever-press responding during ethanol self-administration, extinction, and reinstatement, although there was some variability between experiments in this regard. In addition, this measure was less sensitive to the effects of naltrexone. It should be noted that previous studies have suggested regulation of lever presses and port entries by distinct learning processes (i.e., appetitive vs. consummatory; Balleine, 1992, Balleine and Killcross, 1994).

In the current study we used different olfactory, tactile and visual cues to provide two distinct multimodal contexts. We chose to use a configuration of stimuli, rather than a single discriminative stimulus, to better approximate the distinctiveness of contexts in the real world. Several studies have tested the dependence of conditioned responding on the training environment using similar attributes to form distinct contexts (Frankland et al., 1998, Corcoran and Maren, 2001, Burwell et al., 2004). These studies, along with many others, have found that contextual discrimination involves the hippocampus. Hence it is likely that the hippocampus contributes to the context-dependent reinstatement observed in the current study. This possibility is strengthened by the recent report that

hippocampal inactivation blocks reinstatement of responding for cocaine induced by a context comprised of visual, auditory, tactile, and olfactory stimuli (Fuchs et al., 2004). Although the site of action for the effect of naltrexone on context-dependent reinstatement is not known, the VTA and/or the NAcc are likely, given the contribution of these regions to ethanol's reinforcing effects (Weiss and Porrino, 2002), as well as to reinstatement in general (Kalivas and McFarland, 2003). Recently a role for glutamatergic transmission in the VTA in context-induced reinstatement for heroin was demonstrated by Bossert et al (2005b). Another recent study found that intra-VTA but not intra-accumbens injection of the nonselective opioid antagonist, methylnaloxonium, decreases expression of ethanol-conditioned place preference (Bechtholt and Cunningham, 2005). These findings suggest that the VTA may be a site of action for the inhibition of context-induced ethanol seeking by naltrexone.

Together with current research with i.v. drugs (Alleweireldt et al., 2001, Crombag et al., 2002, Crombag and Shaham, 2002; Fuchs et al., 2004; Bossert et al., 2005b), the present findings provide evidence that the environmental context has a powerful influence on expression of conditioned drug-seeking behavior. Our results also indicate that endogenous opioid release may be part of the mechanism that underlies the influence of context on ethanol seeking. The context-induced reinstatement model described here may prove useful to further investigate the biological mechanisms underlying alcohol relapse.

4.1.2. Cocaine self-administration

The results obtained from the first experiment, indicate that discriminative stimuli associated with and predictive of the possibility of self-administering cocaine can elicit reliable cocaine-seeking behavior in the absence of further drug availability, in accordance with previous findings (Weiss et al. 2000, 2001; Ciccocioppo et al., 2001). Non-contingent presentation of cocaine-associated cues after 3 days in which rats remained on a stable extinction criterion induced drug-seeking behavior lasting up to four test sessions. These behavioral effects cannot

be attributed to non-specific arousal or spontaneous recovery since responding on the inactive lever remained negligible and, more importantly, responding in the presence of stimuli associated with no reward remained at the extinction level in all the test sessions.

In addition, we found that acute pre-treatment with the non-selective opioid receptor antagonist naltrexone attenuates cocaine seeking induced by conditioned stimuli.

Seeking behavior resumed with both cocaine- and sucrose- associated stimuli, whereas cues not associated with rewards had no such effect. Over the dose range tested (0.25-2.5 mg/kg), naltrexone attenuated cue-induced cocaine-seeking behavior, but did not affect the degree of reinstatement to sucrose. This rules out the possibility that naltrexone-induced reduction of lever pressing was due to memory impairment or motor activity abatement, as the animals were still capable of distinguishing the experimental conditions and pressing the lever after naltrexone treatment. It could be argued that differences in the length of reinstatement sessions could account for the different results obtained in the cocaine and the sucrose experiments. This possibility seems unlikely, however, given the pattern of responding during the reinstatement test, which shows the great majority of responses are emitted during the first 30 minutes of the session, as previously reported (Cervo et al., 2006).

Previous studies have shown that the opioid system is also involved in nutritive behavior, but to our knowledge these effects have been observed on natural feeding and self-administration. Indeed, seeking behavior is a distinct process, as demonstrated, for example, by the recent observation that sucrose seeking is decreased by a mGluR 2/3 agonist, which instead has no effect on sucrose self-administration (Bossert et al., 2006). Thus, our interpretation of the selective activity of naltrexone on cocaine seeking induced by conditioned stimuli supports the view that nutritive and non-nutritive reinforcers are processed differentially in the brain, in accordance with previous findings (Carelli et al. 2000). However, it cannot be ruled out that naltrexone preferentially reduces low-level of responding,

as in the case of cocaine reinstatement, as opposed to high level behavioral output as in the case of sucrose-associated cues reintroduction.

The effect of naltrexone indicates that cue-induced reinstatement of cocaine-seeking behavior is influenced, at least in part, by the endogenous opiate system. Together with recent studies showing that acute pretreatment with naltrexone reduces cue-induced alcohol- (Ciccocioppo et al., 2002; Burattini et al., 2006) and metamphetamine- (Anggadiredja et al., 2004) seeking behavior, this evidence suggests a more general, common role of opioid receptors in drug-seeking behavior. Naltrexone is a non-selective opioid antagonist, so we could not distinguish which receptor was involved in the process. However, since dopamine levels in the nucleus accumbens (NAc) are increased by discriminative stimuli previously paired with cocaine availability (Weiss et al., 2000), and μ and δ opioid receptors influence the activity of mesolimbic dopamine neurons (Devine et al., 1993), one possibility is that naltrexone antagonism at μ and/or δ opioid receptors attenuates reinstatement to cocaine-seeking behavior by preventing a cue-evoked increase of dopamine release in the NAc.

However there are also conflicting observations. Studies of reinstatement induced by a priming injection of cocaine in rats have shown that acute administration of naltrexone fails to suppress cocaine-seeking behavior (Comer et al., 1993; Gerrits et al., 2005), and that the μ-agonist etonitazine attenuates cocaine-induced reinstatement (Comer et al. 1993). Interestingly, contradictory results have also been obtained for metamphetamine-seeking behavior. Naltrexone inhibited reinstatement of drug seeking induced by metamphetamine-associated cues, but had no effect on reinstatement induced by drug priming (Anggadiredja et al., 2004). The different effects of naltrexone on cue- and drug-induced reinstatement in both cocaine and metamphetamine experiments might indicate that two distinct neural substrates underlie these processes.

In conclusion, this study provides the first evidence that the endogenous opioid system is involved in the mechanism of cocaine-seeking behavior induced by cocaine-associated stimuli in abstinent rats. In view of the persistence of

motivating effects of drug cues in humans (Childress et al., 1993), further investigation on tolerance to opioid antagonists is advisable.

4.2. Electrophysiological study

The aim of the electrophysiological study was to evaluate the involvement of the endogenous opioid system in LTP at the glutamatergic synapses of the MSN in the NAcc. To do so, the experiments have been performed in control conditions, in the presence of naltrexone (a non-selective opioid receptor antagonist) and in the presence of CTOP (a μ-opioid receptor antagonist), using two stimulation protocols of different intensity.

The results obtained demonstrate that in control conditions both stimulation protocols are capable of eliciting synaptic potentiation. However, the stronger stimulation protocol –HFS (4T)- is more effective for LTP induction.

The experiments performed in the presence of naltrexone show that non-specific antagonism at the opioid receptors only slightly modifies synaptic potentiation. Naltrexone significantly decreased the potentiation induced by HFS (4T), both in LTP and STP, whereas it increased the potentiation induced by HFS (2T), but the increase was only significant in LTP. In parallel, the STP induced by HFS (4T) was significantly shortened by naltrexone, whereas the STP induced by HFS (2T) was significantly prolonged by the same treatment. Thus, the results obtained in the experiments performed in the presence of naltrexone do not clarify the role of endogenous opioids in the potentiation of glutamatergic synapses in the NAcc. Given that naltrexone blocks both μ and κ opioid receptors, it is likely that a different contribution by each receptor on synaptic potentiation could account for the different results obtained with the stimulation protocols used.

In order to evaluate this possibility, an additional set of experiments has been performed in the presence of CTOP, a selective μ opioid receptor antagonist. The results obtained so far with HFS (4T) indicate that LTP is significantly increased by selective blockade of μ opioid receptors, and the increase is greater than that observed in the LTP induced by HFS (2T) when both μ and κ are blocked. This

result is in accordance with the observation that selective activation of μ opioid receptors reduces NMDA currents on MSN (Martin et al., 1997). The increase in LTP observed in the experiments performed in the presence of CTOP might result from the blockade of the negative modulation on NMDA currents exerted by the endogenous β-EP.

4.3. Concluding remarks

The main finding of the present thesis is the involvement of the endogenous opioid system in processes underlying relapse to ethanol and cocaine. Besides, the results reported herein provide evidence that endogenous opioid activity at opioid receptors modulate synaptic plasticity in a brain area relevant to addiction.

The observation that the environmental context can reinstate ethanol-seeking behavior in abstinent animals is in accordance with clinical reports, which find high rates of relapse when drug addicts return to their home environments. The efficacy of naltrexone against reinstatement induced by contextual stimuli conditioned to alcohol availability is in agreement with clinical findings demonstrating its efficacy in the treatment of alcohol relapse in humans. The evidence that naltrexone influences also cocaine reinstatement induced by conditioned stimuli suggests that the endogenous opioid system is involved in the circuitry mediating cocaine-seeking behavior, which overlap with the neural mechanisms underlying alcohol seeking.

The blockade of opioid receptors is hypothesized to prevent reinstatement by indirectly interfering with the activation of the mesolimbic-mesocortical DA system. Antagonism at the μ receptors may, in fact, increase the inhibitory tone on DA neurons in the VTA thereby decreasing the release of DA within forebrain structures likely involved in the reinstatement of drug-seeking behaviors. It should be pointed out, however, that naltrexone has almost equal affinity for μ, δ and κ

receptors, so we could not distinguish which receptor was involved in the process. Further studies with more selective agents will address this issue.

The preliminary results of the electrophysiological study demonstrate that μ-opioid receptors within the NAcc modulate synaptic plasticity in this brain area. However, in order to achieve a better understanding of the role endogenous opioids play in synaptic plasticity in the NAcc, additional experiments will be performed in the presence of k-opioid receptor modulators.

A better understanding of the interactions between mesolimbic DA and opioid systems would greatly enhance our knowledge of the complex mechanisms of addiction, and could lead to improvements in prevention and treatment of relapse.

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