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Divergent Sensitivities to Drugs of Abuse: Neurochemical and Neuroanatomical Characterization of the Roman Rats

Thesis for a PhD degree in Neuroscience

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A la mare i al pare

Abstract

Hundreds of millions of people experiment with drugs of abuse, but only a small percentage of them become addicted. Vulnerability to develop addiction has been associated with impulsivity or novelty-seeking. The Roman rats, genetically selected for high (RHA) or low (RLA) active avoidance acquisition in the two-way shuttle box, appear to be a valid laboratory model of divergent novelty and substance-seeking profiles and differ in the functionality of the dopaminergic system. So far, it is known that RHA rats drink ethanol voluntarily whereas RLA rats show aversion to it. In the present thesis, the Roman rats have been used as a model of differences in vulnerability to addiction. The aim of the thesis was to understand the neurobiological mechanisms that underlie such differences in vulnerability between the two Roman rat strains.

The work has been divided in 3 experimental blocks. First, we studied the behavioral response to an injection of a low dose of ethanol in the Roman rats. Like those humans that have higher risk to develop alcoholism, RHA rats were less sensitive to the effects of low-dose of ethanol. Second, brains of naïve Roman rats were studied in order to characterize several molecular targets of the dopaminergic system and related neuropeptides: dopamine receptor subtypes were quantified by means of receptor autoradiography and mRNA coding for neuropeptides were quantified using *in situ* hybridization histochemistry. When compared to RLA rats, RHA showed higher binding of D_1 and D_3 dopamine receptor subtypes and DYN mRNA expression in the nucleus accumbens (NAc) shell, although they showed lower basal binding of D_3 receptors in the Calleja islands. Moreover, a challenge with a D_3 agonist resulted in greater inhibition of locomotor activity as well as supression of NGFI-A mRNA as measured with *in situ* hybridization in the Calleja magna in RLA rats when compared to RHA rats. These results provide further evidences of the differences in dopamine function between the Roman strains and may represent the neurobiological core of the divergences in novelty-seeking and preference for addictive drugs such as ethanol. Third, behavioral sensitization, a model of behavioral and neuronal plasticity secondary to chronic drug use, was also studied in the Roman rats. Neuronal activity maps with 5 different immediate early genes were made by means of *in situ* hybridization. Amphetamine pre-treated RHA rats showed behavioral sensitization and increased secretogranin and PSD-95 in the NAc core which is suggestive of increased glutamatergic activity at this site. These findings are discussed in the context of the laboratory models of chronic drug use. Pretreatment with amphetamine in RLA rats did not result in behavioral sensitization but induced neuronal adaptations that may be related to the lack of this phenomenon. Moreover, RLA rats that experienced amphetamine for the first time showed activation of the central nucleus of the amygdala (CeA). Activation of the CeA was also seen in mice receiving ethanol and naltrexone, a drug used to prevent relapse in alcoholics. These findings suggest that the CeA may be a relevant brain structure in preventing drug addiction.

Sumari en Català

Només un petit percentatge d'entre tots els individus que experimenten amb drogues d'abús esdevenen addictes. La vulnerabilitat per a desenvolupar un trastorn addictiu està relacionada amb trets de personalitat impulsiva o amb apetència per la novetat. Les soques de rata Romanes, genèticament seleccionades per alta (RHA) o baixa (RLA) adquisició de l'evitació activa en dos sentits, són un model de laboratori vàlid de les divergències en apetència per la novetat i substàncies gratificants que s'observen en humans. A més a més, aquestes soques de rata difereixen en la funcionalitat del sistema dopaminèrgic. Se sap que les rates RHA beuen etanol voluntàriament mentre que les rates RLA mostren aversió. En aquesta tesis, les rates Romanes s'empren com a model de vulnerabilitat addictiva. L'objectiu de la tesis ha consistit en entendre els mecanismes neurobiològics que sustenten les diferències de vulnerabilitat addictiva entre les dues soques de rata Romanes.

El treball s'ha dividit en 3 fases experimentals. En primer lloc, es va avaluar la resposta conductual de les rates Romanes a la injecció d'una dosi baixa d'etanol. Tal i com s'observa en humans amb elevat risc d'esdevenir alcohòlics, les rates RHA van ser menys sensibles als efectes conductuals d'aquesta dosi. En la segona fase, es varen estudiar els cervells de rates Romanes naïve per tal de caracteritzar vàries dianes moleculars del sistema dopaminèrgic i neuropèptids relacionats: es van quantificar diferents subtipus de receptor de dopamina per mitjà de la tècnica d'autoradiografia de receptors així com els nivells d'expressió d'ARNm per diferents neuropèptids mitjançant la tècnica d'hibridació *in situ*. Comparades amb les rates RLA, les rates RHA presenten majors nivells de receptors D_1 i D_3 així com també d'ARNm per la DYN a nivell de l'escorça del nucli accumbens (NAc). En canvi, presenten menors nivells d'expressió del receptor D_3 a nivell de les illes de Calleja. A més a més, quan s'administra un agonista D_3, les rates RLA mostren major inhibició de l'activitat locomotora i supressió de l'expressió del gen NGFI-A a la Calleja magna (mesurat per mitjà de tècniques d'hibridació *in situ*) que les rates RHA. Aquests resultats afegeixen noves evidències de les diferències en la funcionalitat del sistema dopaminèrgic i potser representen l'eix central de l'entramat neurobiològic subjacent a les diferències en apetència per la novetat i preferència per a les drogues d'abús com l'etanol entre les dues soques. En la tercera fase, es va estudiar el fenomen de sensibilització conductual induït per amfetamina en les rates Romanes i es van elaborar mapes d'activitat neuronal per mitjà de tècniques d'hibridació *in situ* amb cinc gens d'expressió immediata. La sensibilització conductual és un model de plasticitat conductual i neuronal induïda per l'ús crònic de drogues. Les rates RHA que van rebre un tractament crònic amb amfetamina van mostrar sensibilització conductual i un augment en l'expressió dels gens de secretogranina i PSD95 al *corus* del NAc. Aquestes troballes es discuteixen en el context d'altres models de laboratori d'ús crònic de drogues. D'altra banda, les rates RLA no van mostrar sensibilització conductual però en canvi van mostrar adaptacions neuronals que poden estar relacionades amb la manca de sensibilització. A més a més, les rates RLA que van rebre amfetamina per primer cop van mostrar activació del nucli central de l'amígdala (CeA). També es va detectar activació del CeA en ratolins que van rebre un tractament amb etanol i naltrexona, fàrmac que s'empra a la pràctica clínica per tal de prevenir les recaigudes en alcohòlics. Aquesta troballa suggereix que el CeA pot ser una estructura del cervell rellevant per tal de frenar el desenvolupament dels trastorns addictius.

LIST OF PUBLICATIONS

PAPER I
Guitart-Masip M, Giménez-Llort L, Fernández-Teruel A, Cañete A, Tobeña A, Ögren S-O, Terenius L, Johansson B (2006)
Reduced acute response to ethanol and neuropeptide mRNAs in relevant brain areas in the alcohol preferring RHA rats.
European Journal of Neuroscience 23, 531-540

PAPER II
Guitart-Masip M, Johansson B, Fernández-Teruel A, Cañete A, Terenius L, Tobeña A, Giménez-Llort L
Divergent anatomical pattern of D3 binding and DARPP-32 mRNA expression in the Roman strains: implications for vulnerability to drug addiction.
Neuroscience, doi:10.1016/j.neuroscience.2006.07.041

PAPER III
Guitart-Masip M, Johansson B, Fernández-Teruel A, Tobeña A, Giménez-Llort L
D3 stimulation in the Calleja islands may mediate locomotor inhibition.
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PAPER IV
Guitart-Masip M, Johansson B, Fernández-Teruel A, Cañete A, Tobeña A, Giménez-Llort L
Induction of behavioral sensitization to amphetamine predicts vulnerability to behavioral sensitization in the Roman High Avoidance Rats.
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PAPER V
Guitart-Masip M, Johansson B, Fernández Teurel A, Cañete A, Terenius L, Tobeña A, Giménez-Llort L
Regional adaptations in PSD-95, NGFI-A and secretogranin gene transcripts related to vulnerability to behavioral sensitization to amphetamine in the Roman rat strains.
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PAPER VI
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Actas Espanolas de Psiquiatria (2006) 34:257-263.

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LIST OF ABBREVIATIONS

AA Alko, Alcohol (rats)

ANA Alko, non Alcohol (rats)

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

BNST Bed nucleus of the stria terminalis

BLA Basolateral amygdala

CeA Central amygdala

DARPP-32 Dopamine and AMPc regulated phosphoprotein of 32 kDa

CCK Preprocholecystokinin

DYN Preprodynorphin

ENK Preproenkephalin

ERK Extracellular signal-regulated kinase

HR High reactive (to novelty rats)

PKA Protein kinase A

LR Low reactive (to novelty rats)

NAc Nucleus accumbens

NGFI-A Nerve growth factor inducible clone A (an immediate early gene)

NMDA N-methyl-D-aspartate

NP Non preferring (alcohol rats)

P Preferring (alcohol rats)

PSD-95 Postsynaptic density 95 kDa (a protein)

PD128907 (+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol (a dopamine D3 receptor agonist)

RHA Roman high avoidance (rats)

RLA Roman low avoidance (rats)

SCH23390 R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (a dopamine D1 receptor antagonist)

Introduction

Drug addiction progress through a characteristic clinical course, depends on specified neuronal mechanisms and its liability is known to be heritable (McLellan *et al.,* 2000). According to the Diagnostic and Statistical Manual of Mental Disorders by the American Psychiatric Association (DSM-IV, the main diagnostic tool in psychiatry), the hallmarks of substance dependence (beside tolerance and withdrawal) are: 1) difficulty to stop or limiting drug use; 2) extremely high motivation to procure and take the drug; and 3) drug taking proceeds despite its negative consequences. Drug dependence is, thus, equated to compulsive drug use. Moreover, addiction is characterized by a chronic course in which addict's repeated attempts to quit the use of drugs are mislead by a high propensity to relapse even after long periods of withdrawal (DeJong, 1994; Hyman and Malenka, 2001). However, the clinical stage of compulsive drug use plus repeated relapses is not reached by everybody having experiences with drugs. In fact, only 15 to 17% of those using drugs develop a behavioral disorder fulfilling the DSM-IV criteria (Anthony *et al.,* 1994). Vulnerability to develop addictive patterns is influenced by a combination of multiple genetic and environmental factors (Kreek *et al.,* 2005). Variation in the core neurobiology of addiction is genetically influenced and vulnerability to drug dependence has a strong genetic component estimated around 40-60% (Crabbe, 2002; Goldman *et al.,* 2005).

Nature Neuroscience devoted a monograph to addiction at the end of 2005 which began with a claim to break stigma and misconception over addicted subjects held by the society (Dackis and O'Brien, 2005). According to the Health Statistics-Key Data (2002), in the European Union, cannabis was the illicit drug with the highest last 12 month prevalence (1-9% varying among member states). Prevalence among younger adults was roughly double the prevalence among all adults. Use of other addictive drugs was around 1% in all member states and less than 5% among younger adults. Data collected in the same period shows that life-time prevalence was 2 to 3 times higher than last 12 month prevalence in most places. Addiction is highly expensive for the society (Dackis and O'Brien, 2005). It is well known, for instance, that alcohol consumption is highly common in the society: according to a report by RAND Corporation (Horlings and Scoggins, 2006), 86% of adult Europeans older than 16 years consumed some alcohol and 15.5% could be considered heavy drinkers. In the European Union alcohol is the third leading risk factor for disease burden after tobacco and obesity and represents 11% of male premature death. Therefore, establishing the mechanisms for vulnerability to addiction is a crucial step in the design of new preventive interventions in the community that dampen the impact of such a social and medical problem.

1. Temperament as a risk factor for addiction: novelty sensation-seeking in rodent models

Current models of personality define several (3 to 5) basic vectors or behavioral dimensions that describe the personality of an individual and cover the rich complexity of human temperament (Eysenck, 1967; Cloninger, 1987; Zuckerman, 1993; Gray and McNaughton, 2000). The terminology to address personality dimensions and the borders between these dimensions vary depending on the model. However, among basic traits, variations in impulsivity together with risk taking, novelty-seeking or sensation-seeking are consistently related to the initiation of drug use as well as the transition from occasional testing to regular use and addiction (reviewed by Dawe and Loxton, 2004; Kreek *et al.*, 2005). Zuckerman (1994a, 1994b) clarified the notions of impulsivity and sensation-seeking and combined them in a supertrait called impulsive sensation-seeking. *Sensation-seeking* is defined by the seeking of varied, novel, complex and intense sensations and experiences, and the willingness to take physical, social, legal and financial risk for the sake of such experiences. *Impulsivity* is the tendency to enter into situations, or rapidly respond to cues of potential reward, without much planning or deliberation and without consideration of potential punishment or loss of reward.

Novelty- or *sensation-seeking* can be studied in rodents by behavioral criteria such as exploration activity in open areas, and this behavioral characteristic is considered an animal model of human sensation-seeking (Bardo *et al.*, 1996; Zuckerman, 1996). Animals that show high response to novelty show higher liability to self-administer drugs of abuse (Piazza *et al.*, 1989) and they show higher levels of dopamine in the nucleus accumbens (NAc) both under basal conditions (Hooks *et al.*, 1992b), as well as during novel or stressful situations (Rouge-Pont *et al.*, 1993). In fact, it has been suggested that novelty-seeking is influenced by the reactivity of the mesolimbic dopaminergic system (Bardo *et al.*, 1996; Zuckerman, 1996). Moreover, marked differences between inbred strains and within subspecies in exploratory behavior in mice suggest a genetic control of this trait (Henderson, 1967).

2. Three main neurobiological approaches to explain addiction

Drug addiction is a complex disorder with interacting environmental factors, drug induced neurobiological changes, comorbility with other psychiatric disorders, personality vulnerabilities and response to stressful demands. There are three major approaches or ways of thinking to try to account in neural terms for the development and maintenance of addiction once individuals have begun to take drugs. They are usually referred to as “incentive sensitization”, “hedonic allostasis” and “habit formation” theories. The first 2 theories are based on changes in the motivational or affective systems of the brain induced by drugs. The last one is mainly based on the automaticity of the behavioral output. They are all based on neuronal adaptations on the pleasure/aversion central systems schematically drawn in figure 1. These theories explain indeed

part of the phenomenology of addiction and they should be seen as complementary, maybe as representative of different stages in the development of the addictive disorders.

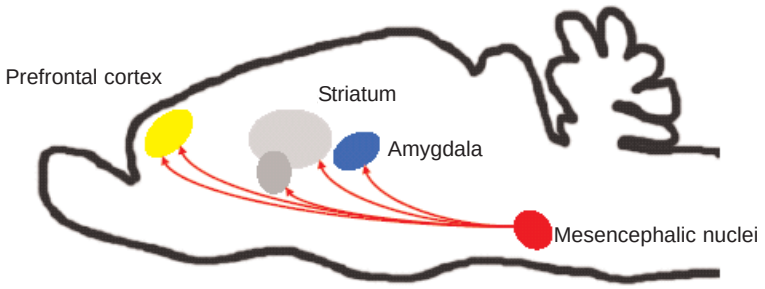

Figure 1: Reward pathways in rodent's brain: key neural sites.

This very schematic figure representing a sagittal slice through the rat brain shows the approximate location of the brain areas that have been related with the processing of reinforcement and reward. In red appear the mesencephalic projection neurons that send their axons towards the forebrain through the median forebrain bundle, reaching the striatum with its dorsal and ventral subdivisions, the amygdala and the prefrontal cortex.

a. *Incentive sensitization*

This theory postulates that, when chronically administered, drugs of abuse induce an increase of the responsiveness (sensitization) of dopaminergic mechanisms that mediate incentive salience (attractiveness) of the drug itself or drug related stimuli. This enhanced dopamine responsiveness would account for the craving that many addicts experience during abstinence and that likely leads to relapse (Robinson and Berridge, 1993, 2001).

b. *Hedonic allostasis*

This theory postulates that the appearance of compensatory mechanisms opposing the effects of the drug leads to a state of allostasis characterized by a brain that is less sensitive to reward. In this situation, the individuals administer the drug in an attempt to compensate for this situation (Koob and Le Moal, 1997; Koob *et al.*, 2004).

c. *Habit formation*

This theory postulates that drug addiction can be understood as a pathological subversion of normal brain learning and memory processes strengthened by the motivational impact of drug-associated stimuli, leading to the establishment of compulsive drug-seeking habits. This transition would emerge from a switch on the neurobiological substrate of the observed behavior (Tiffany, 1990; Robbins and Everitt, 1999; Everitt and Robbins, 2005). This is perhaps the oldest theory of addiction but only recently the neural mechanisms have been specified.

3. The Roman rat lines/strains as an animal model of vulnerability to drug addiction

Similar to what is observed in humans, only in 15-17% of the rats that could self-administer cocaine for long periods of time developed addictive-like behavioral patterns as assessed with the confluence of 3 different addict-like criteria together with high propensity to reinstate self-administration after extinction (Deroche-Gamonet *et al.*, 2004). This finding points out that there are individual factors in rodents that, like in humans, predispose individuals to addiction. The Roman rats are good candidates to study these factors (Driscoll *et al.*, 1998).

a. *Psychogenetic selection of the Roman lines and inbreeding program*

The first report concerning the Roman High- and Low- Avoidance rats (RHA and RLA respectively) appeared in 1965. Wistar *Rattus norvegicus* were psychogenetically selected for their fast (RHA) or extremely low (RLA) acquisition of two-way active avoidance in the shuttle box (Bignami, 1965). Sublines have been established in Switzerland and maintained under continuous selective breeding since 1972 and they have been studied extensively (Driscoll and Bättig, 1982). In 1993 an inbreeding program was started and one inbred colony is currently maintained in the Animal Facilities of the Medical Psychology Unit at the UAB, Bellaterra, Spain (Driscoll *et al.*, 1998; Escorihuela *et al.*, 1999). In the present thesis, RHA and RLA will refer to the outbred Roman rat lines or to findings generalized in both outbred and inbred rats. On the other hand, the use of RHA-I and RLA-I will always refer to the findings in the inbred strains.

b. *General behavioral characteristics of the Roman rat lines*

The acquisition of the two-way active avoidance in the shuttle box is inversely related to fear levels in animals (Fernández-Teruel *et al.*, 1991). Therefore, the Roman rats were selected in a test that measured fear among other variables. Due to this selection, RHA rats, with high avoidance acquisition, show lower emotional reactivity than RLA rats (Fernández-Teruel *et al.*, 1997b; Steimer and Driscoll, 2003). Under circumstances of mild stress like placement in a novel environment, RLA rats show higher fear or emotional reactivity as measured by time spent in freezing or number of defecations (Fernández-Teruel *et al.*, 1997b), and higher endocrine response as measured by a stronger and longer stress induced corticosterone and prolactin release (Steimer *et al.*, 1997; Steimer and Driscoll, 2003). RLA rats also show increased fear response to a strong aversive sound as measured by the startle response (Schwegler *et al.*, 1997), and they show higher number of self-groming episodes, a displacement or conflict activity, when exposed to novelty (Fernández-Teruel *et al.*, 1997b).

On the other hand, RHA rats show a more active behavioral response than RLA rats in several distinct behavioral paradigms: RHA rats show enhanced locomotor response when placed in a novel environment like the open field, the plus maze, the hole board (Fernández-Teruel *et al.*, 1997b; Escorihuela *et al.*, 1999; Fernández-Teruel *et al.*, 2002), or the locomotor test cage

(Giménez-Llort *et al.*, 2005). RHA show stronger and longer lasting attempts to actively remove the source of pressure than RLA rats when exposed to tail-pinch (Giorgi *et al.*, 2003). In tests were there is a transition between two areas with different safety value such as the light/dark box or the plus maze, RHA rats cross the border between the zones at the first attempt whereas RLA rats usually make many attempts (Fernández-Teruel *et al.*, 1997b; Steimer and Driscoll, 2003). Finally, RHA rats show enhanced preference for rewarding substances like saccharine or ethanol (Driscoll *et al.*, 1990; Razafimanalina *et al.*, 1996; Fernández-Teruel *et al.*, 2002).

Based on these and other behavioral and endocrine characteristics, Steimer *et al.* (1997) have defined a two-dimension model of the "temperament" of RLA and RHA rats in which the dimensions are relative between the two lines: a conjunction of high emotionality with passive (reactive) coping style in RLA rats results in increased fearfulness or anxiety; conjunction of low emotionality with an active (proactive) coping style in RHA rats results in animals that can be seen as impulsive or novelty-seekers. Increased impulsivity or novelty-seeking in RHA rats, when compared to RLA rats, is supported by several behavioral evidences and it is the basis of the use of RHA rats as a model for liability to addiction (Driscoll *et al.*, 1998). Perhaps the more conclusive evidence supporting an impulsive or novelty-seeking profile in RHA rats is the finding, only in these rats, of increased amplitude of the visual evoked potential (VEP) as the intensity of the visual stimuli increases (Siegel *et al.*, 1993; Siegel, 1997). The same finding has been reported in humans: those identified as high sensation-seekers with the scales developed by Zuckerman showed increased VEP as a function of increased intensity, whereas a decrease is detected in humans with low scores in the sensation-seeking scales (Zuckerman, 1974).

c. *The mesocorticolimbic dopaminergic system and the impulsive profile in RHA rats*

As pointed out above, impulsivity or novelty-seeking is thought to be influenced by the reactivity of the dopaminergic system. Besides the described differences in the reactivity of the HPA axis, differences in the mesocorticolimbic dopaminergic system between the Roman lines/strains may account for the described behavioral differences (Driscoll *et al.*, 1998). RHA rats show a more pronounced dopamine release than RLA rats in the prefrontal cortex under stress conditions (D'Angio *et al.*, 1988; Giorgi *et al.*, 2003), and this finding correlated with active coping as previously described (Giorgi *et al.*, 2003). However, RLA rats show higher dopamine release in the nucleus accumbens (NAc) than RHA rats under stress conditions (Giorgi *et al.*, 2003). When compared to RLA rats, RHA rats show higher behavioral response to apomorphine (Durcan *et al.*, 1984; Giménez-Llort *et al.*, 2005) and amphetamine (Driscoll *et al.*, 1986; Cañete *et al.*, 2003) a direct and indirect dopamine agonist respectively. RHA rats show a more reactive mesoacumbens pathway than RLA rats as shown by the higher behavioral response and dopamine release into the NAc induced by administration of abused drugs such as amphetamine, cocaine, morphine, and ethanol (Giorgi *et al.*, 1997; Corda *et al.*, 2001; Lecca *et al.*, 2004). The dopaminergic response to these drugs is stronger in the NAc shell when compared to the NAc

core in RHA rats but it does not differ between accumbal subregions in RLA rats (Lecca *et al.*, 2004). Moreover, in the NAc shell, RHA show higher density of D_1 receptors (Corda *et al.*, 1997). As discussed below, release of dopamine in the NAc is a common neuronal substrate for the reinforcing effects of all abused drugs (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988), and more recently the increase has been found circumscribed in the shell subregion (Pontieri *et al.*, 1995). This enhanced responsiveness of the dopaminergic mesoaccumbal system in RHA rats may underlie their enhanced preference for alcohol and other rewarding substances (Razafimanalina *et al.*, 1996; Fernández-Teruel *et al.*, 2002). Finally, RHA rats show behavioral sensitization when they are chronically treated with morphine, amphetamine and cocaine but, under the same circumstances, this phenomenon is not observed in RLA rats (Piras *et al.*, 2003; Corda *et al.*, 2005; Giorgi *et al.*, 2005b).

c. *Other rodent models of vulnerability to drug addiction*

Maybe the best characterized rodent model of vulnerability to drug addiction is represented by the High Reactive (HR) and Low Reactive (LR) to novelty rats (reviewed by Piazza *et al.*, 1998). HR and LR rats display higher or lower novelty-induced locomotor activity than the median in a given rat stock, and they show higher or lower liability to self-administered drugs (Piazza *et al.*, 1989). Both HR and LR rats self-administer psychostimulants during the first day of training for self-administration, but this behavior rapidly extinguishes in LR rats whereas it stabilizes in HR rats (Piazza *et al.*, 1990; Piazza *et al.*, 1991; Piazza *et al.*, 1993a). When compared to LR rats, HR rats show: 1) higher seeking for novel and stressful situations (Dellu *et al.*, 1996); 2) higher behavioral response to administration of psychostimulants (Piazza *et al.*, 1989; Hooks *et al.*, 1991 but see also Pierre and Vezina, 1997); 3) higher basal, cocaine (Hooks *et al.*, 1992b) and stress-induced (Rouge-Pont *et al.*, 1993) dopamine release in the NAc; 4) higher sensitivity to other reinforcing stimuli such as food (Piazza *et al.*, 1998); 5) higher stress-induced corticosterone blood levels. With regards to sensitization with amphetamine, conflicting results have been reported in the HR/LR rats: HR rats show stronger behavioral sensitization when sensitization is context dependent (in other words, induction treatment is paired with the testing conditions) (Hooks *et al.*, 1992a); but sensitization may exclusively appear in LR rats when sensitization is context independent (Piazza *et al.*, 1989).

Table 1 shows a comparison of the HR/LR rats with the RHA/RLA rats in the aspects presented in the text. In most of the comparisons, HR/LR rats comparisons resemble RHA/RLA rats except for the basal dopamine levels in the NAc (HR>LR; RHA=RLA) and the reactivity of the HPA axis (HR>LR; RHA<RLA). HR/LR rats are selected for novelty-induced motor activity, a measure dependent on accumbal dopamine levels (Koob *et al.*, 1981), and this selection criterion may lead to two batches of animals differing in basal accumbal dopamine. Since Roman rats also differ in novelty-induced motor activity and in other measures of the dopamine responsiveness, the difference between HR/LR and RHA/RLA rats is more likely to represent a quantitative difference.

However, the differences regarding the HPA axis seem more relevant and may represent a qualitative difference between HR/LR and RHA/RLA rats. Adrenalectomy abolishes the differences in novelty-induced locomotion between HR and LR rats (Piazza *et al.*, 1998). Moreover, whereas suppression of corticosterone levels decreases amphetamine self-administration in HR rats (Piazza *et al.*, 1994), administration of corticosterone induces the acquisition and maintenance of amphetamine self-administration in LR rats (Piazza *et al.*, 1991). It seems, thus, that greater reactivity of the HPA is a key feature in the phenotype of the drug vulnerable HR rats. It is known that corticosterone participates in the cocaine-induced locomotor effects (Marinelli *et al.*, 1994) and dopamine release in the accumbens shell (Barrot *et al.*, 2000). Moreover, rats learn to self-administer corticosterone and HR rats are more sensitive to the reinforcing effects of this substance (Piazza *et al.*, 1993b). A critical difference in the HPA axis between the two Roman strains seems to be the effectiveness of the feed-back mechanisms: RLA show lower dexamethasone suppression of the HPA axis and lower densities in hippocampal and pituitary glucocorticoid receptors (Steimer *et al.*, 1997). Then, although RHA/RLA rats are not comparable to HR/LR rats in the reactivity of the HPA axis, the actual central sensitivity of the Roman lines/strains to corticosterone and its implication in the known novelty- and drug-induced locomotor activity is not known yet.

Table 1: Comparison between HR/LR rats and RHA/RLA rats

	HR rats compared to LR	RHA rats compared to RHA rats
Locomotor activity induced by novelty	↑	↑
Locomotor response to psychostimulants	↑ (not always)	↑ (not always)
Basal dopamine in the NAc	↑	=
Stress-induced dopamine in the NAc	↑	↓
Stress-induced dopamine in the PFC	unknown	↑
Psychostimulant induced dopamine in the NAc	↑	↑
Reactivity of the HPA axis	↑	↓

Several rat lines have been selected for the amount of ethanol ingested in a free choice paradigm: well characterized examples are the AA/ANA (alcohol preferring Alko, Alcohol and the alcohol non-preferring Alko, Non-Alcohol) rats and the P/NP (alcohol preferring and non- preferring) rats (Eriksson, 1968; Lumeng *et al.*, 1977). Besides differing in ethanol preference, these lines also differ in many other behavioral and neurochemical measures. When compared to NP rats, P rats show higher preference for sweet solutions, higher locomotor activation induced by novelty and low doses of ethanol, and no differences in aversion for bitter solutions (Murphy *et al.*, 2002). When compared to ANA rats, AA rats show higher preference for sweet solutions and lower aversion for bitter solutions (Badia-Elder and Kiefer, 1999) and no differences in ethanol-induced locomotor activity (Päivärinta and Korpi, 1993). In humans, lower sensitivity to ethanol is predictive of higher risk for alcoholism (Schuckit, 1994), but it is not clear whether there is the

same association in rodents. As in the case of the ethanol preferring RHA rats, both AA and P rats display higher ethanol-induced dopamine release in the NAc when compared to ANA and NP rats (Katner and Weiss, 2001; Murphy *et al.*, 2002 respectively). Similarly, both AA and P rats show higher density of μ-opioid receptors in the shell of the NAc than ANA and NP rats (De Waele *et al.*, 1995; Murphy *et al.*, 2002 respectively), but this parameter is not known for the Roman rats. An endogenous ligand of μ-opioid receptors is β-endorphin, whose blood levels increase after ethanol consumption (Oswald and Wand, 2004). Therefore, differences in dopaminergic and opioid function seem to underlie higher ethanol preference in these models.

4. Neurocircuitry underlying drug addiction

The use of animal models has allowed the identification of neurobiological processes that underlie the development of addiction. In this section, an overview of the anatomy and physiology of the circuits that interact with drugs is presented.

a. The mesolimbic and mesocortical dopaminergic system

Although all drugs that humans abuse of differ in their pharmacological profile, they all increase, to a certain extent, the levels of dopamine in one area of the limbic brain, the NAc (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988; Wise, 1998). Rat models of vulnerability to drug addiction, including RHA rats, show increased reactivity of the dopaminergic system. This system is formed by two groups of neurons. One group is located in the substantia nigra pars compacta and projects through the nigrostriatal pathway to the dorsal striatum. The other group of neurons is located in the ventral tegmental area (VTA) and projects through the mesolimbic and mesocortical pathways. The mesolimbic pathway reaches the NAc and the amygdala, and the mesocortical pathway reaches the prefrontal cortex (PFC). A schematic representation of the main dopaminergic bundles is shown in figure 2. Dopamine is a modulatory neurotransmitter that mainly modulates the response of neurons to glutamate and GABA, the main excitatory and inhibitory neurotransmitter, respectively (revised by Nicola *et al.*, 2000 and West *et al.*, 2003). Dopamine released by neurons of the nigrostriatal pathway has a prominent role in modulation of movement and learning of motor skills as evidenced by the appearance of Parkinson disease when these neurons die (reviewed by Berke and Hyman, 2000; Packard and Knowlton, 2002). Dopamine neurons of the VTA signal reward or reward related stimuli (Schultz, 1998). Dopamine, in the NAc, mediates incentive salience and reward learning (Berke and Hyman, 2000; Berridge and Robinson, 2003), and boosts approach component of goal directed behaviors (Cardinal *et al.*, 2002). In the prefrontal cortex, dopamine modulates cognitive processes related to goal directed behavior (Tzschentke, 2001). However, the hedonic impact of the reward itself is independent on dopamine (Berridge and Robinson, 1998) and has been related to the opioid system which is widespread around the brain (Glass *et al.*, 1999; Kelley *et al.*, 2002).

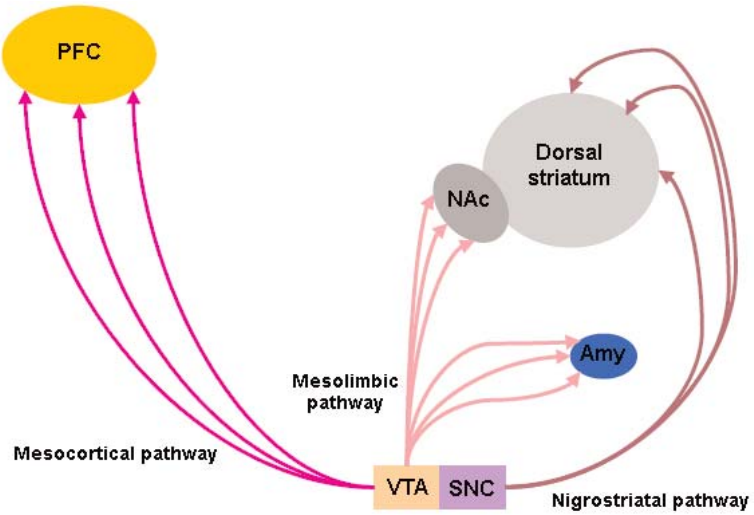

Figure 2: The dopaminergic system.

This schematic figure shows the main dopaminergic pathways arising from the two main groups of neurons in the mesencephalon that project to the forebrain. One group is located in the substantia nigra pars compacta (SNC) and projects through the nigrostriatal pathway to the dorsal striatum. The other group of neurons is located in the ventral tegmental area (VTA) and projects through the mesolimbic and mesocortical pathways. The mesolimbic pathway reaches the nucleus accumbens (NAc) and the amygdala (Amy), and the mesocortical pathway reaches the prefrontal cortex (PFC).

b. *Anatomical and molecular aspects of the corticostriatal systems*

In the previous section, the dopaminergic system was presented as having different effects in independent brain areas. In fact the cortex and the striatum work together in circuits arranged in parallel loops that go from the cortex to the striatum (corticostriatal systems) and return to the cortex through the pallidum and the thalamus (Alexander *et al.*, 1986; Bolam *et al.*, 2000). The group of nuclei between the cortex and the thalamus are known as the basal ganglia. The striatum is the first relay station of the basal ganglia and receives excitatory inputs from the entire cortex and is connected to the output structures of the basal ganglia, i.e. the substantia nigra reticulata and entopeduncular nucleus (revised by Albin *et al.*, 1995). Due to the high content of dopamine and dopamine receptors in the striatum (Mansour *et al.*, 1990; Mansour *et al.*, 1991), and the importance of dopamine in movement and reinforced behavior, the striatum has been an important focus of research. The striatum can be divided in terms of embryology, anatomy and physiology in a dorsal and a ventral part, the latter including the NAc (De Olmos and Heimer, 1999; Heimer, 2003). The dorsal striatum receives its cortical input from somatosensory and associative cortices. On the other hand, the ventral portion receives converging input from the hippocampus, the basolateral amygdala and the prefrontal cortex (Voorn *et al.*, 2004). The histological and physiological characteristics of the dorsal striatum and its integration in the basal ganglia systems are better characterized. Therefore, a simplified description of the histological and physiological organization of the dorsal striatum is summarized in box 1 and in figure 3 as an illustrative example for the whole system.

Box 1: Simplified circuitry of the basal ganglia through the dorsal striatum.

To achieve its function, the dorsal striatum receives converging inputs from many cortical areas that form a functional mosaic of parallel circuits with topographical organization that is maintained in the striatal projection areas (Deniau *et al.*, 1996). 90% of the neurons in the striatum are GABAergic projection spiny neurons that are subdivided into two groups (Gerfen, 1992). One group of spiny neurons expresses D_1 receptors, substance P and the opioid peptide dynorphin. These neurons conform the so called direct pathway and directly project to the output structures of the basal ganglia: the substantia nigra reticulata (SNR) and the entopeduncular nucleus (EP). The other group of striatal neurons expresses D_2 receptors and the opioid peptide enkephalin. These neurons comprise the so called indirect pathway that successively involves GABAergic neurons in the globus pallidum (GP) and glutamatergic neurons in the subthalamic nucleus (STN) that eventually project to the output structures (Bolam *et al.*, 2000). The STN also receives direct excitatory input from motor, premotor and prefrontal areas of the cortex (Maurice *et al.*, 1998). The output nuclei of the basal ganglia such as the SNR project to the thalamus and reenter the corticostriatal loops but they also project to the brainstem premotor areas (BSPM) (Alexander *et al.*, 1986; Bolam *et al.*, 2000). The GABAergic neurons of the output structures tonically fire under basal conditions and maintain inhibition of the thalamus and the brainstem premotor centers (Deniau *et al.*, 1978; Chevalier and Deniau, 1990). As suggested by Kolomiets *et al.* (2003) or Grillner *et al.* (2005), activation of neurons in the two striatal pathways and neurons in the subtalamic nucleus results in selective inhibition of a group of output neurons. If this inhibition is achieved, it has a functional consequence (behavioral output, modulation of cognitive processes, etc…). The striatum, thus, works as a gate for competing cortical behavioral or cognitive signals.

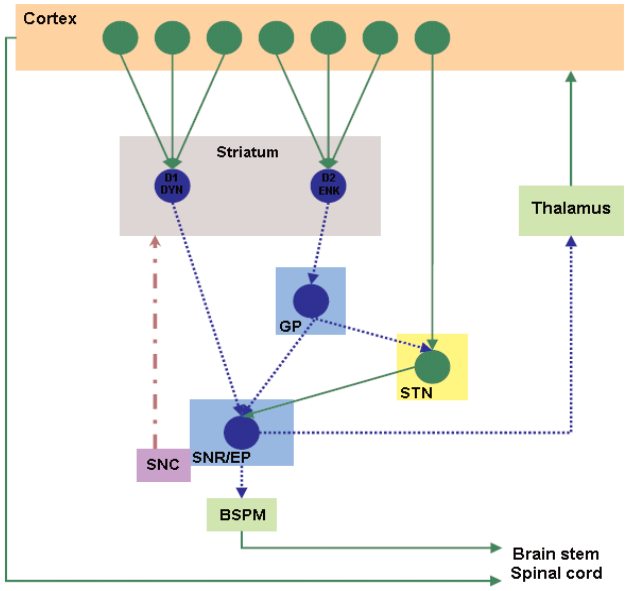

Figure 3:

Excitatory projections are shown by green lines, inhibitory projections by dotted blue lines, and dopaminergic projections by discontinuous lines. Adapted from Bolam et al., (2000)

How do the cells in the striatum work as a gate? Striatal neurons show two different electrophysiological states: a hyperpolarized or silent state (also referred as “down state”) maintained by rectifying K^+ currents, and a more depolarized state (also named “up state”) in which neurons may fire action potentials (Wilson and Kawaguchi, 1996). To reach the “up state”, neurons must be activated by convergent glutamate (cortical or thalamic) signals (O’Donnell and Grace, 1995). When neurons are in the silent state, dopamine, by activating D_1 receptors, promote the K^+ rectifying currents and, thus, suppression of excitability. However, near the depolarized state, D_1 receptor activation enables and maintains the steady up state. Then, dopamine acts as a coincidence detector, enhancing glutamate induced currents when many excitatory synapses coincide in time and space. Moreover, D_1 receptors are coupled to mechanisms of synaptic plasticity that eventually may change synaptic strength and underlie learning processes. This model of dopamine and glutamate interaction in the striatum is reviewed by, among others, Nicola *et al.* (2000), West *et al.* (2003) and Kelly (2005). Less is know about the electrophysiologic consequences of D_2 receptors expressed by spiny cells (Nicola *et al.*, 2000).

More than 90% of the striatal neurons are spiny GABAergic neurons. D_1 and D_2 receptors are generally expressed by different spiny cells that form the direct and indirect pathways respectively (Gerfen, 1992). However, virtually all spiny neurons express DARPP-32 (Svenningsson *et al.*, 2004), an intracellular protein with several phosphorylation sites. A schematic representation of each of these neurons and its molecular signaling pathways is shown in figure 4. The importance of this signaling protein for the field of addiction was shown by the lack of behavioral effects of abused drugs in the knock out mice (Svenningsson *et al.*, 2003). D_1 and D_2 receptors are coupled to adenyl cyclase and activate or inhibit cAMP dependent protein kinase (PKA) respectively. When activated by PKA phosphorylation, DARPP-32 becomes a potent inhibitor of protein phosphatase 1 and prevents dephosphorylation of PKA substrates and PKA itself. So, through DARPP-32 modulation, dopamine achieves amplification of its cellular signaling effects (Nishi *et al.*, 1997; Fienberg *et al.*, 1998; Nishi *et al.*, 2000). Glutamate may alter DARPP-32 phosphorylation at different sites (Nishi *et al.*, 2005). The exact nature of dopamine/glutamate interactions in DARPP-32 modulation is not clear yet, though DARPP-32 is a clear candidate cellular site in which this interaction takes place.

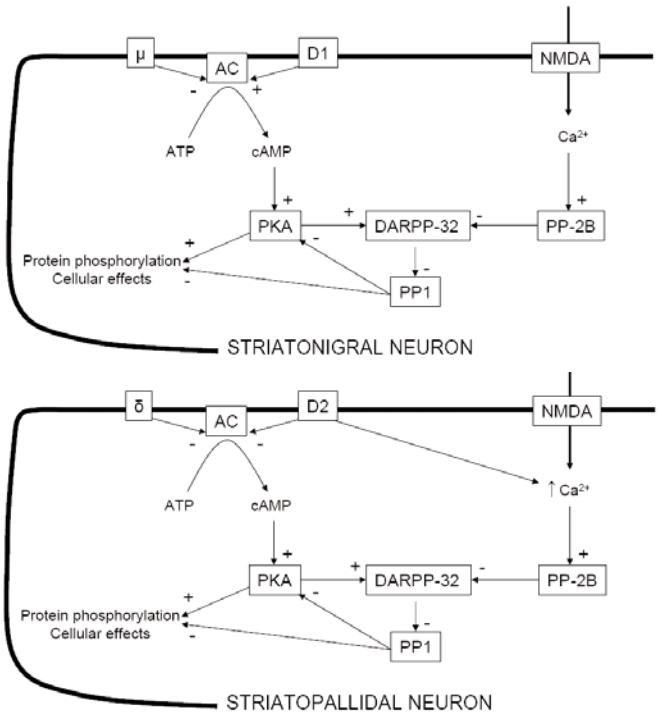

Figure 4: Signaling pathways and dopamine/glutamate interaction in striatal spiny cells.

This figure depicts the signaling pathways mediating the major effects of dopamine, glutamate and opioids in the two subtypes of striatal spiny neurons. In striatonigral neurons, activation of D1 receptors activates the adenylyl cyclase which increases cAMP and activates protein kinase A (PKA). This protein phosphorylates many other proteins which ultimately have cellular effects. PKA and its substrates are dephosphorylated by protein phosphatase 1 (PP1). Therefore, PP1 brings the effects of D1 stimulation to an end. By activating DARPP-32, PKA keeps PP1 inhibited. Stimulation of opioid receptors suppresses PKA activity and, by inhibiting DARPP-32, relives PP1 from inhibition and promotes inactivation of PKA and its substrates. Activation of NMDA glutamate receptor and the subsequent increase in intracellular Ca2+ activates protein phosphatase PP-2B (or calcineurin) which also suppresses DARPP-32 and relives PP1 from inhibition. In striatopallidal neurons, activation of D2 receptors inhibits DARPP-32 through two synergic mechanisms: 1) inhibition of adenylyl cyclase, and 2) increase in intracellular Ca2+.
Adapted from Svenningsson et al., (2004)

As suggested by Berke and Hyman, 2000, it may be said that dopamine, through its interaction with glutamate, facilitates action and regulates learning in processes involving the striatum. However, in the striatum, dopamine interacts with many other molecules. Some of them, like the opioid peptides (dynorphin and enkephalin) and cholecystokinin, have been related to vulnerability to addiction in the HR/LR rats (Lucas *et al.*, 1998). Moreover, the relation of opioid peptides with alcoholism has already been noticed. The striatum is enriched in opioid receptors (Mansour *et al.*, 1987). Activation of κ-receptors (presumably located presynaptically on the dopamine terminals) by dynorphin peptides leads to a decrease in dopaminergic transmission (Spanagel *et al.*, 1992). Moreover, it has been suggested that dynorphin levels upregulate as a result of hyperdopaminergic activity in an attempt to dampen cellular response to dopamine (Steiner and Gerfen, 1998). Cholecystokinin reaches the striatum from pyramidal neurons and dopaminergic neurons (reviewed by Hökfelt *et al.*, 2002), and it has been related to addictive behaviors (reviewed by Rotzinger and Vaccarino, 2003).

c. The nucleus accumbens and its place in the motive circuit

The corticostriatal loops that flow through the NAc together with the VTA conform what has been termed the motive circuit. This circuit is implicated in the translation of biological relevant stimuli into adaptative behavioral responses (Kalivas *et al.*, 1993). The repeated use of addictive drugs induce neurochemical and structural changes in this circuit which results in behavioral sensitization (Pierce and Kalivas, 1997) and the increased craving and drug seeking that predispose addicts to relapse (Kalivas *et al.*, 2005; Kalivas and Volkow, 2005). The NAc can be divided anatomically, histochemically, pharmacologically, and functionally in distinct subareas: the shell and the core (reviewed by Pennartz *et al.*, 1994; Zahm, 2000). Whereas the core shares anatomical and histochemical characteristics with the rest of the striatum, the shell is a transitional area and also share features with the central nucleus of the amygdala (CeA) (Koob *et al.*, 1998; Zahm, 1999). The ventral striatum is one of the areas where D_3 receptors are expressed (Sokoloff *et al.*, 1990).

Like other striatal areas, the NAc shell takes part in the reentering corticostriatal loops; it projects to the ventral pallidum (output structure of the basal ganglia) and through a relay in the thalamus the information flows again to the cortex (De Olmos and Heimer, 1999; Zahm, 2000). As reviewed by Zahm *et al.* (1999), and shown in figure 5, the organization of the parallel corticostriatal loops is spiral-shaped so that neural information is shunted directly from the shell to the core. This spiral organization is also seen from the core to more dorsal striatal areas. However, the shell is the only striatal area projecting to the lateral hypothalamus, an area to which the CeA also projects (Zahm *et al.*, 1999). The lateral hypothalamus has direct control over hypothalamic and brainstem pattern generators (Swanson, 2000). Striatal neurons also project to the mesencephalon, where the dopaminergic neurons are set, and both directly and indirectly control their function. This regulatory system is also arranged in a spiral way so that the NAc shell and the ventromedial

ventral pallidum control the dopamine that is released in the NAc core and adjacent parts of the striatum. These latter areas, in turn, reach dopamine neurons projecting to much of the dorsal striatum (Haber *et al.*, 2000; Zahm, 2000). As already mentioned, the NAc receives information from the prelimbic prefrontal cortex, the hippocampal formation and the basolateral amygdala. Other prefrontal areas innervate the accumbens (Pennartz *et al.*, 1994; Zahm, 2000). The anterior cingulate cortex, an area involved in discriminative learning, projects to the NAc core and is necessary for animals to show locomotor approach to conditioned stimuli (Cardinal *et al.*, 2002).

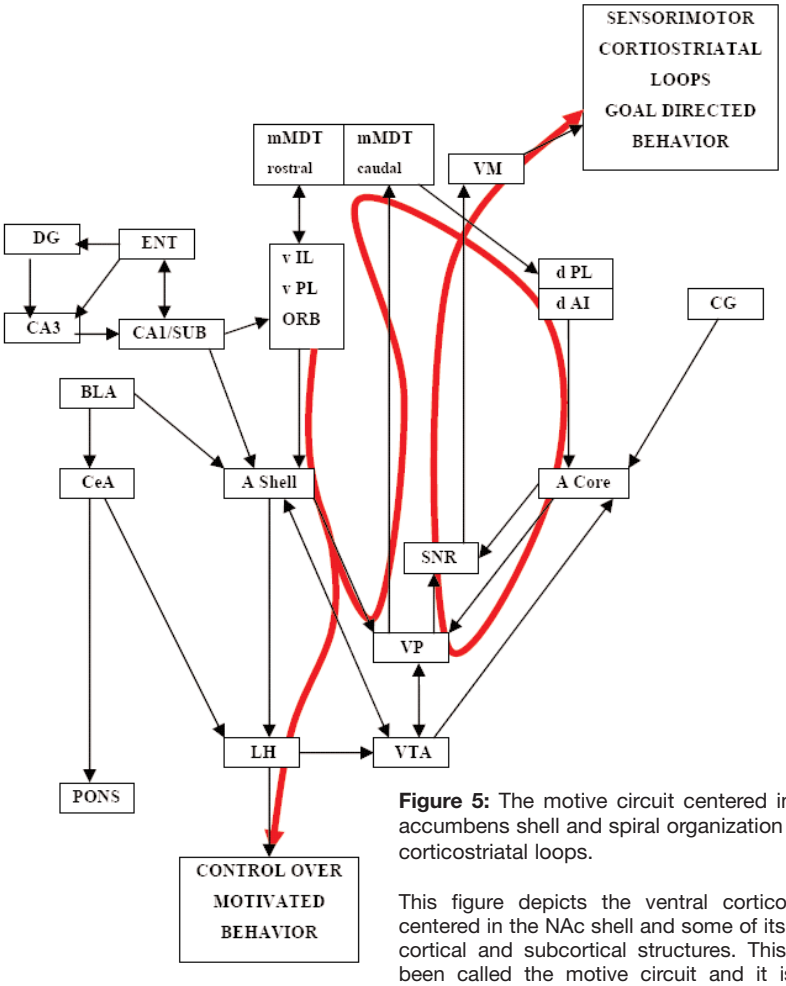

Figure 5: The motive circuit centered in the nucleus accumbens shell and spiral organization of the ventral corticostriatal loops.

This figure depicts the ventral corticostraital loops centered in the NAc shell and some of its relations with cortical and subcortical structures. This circuitry has been called the motive circuit and it is believed to translate the incentive salience (that is, motivational impact) of environmental stimuli to goal directed behavior. This figure is simplified so that not all the possible connections are shown, projections from and to the BLA, the CeA and the CA1/subiculum not related to the NAc shell have not been systematically considered. The red thick line represents the flow of information processing through different systems to generate a behavioral output. Dopamine controls the probability of cortical inputs to generate action potentials in the accumbal spiny neurons. Abbreviations: medial dorsal thalamus (mMDT); ventromedial thalamus (VM); ventral infralimbic cortex (vIL); ventral prelimbic cortex (vPL); orbitofrontal cortex (ORB); dorsal prelimbic cortex (dPL); dorsal agranular insular cortex (dAI); anterior cingulate cortex (CG); enthorhinal cortex (ENT); dentate gyrus (DG); subiculum (SUB); basolateral amygdala (BLA); central amygdala (CeA); nucleus accumbens (NAc); substantia nigra reticulata (SNR); ventral pallidum (VP); lateral hypothalamus (LH); ventral tegmental area (VTA). Some projections omitted are the projection from the BLA to the NAc core, the projections from PFC cortex to the CeA and the bidirectional connections between the PFC and the BLA. The dopaminergic influence of VTA over cortical and amygdalar areas is also omitted to simplify.

It is known that one distinctive feature of the NAc shell is its involvement in unconditioned behaviors like feeding, maternal behavior, defense or psychostimulant induced locomotion (Kelly *et al.*, 1975; Stratford and Kelley, 1997; Reynolds and Berridge, 2002; Li and Fleming, 2003). On the other hand, the NAc core is involved in the control of conditioned locomotor approach and mediates the motivational impact of conditioned stimuli (Cardinal *et al.*, 2002). This latter function depends on glutamatergic inputs rather than dopaminergic ones (Di Ciano *et al.*, 2001; Di Ciano and Everitt, 2001). Although mesolimbic dopamine does not mediate conditioned responses (Robbins *et al.*, 1989), dopamine in the NAc shell invigorates conditioned responses depending on the NAc core (Parkinson *et al.*, 1999). The accumbens shell has a negative control over feeding, an unconditioned behavior, through the lateral hypothalamus. Therefore, the accumbens shell has been suggested to serve as a fast adaptative switch between different goal directed behavioral strategies (Kelley *et al.*, 2005). Electrophysiological evidences support this notion. Most of the accumbal neurons recorded during instrumental behavior show excitation in firing activity either before the response or short after the response, and another subset of neurons show inhibition short after the response when the actual "consumption" takes place (Carelli and Deadwyler, 1994; Carelli *et al.*, 2000; Nicola and Deadwyler, 2000; Carelli and Wondolowski, 2003). Moreover, neurons in the NAc have been identified to encode the rewarding value of orosensorial stimulation and firing inhibition has been directly or indirectly associated with consummatory motor generation (Roitman *et al.*, 2005; Taha and Fields, 2005, 2006). As suggested by Taha and Fields (2005), the neurons showing inhibition could project to the lateral hypothalamus and, by releasing inhibition, they could initiate consummatory (motivated) behaviors (or hormonal control). It has been suggested that the NAc is constituted by several distinct neuronal ensembles (Pennartz *et al.*, 1994). This view is supported by electrophysiological evidences that demonstrate different neuronal populations involved in behaviors directed to natural (food and water) and drug rewards (Carelli *et al.*, 2000; Carelli and Wondolowski, 2003). By contrast, accumbal neurons exhibited similar firing patterns for different types of water and food rewards (Carelli *et al.*, 2000) regardless of the palatability or reward value (Roop *et al.*, 2002). In a very interesting review of this issue, Carelli and Wightman (2004) dropped the idea that neurons activated by drugs could be the same that underlie sexual behaviors (an old notion, though devoid of precision).

d. The amygdala: a neglected but emergent neuronal structure in addiction circuitry

The amygdala is a heterogeneous structure constituted by a cortical-like glutamatergic projecting nuclei, like the lateral and basolateral nuclei, and subcortical nuclei constituted by GABAergic projecting neurons, like the central and the medial nuclei (Swanson and Petrovich, 1998; Alheid, 2003). The cortical-like nuclei project, among others, to the subcortical one in a complicated but functional fashion (Pitkänen *et al.*, 1997; Swanson and Petrovich, 1998). The best characterized function of the amygdala is its role in acquisition and expression of learned fear in circuitry implicating the lateral amygdala, the basolateral amygdala (BLA), and the CeA in a serial connection (LeDoux, 1996; LeDoux, 2000). However, the amygdala, especially the BLA and CeA,

have been associated with the regulation of reward related behaviors in a parallel, independent, way (Cardinal *et al.*, 2002; Balleine and Killcross, 2006). The BLA projects to the NAc core and it is necessary for transferring the motivational value to environmental stimuli (Cardinal *et al.*, 2002; Di Ciano and Everitt, 2004). The BLA has reciprocal connections with prefrontal areas and they work together in regulating emotional behaviors (Cardinal *et al.*, 2002). The CeA massively projects to the lateral portion of the bed nucleus of the stria terminalis (BNST) (Dong *et al.*, 2001) and together they form the lateral portion of the extended amygdala (Swanson and Petrovich, 1998). The lateral extended amygdala strongly projects to the mesencephalon and the brain stem and has access to autonomic and motor behavioral generators (Swanson and Petrovich, 1998; Sah *et al.*, 2003). The CeA also projects to the lateral hypothalamus and the VTA (Zahm *et al.*, 1999). The CeA is necessary for the somatic expression of negative emotions like fear (LeDoux, 2000) and positive emotions such as approach to a stimulus paired with reward (autoshaping) (Cardinal *et al.*, 2002). However, CeA is not necessary for rats to assign a motivational value to unconditioned stimuli (Hatfield *et al.*, 1996). The CeA also participates in homeostatic regulation like food intake (Glass *et al.*, 1999), as well as drinking and salt appetite (Johnson *et al.*, 1999). Koob *et al.* (1998) have suggested that the NAc shell is part of the extended amygdala and altogether, the NAc shell and the lateral extended amygdala (the CeA and the lateral BNST), may represent a common anatomical substrate for acute drug reward and the negative effects of compulsive drug administration on reward function.

5. Drug induced behavioral and neuronal plasticity

As discussed previously in the introduction, only a small proportion of those that experiment with drugs of abuse get hooked and develop addiction. There is a general agreement that there must be biological processes that lead from sporadic drug use to addiction. The nature of these processes is the subject of an intense debate. Animal models have made possible the identification of crucial neurobiological mechanisms and much of the efforts have been focused on the VTA and the NAc, but new findings are illuminating new brain territories. The literature is huge and it is beyond the scope of this section to perform a systematic review. I will try, instead, to summarize part of this debate.

a. *Drug induced molecular adaptations in the striatum*

Drugs of abuse have an acute pharmacologic effect on the brain and repeated use progressively leads to stable molecular and cellular changes that modify the way in which brain controls behavior (Koob *et al.*, 1998; Berke and Hyman, 2000; Nestler, 2001; Kelley, 2004; Kalivas and Volkow, 2005). Figure 6 shows a schematic spiny neuron with the molecular changes that have been described after chronic drug use. One powerful mechanism to achieve stable and permanent changes is the known drug-induced activation of transcription factors that initiate a genomic response in neurons (Nestler and Aghajanian, 1997; Nestler, 2001). Acute drug

administration induces the transient expression of several Fos genes in the striatum codifying for proteins that form dimers (AP1 complexes) and control the expression of a long list of genes (Nestler, 2001). Most of these gene responses extinguish with repeated drug exposure (Hope *et al.*, 1992; Persico *et al.*, 1993; Moratalla *et al.*, 1996; Turgeon *et al.*, 1997). However, extracellular signaling-regulated kinase (ERK) phosphorylation and NGFI-A induction are required for induction of behavioral sensitization (Valjent *et al.*, 2005; Valjent *et al.*, 2006). Moreover, ΔFosB protein is stable and accumulates in the striatum after chronic drug treatments (Hope *et al.*, 1994; Moratalla *et al.*, 1996). Increased ΔFosB expression in the NAc induced sensitized behavior to cocaine administration (Kelz *et al.*, 1999), increased incentive for cocaine (Colby *et al.*, 2003), and increased rewarding effects of morphine (Zachariou *et al.*, 2006). ΔFosB overexpression seems an interesting molecular candidate in the search of the neurobiological phenomena that makes the difference and keeps subjects on taking drugs once they have begun (Nestler *et al.*, 2001; Kalivas and Volkow, 2005). As already mentioned though, both HR and LR rats self-administer drug the first session but, from the second day on, only HR rats keep on taking it. However, accumulation of ΔFosB can be measured only upon chronic drug treatments. Therefore, because of this time scale, ΔFosB overexpression is unlikely to account for the differences in drug self-administration observed between the HR and LR rats.

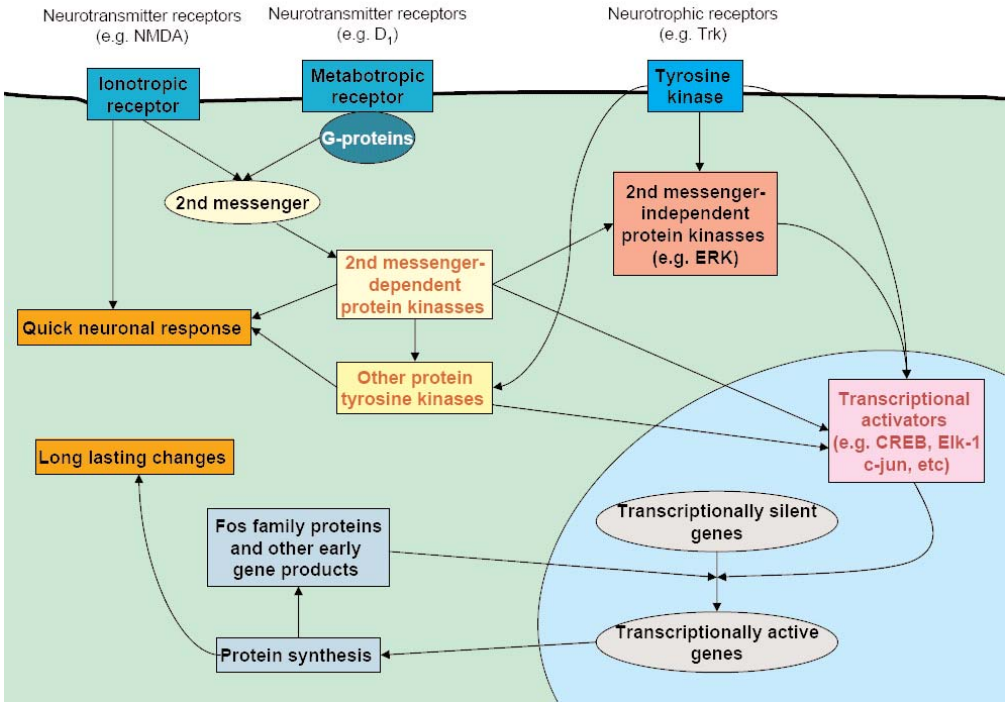

Figure 6: Regulation of gene expression by stimulation of different receptor classes on neurons.

This figure shows an integrated picture of how stimulation of different class of receptors expressed by neurons achieves a quick or a long lasting effect on neuronal function. The long lasting effects require synthesis of proteins. Changes in neurotransmission lead to changes in intracellular signaling pathways that regulate transcription of those genes required for new protein synthesis. *Adapted from Nestler (2001).*

b. *Hedonic dysregulation*

A clue to the neurobiological factor that makes the difference between those that get addicted when experimenting with drugs of abuse comes from the theoretical framework developed by Koob and coworkers. They have systematically shown that the rewarding systems of the brain are less sensitive during withdrawal of all kind of abused drugs, as measured with intracranial self-stimulation threshold in the lateral hypothalamus (reviewed by Koob *et al.*, 1998). This observation led to the hypothesis that the motivational source for drug self-administration must be dual: search for positive reinforcement and relief of negative emotional state (Koob and Le Moal, 1997). Repeated chronic amphetamine and cocaine administration enhances the function of the constitutive transcription factor CREB in the NAc (Nestler, 2001). Enhanced CREB function decreases the rewarding effects of drugs (Carlezon *et al.*, 1998) and natural rewards such as sucrose (Barrot *et al.*, 2002). Enhanced CREB is also responsible for dynorphin upregulation (Carlezon *et al.*, 1998), which decreases dopamine release (Spanagel *et al.*, 1992), induces dysphoria (Spanagel *et al.*, 1994; Hyman, 1996) and dampens cellular responsiveness (Steiner and Gerfen, 1998). Enhanced CREB function is, thus, a homeostatic mechanism that diminishes drug effects in the brain. However, CREB and dynorphin function return to baseline after withdrawal and they seem to be involved in the aversive effects experienced by addicts during early phases of withdrawal (Nestler, 2001), as well as decreased interest for other sources of reward. The same is true for the observed decrease in dopamine release during withdrawal (Weiss *et al.*, 1992), as well as the increased reactivity of the HPA axis and expression of CRH mRNA in the extended amygdala (Zhou *et al.*, 1996). Decreased reward function may play a role in the "decision" that HR and LR rats take the second self-administration session. Decreased reward function has been suggested to be a determinant factor in an animal model of transition to addiction: rats that have long access to drug (long training sessions) escalate drug intake over days whereas drug intake remains stable in rats that have short access to the drug (Koob *et al.*, 2004). Consistent with a model of transition to addiction, long access rats develop compulsive cocaine intake: they show increased drug seeking after abstinence in models of relapse (Ahmed *et al.*, 2000; Ferrario *et al.*, 2005); and they do not stop taking cocaine although they receive contingent electric shocks that suppressed cocaine taking in the same rats before they went through long access sessions (Vanderschuren and Everitt, 2004). However, the differences in brain reward can not underlie relapse since reward function recovers after long withdrawal.

c. *Disentangling the circuitry underlying relapse*

Rats that have extinguished the self administration behavior and are kept abstinent for a long period may show reinstatement of drug seeking after a non-contingent administration of the drug, drug conditioned stimuli or stress. This is considered to model the circumstances that induce craving and subsequent relapse in humans. Decades of research has made it possible to gain quite a complete picture of the neurobiological substrates underling relapse to heroin- and cocaine-seeking (reviewed by Shalev *et al.*, 2002). Increased glutamate release of prefrontal origin

in the NAc core is a common feature that provoking situations achieve to induce reinstatement of drug seeking (Kalivas *et al.*, 2005; Kalivas and Volkow, 2005). Glutamate receptor expression in the NAc is increased up to 90 days after withdrawal from cocaine self-administration in rats (Lu *et al.*, 2003). Cocaine-induced reinstatement requires integrity of the VTA, PFC and NAc core (McFarland and Kalivas, 2001), and cocaine-induced dopamine release in the prefrontal cortex induces glutamate release in the NAc core (Kalivas and Volkow, 2005). Reinstatement induced by conditioned stimuli depends on the integrity of the BLA (Meil and See, 1997; Kantak *et al.*, 2002), probably through its interaction with the NAc core (Di Ciano and Everitt, 2004). Stress induced reinstatement is dependent on a neuronal pathway that projects from the CeA to the BNST and uses CRH as a neuromodulator (Erb and Stewart, 1999; Erb *et al.*, 2001). However, stress induced reinstatement must recruit the PFC, probably through the NAc shell (McFarland *et al.*, 2004). This suggests that stress activates the extended amygdala and this latter structure eventually activates the pathway running from the PFC to the core.

d. *Molecular adaptation behind behavioral sensitization*

Behavioral sensitization is the long-lasting increase in psychomotor effects of drugs after repeated administration in humans and laboratory animals (Robinson and Becker, 1986; Stewart and Badiani, 1993). The appearance of behavioral sensitization is associated with increased incentive motivation for the drug (Vezina, 2004), and it is a model of the behavioral and neuronal plasticity induced by chronic drug effects that may underlie craving and relapse in abstinent addicts (Robinson and Berridge, 1993, 2001). Pharmacological evidence supports the relationship between sensitization and reinstatement of drug use (De Vries *et al.*, 1998; De Vries *et al.*, 2002). Sensitization induced by the non-contingent administration of drugs in laboratory animals has been widely used for the study of the neurological phenomena underlying behavioral sensitization (Pierce and Kalivas, 1997; Wolf, 1998; Vanderschuren and Kalivas, 2000).

The neuronal correlates of behavioral sensitization strongly depend on the drug used as well as on the conditions surrounding drug administration (Vanderschuren and Kalivas, 2000). The induction of psychostimulant sensitization is dependent on glutamate neurotransmission in the VTA (Pierce and Kalivas, 1997; Wolf, 1998; Vanderschuren and Kalivas, 2000). The expression, better observed after relatively long withdrawal periods (Robinson and Becker, 1986; Pierce and Kalivas, 1997), is dependent on dopamine and glutamate transmission in the NAc (Vanderschuren and Kalivas, 2000). Expression is normally accompanied by increased dopamine release in the NAc (Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000), but it has been observed in animals with unchanged dopamine release at this site (Berke and Hyman, 2000). Withdrawal from chronic cocaine treatment induces a decrease in the activity of the glutamate/cystine exchanger that results in low extracellular glutamate and increased glutamate release in subsequent cocaine administration (Baker *et al.*, 2003). Recent works addressing dopamine release in sensitization considered the shell/core subdivisions and found increased dopamine release selectively in the

core of the NAc (Cadoni *et al.*, 2000; Giorgi *et al.*, 2005a), the same is true for increased glutamate release (Pierce *et al.*, 1996). Behavioral sensitization induces increased density of dendritic spines in the NAc and prefrontal cortex (Robinson and Kolb, 1997; Li *et al.*, 2004). Although Kalivas (2005) stressed that sensitization and reinstatement differ in the relative importance of dopamine, a comparison of the neuronal mechanisms underlying sensitization clearly evidences an overlap with those underlying reinstatement to drug seeking. Moreover, sensitization enhances reinstatement of cocaine-seeking when AMPA, a glutamate agonist, is infused into the NAc (Suto *et al.*, 2004).

We already know that, contrary to RLA rats, RHA rats show behavioral sensitization when they are chronically administered with amphetamine (Corda *et al.*, 2005), cocaine (Giorgi *et al.*, 2005a) and morphine (Piras *et al.*, 2003). Therefore, these rats emerge as a valuable tool to study the neurobiology of behavioral sensitization as a model of drug induced neuronal plasticity underlying relapse. They offer the opportunity not only to further characterize the described phenomena in RHA rats, but also to disentangle the biological phenomena that block the emergence of sensitization in RLA.

6. Study of the neuroanatomical substrate of behavior with immediate early genes or other genes regulated by neuronal activity

Immediate early genes (IEG) are a class of genes that are rapidly up-regulated following neuronal stimulation and are, therefore, extensively used to perform functional mapping studies of the brain after a given stimulation or behaviorally relevant situation (reviewed by Farivar *et al.*, 2004 and Guzowski *et al.*, 2005). IEG encode a diverse range of proteins including regulatory transcription factors, structural and scaffolding proteins, signal transduction proteins, growth factors, proteases, and enzymes (Guzowski *et al.*, 2005). The most common ways to use IEG to map neural circuits is the use of immunohistochemistry or *in situ* hybridization to detect IEG protein or mRNA, respectively. IEG levels are measured by means of densitometric methods, especially with the use of isotopic *in situ* hybridization, or by means of cell counts (Guzowski *et al.*, 2005).

More than 30 genes were found to be upregulated upon activation of D_1 receptors in the striatum (Berke *et al.*, 1998). However, *c-fos* and NGFI-A (also known as *zif*268, Krox-24 or Egr1) are among the most used IEG (Farivar *et al.*, 2004). *c-fos* has low basal expression in most neural systems, its up-regulation is readily detectable and mRNA picks up after about 20-60 minutes and falls to basal levels by about 2 hours (Zangenehpour and Chaudhuri, 2002). NGFI-A has high level of expression in many neural systems (Knapska and Kaczmarek, 2004). NGFI-A is up-regulated in neurons with stimuli that also induce c-*fos*, but its down-regulation can also be studied (Farivar *et al.*, 2004). NGFI-A plays a critical role in several memory and learning tasks due to its role in neuronal plasticity (Knapska and Kaczmarek, 2004); it especially plays a critical role in reconsolidation processes (Lee *et al.*, 2004). Although it is clearly established that acute

administration of psychostimulants like amphetamine and cocaine induce expression of IEG like c-*fos* and NGFI-A in the striatum and several cortical areas (Bhat *et al.*, 1992; Moratalla *et al.*, 1992; Persico *et al.*, 1993; Uslaner *et al.*, 2001), this response undergoes tolerance with chronic treatments (Hope *et al.*, 1992; Persico *et al.*, 1993; Steiner and Gerfen, 1993).

Secretogranin and PSD-95 genes were also considered interesting to study since they are regulated by neuronal activity. Secretogranin is a secretory protein stored with other neuropeptides (Fischer-Colbrie *et al.*, 1987) that can be used as a presynatic marker (Iwazaki *et al.*, 2004). Secretogranin gene transcript is regulated by neuronal activity and accumulates upon chronic neuronal stimulation (Shen and Gundlach, 1996). Therefore, contrary to c-*fos* and NGFI-A, secretogranin is more likely to detect effects of chronic treatments when mapping neuronal activity (Kuzmin and Johansson, 1999). PSD-95 is a scaffolding protein enriched in the glutamatergic postsynaptic density that binds to the plasma membrane AMPA and NMDA glutamate receptors, other receptors, and proteins related to postsynaptic transmission among other proteins (Kennedy, 2000). As shown in figure 7, PSD-95 forms complexes with a transmembrane protein and a synaptically released protein, and when these complexes are formed, AMPA mediated glutamate transmission is increased (Fukata *et al.*, 2006). In different neuronal models, PSD-95 mRNA and protein levels were up-regulated upon neuronal activity (Skibinska *et al.*, 2001; Bao *et al.*, 2004; Van Zundert *et al.*, 2004). Therefore, this gene emerges as a marker of neuronal activity at the glutamatergic synapses.

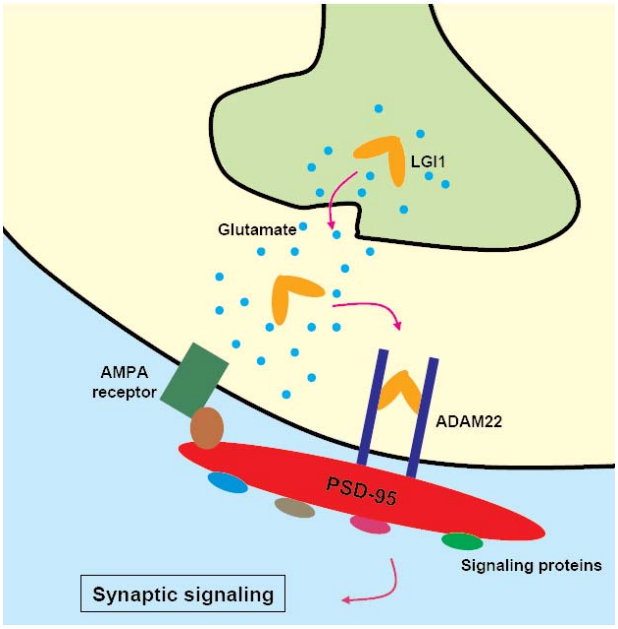

Figure 7: PSD-95 and synaptic transmission.

PSD-95 is a scaffolding protein associated to AMPA glutamate receptors and multiple signaling proteins. LGI1, a secreted protein thought to be secreted by the presynaptic neuron, is an oligomer that binds to two proteins of ADAM22 on the surface of the postsynaptic membrane through interaction with PSD-95. When LGI1 is binding ADAM22, the synaptic transmission is strengthened. Adapted from Snyder (2006).

Aims of the Present Thesis

The present thesis was aimed to study biological factors of vulnerability to addiction in a rat model. For this purpose several behavioral and neurochemical variables were studied in the Roman rats, a potential rodent model of differences in vulnerability to addiction.

Three main aims were defined:

1. To study the behavioral response to acute administration of low doses of ethanol in the Roman strains.
2. To characterize several molecular targets of the dopaminergic system and related neuropeptides in these rats.
   a. To quantify the basal expression levels of D_1, D_2 and D_3 dopamine receptor subtypes and DARPP-32 mRNA.
   b. To study the behavioral and neurochemical response to a challenge with a selective D_3 agonist.
   c. To quantify the basal expression levels of preprodynorphin (DYN), preproenkephalin (ENK) and preprocholecystokinin (CCK).
3. To study behavioral sensitization to amphetamine in the Roman rats and to identify brain areas implicated in their differential vulnerability.
   a. To characterize the induction and expression of behavioral sensitization.
   b. To make a map of neuronal activity with different IEGs upon a challenge with amphetamine in sensitized or control rats.

The study of effects of naltrexone on ethanol induced neuronal activity are integrated in this thesis as a collateral aim because it reinforces the results obtained in aim number 3, revealing a possible role of the central amygdala in the vulnerability to addiction.

Brief Description of Materials and Methods

Table 2: Experimental methods used in the different papers

	Animals	Behavioral procedure	Histochemical technique
Paper I	Roman strains	Hole board test* (Acute response to ethanol)	*In situ* hybridization* (DYN, ENK, CCK)
Paper II	Roman strains	None	*In situ* hybridization (DARPP-32) Receptor autoradiography D_1, D_2, D_3
Paper III	Roman strains	Locomotor activity (Acute response to PD128907)	*In situ* hybridization (NGFI-A)
Paper IV	Roman strains SD-OFA rats	Locomotor activity (Induction of behavioral sensitization)	None
Paper V	Roman strains SD-OFA rats	Locomotor activity (Expression of behavioral sensitization)	*In situ* hybridization# (NGFI-A, DYN, ENK, Secretogranin, PSD-95)
Paper VI	NMRI mice	None (Administration of ethanol, naltrexone and acamprosate)	*In situ* hybridization (NGFI-A)

This table summarizes the different experimental procedures (animals used and kind of experiments) generally described in this section and fully described in each of the papers. * The animals used in the behavioral experiments were not the same as the animals used in histochemical experiments. #Histochemical studies were only performed on the brains of the Roman rats.

1. Animals

The subjects of the present study were the Roman rats. As shown in table 2, in some studies, SD-OFA rats were included as standard rats to compare the behavioral phenotypes observed in the Roman rats. The last study included in the present thesis was performed on mice because it belongs to another project and is included in this thesis as a collateral aim.

Rats were used in all the experiments described in this thesis except in the experiments described in paper VI where NMRI (Naval Medical Research Institute) mice were used. In papers I-V, Roman rats (see introduction) bred in the animal facilities of the Medical Psychology Unit were used. In paper IV and V, Sprague-Dawley-OFA (SD-OFA) rats supplied by the General Animal Facilities at the UAB (Bellaterra) were also used. These animals were used in the Medical Psychology Unit (Bellaterra) and were housed in the same conditions as the Roman rats for 2 weeks before the experiments began. NMRI mice were supplied by Charles River (Uppsala; Sweden) and were used in the Center for Molecular Medicine (Stockholm) where they arrived 5 days before experiments began. For more details about the animals, look at the respective papers.

All experiments were conducted in accordance with guidelines and protocols approved by the European Economic Community (86/609/EEC Council) regarding the care and use of animals for experimental procedures and by the Ethics Commission of the Autonomous University of Barcelona.

2. Behavioral procedures

a. *The hole board test*

The hole board apparatus used in the present thesis consisted in a white 66 x 66 x 47 cm wooden box divided into 16 equal squares and containing four holes (diameter: 3,7 cm) on the floor. Four identical objects (plastic balls partially hidden in metal containers) were placed under the holes. Each animal was placed individually in the centre of the hole-board and was allowed to explore it during 5 minutes. The measures done were: 1) horizontal crossings; 2) vertical rearing activities; 3) number of head-dips; 4) number of different explored holes; 5) time spent head-dipping; 6) latency to self-grooming; 7) number of grooming episodes; and 8) time spent in grooming.

This test performed as described above allows the measurement of novelty-seeking behavior (as measured by head-dipping variables) independently from measurements of locomotor activity (horizontal crossings and vertical activity) (Escorihuela *et al.*, 1999). For this reason, we used this test to characterize the acute response of the Roman rats to a low dose of forced ethanol (0.25g/Kg; i.p.). For more details see paper I.

b. *Measures of locomotor activity*

Locomotor activity was determined in two different ways depending on the experiments. In the experiments described in paper III, 4 animals were placed in single plexiglass test cages (dimensions: 40 x 40 x 40 cm) and locomotor activity was determined by means of light-beam breaks (Panlab S.L.). In the ones described in paper IV and V, animals were placed in the same plexiglass test cages described above and were simultaneously recorded with video. The videotapes were analyzed using a video-computerized system (SMART, Panlab S.L.) which detects the position of the animal at each moment, draws its trajectory and calculates the total distance (in cm) covered by the animal during a certain period of time.

In paper III we studied the locomotor activity induced by novelty in RHA and RLA rats and its modulation by the putative D_3 agonist PD128907. RHA-I and RLA-I rats were placed in the locomotor cage after administration of saline or either 0.01 or 0.1 mg/Kg of PD128907. Locomotor was measured for 1 hour and afterwards rats were killed and their brains collected.

In papers IV and V we studied both the induction and the expression of behavioral sensitization with amphetamine, in each of the RHA, RLA and SD-OFA rats. Before the beginning of the

sensitization regime, rats were counterbalanced according to their response to saline injection in order to avoid differences in the basal activity between treatment groups of the same strain. We submitted RHA, RLA and SD-OFA rats to an 11 days treatment with either 1mg/Kg of amphetamine or vehicle. Every second day (days 1, 3, 5, 7, 9, and 11) rats were habituated for 1 hour to the locomotor test cage and then received the treatment and were placed for 2 hours in the test cage. The other days (days 2, 4, 6, 8, and 10) rats received the treatment and were placed back in the home cage. Rats were left for a 14 days withdrawal period and were only manipulated for the routine animal department cleaning procedures. Thereafter, all rats were challenged with 0.25mg/Kg of amphetamine regardless of the induction treatment (amphetamine or saline). The challenge session was divided in 3 phases in which locomotor activity was measured for 1 hour: spontaneous activity after placement in the test cage; reactivity to a saline injection; challenge proper with amphetamine.

3. Histochemical procedures

Histochemical techniques used in the experiments described in papers I, II, III, V and VI were performed at Karolinska Institute (Sweden). In all instances, animals were killed by decapitation and the brains rapidly removed and frozen by contact with dry ice. Afterwards, brains were stored at -80^{o}C until they were processed.

a. *Cryostat sectioning*

The brains were placed in the cryostat for 15 to 20 minutes to increase their temperature from -80 ºC to -20 ºC and were mounted onto a holder in the cryostat. 14 µm-thick coronal sections were cut and thaw-mounted onto SuperFrost Plus (Menzel-Gläser, Braunschweig, Germany) slides. Slides were frozen again and stored at -20 °C until used. Equivalent sections for all brains in the same experiment were collected at different levels according to Paxinos and Watson (1998) atlas. This allowed us to map different brain areas along the rostrocaudal axis (see in the papers for the exact levels chosen in each experiment). For the identification of the different brain structures, adjacent sections to those used for *in situ* hybridization or autoreceptor experiments, they were stained with cresyl violet as described by Johansson *et al.* (1994).

b. *In situ hybridization*

An *in situ* hybridization procedure was used in order to analyze the levels of mRNA that had to be studied. An oligodeoxyribonucleotide probe complementary to rat mRNAs coding for the gene that we wanted to study in each experiment (see the respective paper for details about the oligonucleotide sequence and synthesis) was selected and was labeled at the 3´- end with [^{33}P]-dATP (300 Ci/mmol; NEN, Perkin Elmer) using terminal deoxynucleotidyl-transferase (Amersham).

The slides with the cryostat sections were dried in front of a fan for 30 to 45 minutes and thereafter

they were incubated for 16-20 h at 42°C with a hybridization cocktail containing approx. 10^6 cpm of the labelled oligonucleotide probe. Following hybridization, the sections were washed four times in 1 x SSC (1 x SSC=0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) at 55°C for 15 min each time, rinsed in water at room temperature for 1 min, dehydrated through EtOH (60%, 95% and 100% 1 min each), and air-dried. Brain sections were exposed to Kodak Biomax film (Amersham) for 2-12 days. The specificity of the oligonucleotide was checked on one slide by the addition to the hybridization cocktail of a 225x excess of unlabelled probe. This manipulation blocked the signal, whereas the signal was not influenced by a 225x excess of a non-related oligonucleotide

c. *Receptor autoradiography*

The protocol of receptor autoradiography was slightly different depending on the radiolabeled ligand used: [^{3}H]SCH 23390 for D_1 receptors, [^{3}H]raclopride for D_2 receptors, and [^{3}H]PD128907 for D_3 receptors (see paper II for more details). Slides were dried for 60 min at room temperature and then incubated for a variable period of time (varying from 60 to 150 minutes) at room temperature with a buffer specific for each radiolabeled ligand and containing a specific amount of it. When needed, non-labeled ligands were added to the incubating buffer to block the binding of the radiolabeled ligand to receptors sites that were not aimed to be studied. After incubation, slides were washed several times for a determined period of time (depending on the ligand) in ice-cold buffer, they were briefly rinsed once in ice-cold distilled water, and eventually they were dried at 4ºC over a strong fan. Slides were exposed for a period of time depending on the ligand to Hyperfilm-^{3}H (Amersham) together with plastic standards (Amersham) at 4ºC.

To measure the non-specific binding, a slide adjacent to the one incubated with radiolabeled ligand was incubated in the same conditions but with the addition of (+) butaclamol (Sigma) to the incubation buffer. (+) Butaclamol is a ligand structurally unrelated to the radioligands used and was added to the buffer in a concentration about 1000 times the dissociation constant at the receptor that was studied.

d. *Analysis of autoradiograms*

A Macintosh computer using the public domain NIH Image program (US National Institutes of Health; see http://rsb.info.nih.gov/nih-image) was employed for the analysis of the autoradiograms. Optical densities, expressed in grey levels, were measured on both cerebral hemispheres at the desired areas and the corresponding background was subtracted for each measurement. Data of both hemispheres was pooled for each animal. During the whole analytical procedure, analysis in individual batches and measurements by researchers blinded to the experimental conditions were used in order to avoid methodological bias.

4. Statistical analysis

In each experiment the data obtained was expressed in different units: number of horizontal crossings or head-dips among others for the hole board test; cm for the locomotor tests; optical density in arbitrary units for the *in situ* hybridization experiments; and fmol receptor/mg protein for the receptor autoradiography experiments. In all instances, the results are expressed as a mean + or - SEM. Data was analyzed using Student's t test or ANOVA depending on the number of factors and the number of groups within a factor. The factors considered were "strain" (mainly RHA and RLA but in some cases also SD-OFA) and "treatment" when the effect of a pharmacological manipulation (either acute or chronic) was studied. For the measurements of locomotor activity, data was analyzed considering the effect of time over the measures and then, a repeated factor was added to the standard ANOVA. When adding a repeated factor, one must check the sphericity of the distribution. In case sphericity was not achieved, the effects of the repeated factor and its interactions with other factors were analyzed using Huynh-Feldt corrected test that adjusts the degrees of freedom to the average tests of significance (online manual SPSS, version 12). When appropriate, multiple group comparisons were performed using the *post hoc* Duncan's tests.

Results Summary

1. Differential response to acute ethanol in the Roman strains (Paper I)

RHA-I rats showed higher novelty-seeking behavior (number of head dips) and locomotor activity (number of horizontal crossings) than RLA-I rats when they were injected with saline. However, RLA-I rats showed an increase of novelty-seeking behavior after being injected with 0.25 g/Kg ethanol, whereas RHA-I rats did not. The increase in novelty-seeking behavior after ethanol administration in RLA-I rats was independent of any increase in locomotor activity as ethanol did not modify this latter variable.

2. Basal differences in the anatomical pattern of D_1 and D_3 binding and DARPP-32 mRNA in the Roman strain (Paper II)

Receptor autoradiography experiments using [^{3}H]SCH23390, [^{3}H]raclopride and [^{3}H]PD128907 were performed to detect dopamine D_1, D_2, and D_3 receptor subtype binding, respectively. When compared to RLA-I rats, RHA-I rats showed higher D_1 and D_3 binding in the medial and ventral subdivisions of the NAc shell than RLA-I. Moreover, RHA-I rats showed higher D_1 binding in the lateral hypothalamus and the tail of the caudate putamen, and higher D_3 in ventral striatal areas besides the NAc. On the other hand, RLA-I rats showed higher D_3 binding than RHA-I rats in the Calleja islands. Finally, no differences between the two Roman strains were found in any of the measured areas in D_2 binding.

Quantification of DARPP-32 mRNA by means of *in situ* hybridization revealed higher expression of this gene transcript in RHA-I rats than in RLA-I rats in several limbic areas: prelimbic cortex, rostral and medial cingulate cortex, dentate gyrus, a restricted subdivision of the caudal striatum (see paper II for exact location) and central nucleus of the amygdala.

3. Further characterization of the D_3 dopamine receptor system in the Roman strains: behavioral and neurochemical response to a challenge with a selective D_3 agonist (Paper III)

We measured novelty induced locomotor activity in RHA-I and RLA-I rats after administration of saline, 0.01 or 0.1 mg/Kg of the putative D_3 agonist PD128907. When treated with saline, RHA-I rats showed higher locomotor activity during the first 10-minute interval than RLA-I rats which is indicative of higher locomotor activity induced by novelty in the former strain. Moreover, RLA rats were more sensitive to the D_3 receptor agonist administration: the low dose of the agonist only suppressed locomotor activity in RLA rats; the high dose was effective in both strains but the suppression of locomotor activity was stronger in RLA rats.

In the same animals, we also looked at the expression level of NGFI-A mRNA in several brain areas including the Calleja magna (the biggest Calleja island with a consistent anatomical location) and the striatum. Administration of PD128907 caused a higher suppression of NGFI-A mRNA in the Calleja magna of RLA-I rats when compared to RHA-I rats. The high dose suppressed NGFI-A in the Calleja magna of both strains but this effect only reached statistical significance in RLA-I rats. The low dose of agonist suppressed NGFI-A at this location in RLA-I rats although this effect was not statistically significant. A positive correlation between NGFI-A mRNA expression levels in the Calleja magna and total locomotor activity performed during the test was found in both strains. In the striatum, the low dose of agonist had no effect in NGFI-A mRNA expression in any strain, whereas the high dose suppressed NGFI-A mRNA expression in restricted subdivisions of the dorsal striatum in both strains. No suppression of NGFI-A mRNA was seen in the NAc.

4. Basal differences in DYN, ENK and CCK mRNA expression levels in the Roman strains (Paper I)

Quantification of the opioid peptides mRNA by means of *in situ* hybridization in the brain of naive rats revealed higher expression in RHA-I rats than in RLA-I rats of DYN gene transcript in the medial and ventral portions of the NAc shell, and of ENK gene transcript in the caudal portion of the anterior cingulate cortex. However, RLA rats showed higher expression of ENK gene transcripts in the rostral dorsolateral caudate putamen.

In situ hybridization with an oligonucleotide complementary to CCK mRNA revealed that RHA-I rats had higher expression of CCK mRNA than RLA-I rats in the agranular insular cortex, layer 2 of the cingulate cortex, layer 1 and 2 of the motor cortex and in CA3 of the dorsal hippocampus. In this latter area, we found dots of CCK staining outside the pyramidal layer. We measured the number of spots and found that RLA-I rats showed a higher number of these dots than RHA-I rats.

5. Divergent induction and expression of behavioral sensitization in the Roman strains and comparison to SD-OFA rats (Paper IV and V)

a. *Induction of behavioral sensitization*

Induction of behavioral sensitization was studied during 11 days. On the previous day (day 0), all rats were tested for their basal locomotor activity (spontaneous activity) for counterbalancing purposes. On this day, the three strains showed the habituation pattern already described in our laboratory (Giménez-Llort *et al.*, 2005). Namely, SD-OFA rats developed less total motor activity than the Roman rats. RLA-I and RHA-I rats could only be distinguished during the first 10-minute interval and therefore, when the total amount of spontaneous locomotor activity was considered there was a lack of differences between RHA-I and RLA-I.

Changes in spontaneous activity during the induction of behavioral sensitization were studied by measuring it for 1 hour before administration of the respective treatment every second day. There

was not any statistically significant difference between treatment groups of the same strain and, therefore, the data concerning spontaneous activity was pooled in strains. However, the 3 strains showed a different evolution of their spontaneous motor activity as the induction treatment progressed. SD-OFA rats decreased their spontaneous activity the second time they were placed in the test cage and their spontaneous activity did not decrease any further as the induction treatment progressed. RHA-I rats did not show changes in their spontaneous activity although they were repeatedly placed in the test cage as induction treatment progressed. RLA-I showed increased spontaneous activity on day 5 and 7 with a posterior decrease on day 9. Except from day 0, before sensitization treatment began, RLA-I rats showed higher spontaneous activity than RHA-I rats, and these latter rats showed higher spontaneous activity than SD-OFA rats.

During the induction procedure, administration of amphetamine (1mg/Kg) induced more locomotor activity than saline. Moreover, amphetamine always induced greater locomotor activity in RLA-I rats than in RHA-I rats, and greater in the latter strain than in SD-OFA rats. Locomotor activity induced by amphetamine or saline in SD rats was not modified although the treatment was repeated 11 times. However, repeated administration of saline in RLA-I and RHA-I rats or amphetamine in RHA-I rats resulted in an increased motor activity compared to the respective motor activity on day 1 (RLA-saline in day 5, 7 and 11; RHA-saline in days 3, 5, 7 and 11; RHA-amphetamine in days 7, 9 and 11). The increases in induced locomotor activity observed after saline injections in RLA-I and RHA-I rats could be due to repeated exposition to the activity cage. If this was the case, a statistic analysis that included spontaneous activity as a covariant would allow us to see only those changes in induced locomotor activity that are independent of the repeated exposure to the activity cage. In fact, when such an analysis was performed, only RHA rats that received amphetamine showed an increase in induced motor activity on day 9 and 11 when compared to day 1. Therefore, it seems that the changes observed during induction treatment with saline in the Roman strains were due to a factor already present during the habituation and independent of the treatment itself.

b. *Expression of behavioral sensitization*

Once the induction of behavioral sensitization was finished, animals remained undisturbed for 14 days. After this withdrawal period, all rats were challenged to detect behavioral sensitization. Animals were treated equally in 3 phases in which locomotor activity was measured for 1 hour: spontaneous activity after placement in the test cage; reactivity to a saline injection; the actual challenge with amphetamine (0.25mg/Kg). Spontaneous activity or the reactivity to a saline injection was not modified by the induction treatment (either amphetamine or saline) in any of the strains. However, the locomotor activity induced by the amphetamine challenge was increased (behavioral sensitization) in RHA-I and SD-OFA rats that received amphetamine as induction treatment when compared to the animals of the same strain that received saline as induction treatment. The response to the amphetamine challenge was not modified by the induction

treatment (lack of behavioral sensitization) in RLA-I rats. When the challenge with amphetamine that lasted for 1 hour was analysed at 10-minute intervals, SD-OFA rats that received amphetamine as induction treatment developed greater motor activity to the challenge than their controls (induction with saline) only in the second 10-min interval. By contrast, in RHA-I rats, the expression of behavioral sensitization was longer, as the enhanced motor activity in the group that received amphetamine during induction persisted for 50 minutes (statistically significant in the first, second, third and fifth 10-minute intervals). After the challenge with amphetamine, RLA-I rats developed greater motor activity than RHA-I rats, and the latter more than SD-OFA rats.

6. Differential neuronal activity map with immediate early genes upon a challenge with amphetamine in sensitized and control Roman rats (Paper V)

The expression levels of NGFI-A, DYN, ENK, secretogranin and PSD-95 mRNA were measured in multiple brain areas 1 hour after the challenge with 0.25 mg/Kg in the experiment described above only in the Roman rats.

Compared to RHA-I rats that received saline during induction, RHA-I rats pretreated with amphetamine showed: 1) increased expression levels of NGFI-A in the rostral dorsomedial striatum, the rostral ventral striatum and the piriform cortex; 2) increased DYN mRNA in the medial subdivision of the rostral striatum; 3) increased ENK mRNA expression in the medial subdivision of the rostral striatum; and 4) higher secretogranin and PSD-95 mRNA in the NAc core.

Compared to RLA-I rats that received saline as induction treatment, RLA-I rats pretreated with amphetamine showed: 1) higher expression levels of NGFI-A in the rostral medial striatum and the rostral ventral striatum; 2) lower level of NGFI-A in the central nucleus of the amygdala; 3) increased ENK mRNA expression in the central subdivision of the caudal striatum; 4) increased secretogranin mRNA expression in the infraorbital cortex and in CA3 field of the ventral hippocampus; and 5) decreased PSD-95 mRNA in the NAc core.

7. Effects of naltrexone on alcohol induced neuronal activity measured with immediate early genes in mice (Paper VI)

NMRI mice were injected with either saline or 15 mg/kg naltrexone and half an hour later they received an injection of either saline or 2 g/kg ethanol. Brains were collected half hour after the second injection to perform an *in situ* hybridization with NGFI-A. Mice treated with ethanol or naltrexone alone showed an increase in NGFI-A mRNA levels when compared to vehicle-treated mice in the CeA. The combination of these two treatments had a synergic effect and induced NGFI-A mRNA expression to levels higher than those observed in mice treated with either of the drugs alone.

Discussion

1. Differential behavioral response to acute ethanol and its place in the divergent sensitivity to drugs of abuse between the Roman rats

The results obtained in the present thesis show that RHA-I and RLA-I differ in the acute response to low doses of ethanol. Although this result and its implication for the validity of RHA-I rats as an animal model for vulnerability of alcoholism has extensively been discussed in paper I, a brief discussion will be summarized here.

In the hole board test, RHA-I rats showed higher locomotor and exploratory activity than RLA-I rats when they were administered with saline as it has already been observed using this test in the inbred Roman strains (Escorihuela *et al.*, 1999; Fernández-Teruel *et al.*, 2002), as well as in the outbred rats (Fernández-Teruel et., 1997). However, after receiving a low dose of ethanol ip (0.25 g/Kg), RLA-I rats showed an increase in exploratory activity independent of locomotor activity whereas RHA-I did not show any effect of such a dose. The lack of effect in RHA-I cannot be attributed to a ceiling effect because RHA-I rats with early life manipulations have shown higher rates of exploratory behavior in the hole board test than those observed in the present thesis (Fernández-Teruel *et al.*, 2002). Therefore, it was concluded that the alcohol-preferring RHA rats were less sensitive to administration of a low dose of ethanol than the alcohol non-preferring RLA rats.

In humans, low sensitivity to ethanol has been suggested to be a risk factor for alcoholism (Schuckit, 1994). In this regard, the alcohol preferring RHA-I rats would be less sensitive to ethanol than the alcohol non-preferring RLA-I rats: we already knew that RHA-I rats are less sensitive than RLA-I rats to the hypnotic effects of high doses of ethanol (Fernández-Teruel *et al.*, 1997a); now, lower sensitivity to administration of a low dose of ethanol has been added to the phenotype of these alcohol preferring rat strain. However, Murphy *et al.* (2002) suggested that the ethanol preference in the alcohol preferring P rats was related to the higher response to the low-dose locomotor stimulant effects when compared to the alcohol non preferring NP rats. Following the psychomotor stimulant theory of addiction (Wise and Bozarth, 1987), Murphy and coworkers interpreted that low ethanol doses with locomotor activation effects induce activation of mesolimbic dopaminergic system. In this context, locomotor activity represents a model of the euphoric effects and rewarding properties of ethanol. However, a survey of the literature as presented in paper I demonstrates that low doses of ethanol does not always induce locomotor activity in commonly studied rat strains, maybe because of the implication of other neurotransmitter systems besides dopamine in the effects of ethanol. Although P rats are normally more sensitive than NP rats to low doses of ethanol (Murphy *et al.*, 2002), this is not always the case (Criswell *et al.*, 1994). Other alcohol preferring strains like the AA rats or the Fawn

hooded rats also show no effects of low doses of ethanol (Päivärinta and Korpi, 1993; Criswell *et al.,* 1994). As concluded in paper I, there is a lack of consistent association between the preference of ethanol and the sensitivity to low psychostimulant doses of the drug in rodent models. The lower sensitivity to the psychomotor activating doses of ethanol is another trait to be added to the novelty- and incentive- seeking profile which defines RHA rats. This result gives further validity to the RHA-I rats to model vulnerability to addiction since they are more novelty-seekers and they also show lower response to ethanol, both considered risk factors for drug addiction in humans, particularly for ethanol.

2. Basal neurochemical and neuroanatomical characterization of the brain of the Roman rats: implications for drug addiction

The results of this thesis also demonstrate differences in the expression levels of several molecular targets of the dopaminergic system and related neuropeptides. Among all mapped areas, the NAc shell is the area that concentrates most differences, namely differences in D_1, D_3 receptors subtypes and DYN mRNA. Moreover, a challenge with a D_3 agonist resulted in different behavioral and neurochemical responses between RHA-I and RLA-I. An individualized discussion of these neuroanatomical, molecular and functional results is found throughout paper I, II and III and will be summarized here. The discussion will be extended in an attempt to bring all the results to an anatomical model and to relate them to the differences in vulnerability to addiction between the Roman rats.

a. *D_1 and D_2 dopamine receptor subtypes*

The use of receptor autoradiography allowed us to measure D_1 and D_2 binding in up to 24 areas. Among all these areas, differences were restricted to 3 areas. Inbred RHA-I rats showed higher binding of D_1 than inbred RLA-I rats in the NAc shell, the lateral hypothalamus and the tail of the caudate putamen, while no differences were found between the Roman strains in D_2 binding. These results are in accordance with a previous study in tissue homogenates that reported the same difference in the accumbens shell between outbred RHA and RLA rats (Corda *et al.*, 1997). As discussed in the introduction, RHA rats show higher dopaminergic function as assessed in behavioral paradigms and microdialysis experiments. Although dopamine receptors could be down-regulated as a compensatory mechanism, the fact that D_1 receptors are up-regulated in the NAc shell suggests that the higher dopamine function at this anatomical site is an important feature shaping the behavioral phenotype of RHA-I rats as novelty-seeker animals. The lateral hypothalamus is the site through which the medial forebrain bundle runs from the mesencephalon to the forebrain (Paxinos and Watson, 1998). The significance of higher D_1 binding at this site is, however, unknown.

b. *D_3 receptor subtype*

The Roman strains showed a differential distribution of dopamine D_3 receptors subtypes: when compared to each other higher D_3 receptor binding in the ventral striatum including the NAc shell was measured in RHA-I rats, whereas higher D_3 receptor binding in the Calleja islands was measured in RLA-I rats. Pharmacological studies with selective D_3 agonists and antagonists have suggested that D_3 stimulation has inhibitory effects on locomotion (Richtand *et al.*, 2001). In fact, it is known that stimulation of D_3 receptors inhibits locomotor activity induced by novelty (Pritchard *et al.*, 2003). Deletion of D_3 receptors in knock-out mice (Accili *et al.*, 1996; Xu *et al.*, 1997) or down-regulation with antisense oligonucleotides in wild type animals (Ekman *et al.*, 1998; Menalled *et al.*, 1999) induces an increase in locomotor activity induced by novelty. RLA-I show much greater locomotor inhibition and enhanced yawning behavior than RHA-I rats when treated with low doses of the direct dopamine agonist apomorphine (Giménez-Llort *et al.*, 2005), which could be related to higher D_3 receptor function in RLA-I rats. As discussed previously, RHA lines/strains of rats show higher levels of exploratory behavior in tests of novelty-seeking when compared to RLA lines/strains of rats (Fernández-Teruel *et al.*, 1997b; Escorihuela 1999; Steimer and Driscoll, 2003; Giménez-Llort *et al.*, 2005). Therefore, a simple association between higher D_3 receptor binding and decreased locomotor activity cannot be held as enhanced novelty induced locomotor activity in RHA-I rats is associated with higher D_3 binding in the ventral striatum. Probably, regional differences of D_3 receptor expression may underlie some of the behavioral differences between the two strains.

In the Calleja islands as well as in the nucleus accumbens, D_3 and D_1 receptors are coexpressed by the same cells in most instances (Schwartz *et al.*, 1998). Evidences supporting a different role of D_3 receptors depending on the brain area where they are expressed are summarized in paper II and III. In the experiments described in paper III, stimulation of D_3 receptors with PD128907 induced higher locomotor inhibition in RLA-I rats expressing higher D_3 in the Calleja islands. Moreover, stimulation of D_3 receptors had a stronger neurochemical effect in the Calleja magna of RLA-I rats as assessed with NGFI-A expression. Finally, locomotor activity induced by novelty correlated with the measure of neuronal activity in the Calleja magna in both RLA-I and RHA-I rats. These findings were interpreted as convincing evidence supporting that D_3 stimulation of the Calleja islands play a role in controlling locomotor activity under circumstances that induce mild stimulation of the mesolimbic dopaminergic system. Therefore, the higher behavioral activation induced by novelty in RHA-I rats may be due to the lower levels of D_3 binding in the Calleja islands when compared to RLA-I rats.

On the other hand, the differences in D_3 receptors found in the ventral striatum would have quite different consequences. The highly selective D_3 receptor antagonist SB-277011-A decreases the reactivity to drug-associated stimuli as well as the motivation to self-administer cocaine under schedules where the response requirements are high (reviewed by Heidbreder *et al.*, 2005; Le Foll

et al., 2005). Moreover, the D_3 receptor agonist 7-OH-DPAT increased the reinforcing properties of cocaine although it decreased dopamine levels in the NAc (Parsons *et al.*, 1996). There is evidence for the existence of a crosstalk between D_1 and D_3 receptors in the ventral striatum (Ridray *et al.*, 1998; Karasinska *et al.*, 2005). As extensively discussed in paper II and III, the study of D_3 receptors' role in behavioral sensitization also support the view that stimulation of accumbal D_3 receptors elicits behavior. In this context, RHA-I rats would have a stronger dopamine modulation over spiny neurons in the NAc shell when compared to RLA-I. Therefore, differences in accumbal D_3 receptors seem to contribute to the novelty-seeking profile shown by RHA-I rats.

c. *DARPP-32 mRNA*

DARPP-32 mRNA expression differed between the two Roman strains, RHA-I rats showing greater gene expression than RLA-I rats in the prelimbic cortex, the cingulate cortex, the dentate gyrus, and the dorsomedial subdivision of the caudal striatum. In order to perform a reliable interpretation of the functional implication of these findings, it would be necessary to address the phosphorylation state of DARPP-32 in limbic areas (Svenningsson *et al.*, 2004). Our methodology, quantifying DARPP-32 mRNA using *in situ* hybridization, leaves out important post-translational regulation of DARPP-32 activity. However, strain differences in expression which are always in the same direction and mainly restricted to limbic areas seem to have a functional significance.

d. *Opioid peptide mRNA*

Opioid peptides mRNA levels differed between RHA-I and RLA-I in the striatum: DYN mRNA levels were higher in the nucleus accumbens and ENK mRNA levels were lower in the rostral dorsolateral caudate putamen of RHA-I rats. As discussed in the introduction, the opioid peptides have a reciprocal interaction with dopamine. In paper I, these findings were interpreted in the light of the evidences that changes in opioid peptide gene expression in the striatum are likely to be compensatory: an effect rather than a cause. As discussed several times in this thesis, higher reactivity of dopamine system can be measured in RHA rats. In this regard, higher DYN in the NAc shell can be clearly interpreted as a neuronal correlate of increased dopaminergic reactivity at this site and it is added to the differences in D_1 and D_3 receptors. HR rats also show higher DYN mRNA levels than LR rats in the nucleus accumbens and the striatum (Lucas *et al.*, 1998) and basal and induced DA levels at this site (Hooks *et al.*, 1991). As discussed in the introduction, DYN peptides have a dysphoric effect and higher DYN expression may lead to decreased basal reward function (Koob *et al.*, 1998). In an attempt to avoid this situation of lowered reward function, RHA and HR rats may take the drug (ethanol or amphetamine respectively) when they have the opportunity. Thus, higher DYN mRNA levels may contribute to the vulnerability to addiction observed in these animals. Similarly to the compensatory role described for DYN, higher ENK mRNA levels in the rostral dorsolateral caudate putamen in RLA-I could be a correlate of a lower dopaminergic input in these rats. This could be related to the lower score of motor stereotypes induced by high doses of apomorphine (Durcan *et al.*, 1984; Giménez-Llort *et al.*, 2005) and

amphetamine (Driscoll *et al.*, 1986; Cañete *et al.*, 2003) in RLA rats when compared to RHA rats.

We also found higher ENK mRNA in the caudal portion of anterior cingulate areas of RHA-I rats. This area belongs to the rat medial prefrontal cortex which conforms the visceromotor network and works together with the viscerosensory network located in the orbital prefrontal cortex (Öngür and Price, 2000). It receives nocioceptive information and coordinates autonomic responses (Gray and McNaughton, 2000; Vogt *et al.*, 2004). It is known that the cingulate cortex projects to the accumbens core (Zahm and Brog, 1992). This projection from the anterior cingulate to the accumbens core is necessary for conditioned locomotor approach or autoshaping when more than one stimulus is on play (Cardinal *et al.*, 2002). Much more research is needed to clarify which are the neurons that express ENK mRNA in the cingulate cortex and their physiological role. However, one part of the network related to the stimuli-response association is richer in ENK-derived peptides in RHA-I rats compared to RLA-I rats. This neurochemical difference may have some relevance in the final subjective experience of these rats when interacting with drugs and drug related stimuli.

e. *Cholecystokinin*

Striking between-strain differences were found in CCK mRNA expression in superficial layers of the prefrontal agranular insular cortex, the anterior cingulate cortex, the motor cortex and the dorsal hippocampus. In this latter area RHA-I rats showed higher expression of CCK mRNA in the pyramidal layer while RLA-I rats had more spots of CCK staining in the other two layers. In paper I, these differences were interpreted as representing different anatomical distribution of the CCK neuronal networks. CCK-interneuron activity may be superimposed on the synchronized firing pattern of pyramidal and parvalbumin-containing cells and drive mood and emotional influences both in the hippocampus and supposedly in the isocortex (Freund, 2003). Consequently, functional differences of the areas where the two strains differ in CCK may be expected and may underlie, in part, the differences in "temperament" that have been described by Steimer *et al.* (1997) and presented in the introduction, especially with regard to the coping style. However, the Roman strains do not differ in the CCK mRNA expressed by dopaminergic neurons. Therefore, the differences in the dopaminergic function can not be accounted by different CCK regulation of dopamine neurons firing as was suggested for HR/LR rats (Lucas *et al.*, 1998).

f. *The nucleus accumbens shell and a brain model of vulnerability to addiction*

In figure 8, the representation of the motive circuit shown in the introduction has been complemented with numbers that indicate each basal neurochemical finding. It can be appreciated that the NAc shell concentrates basal differences in D_1 and D_3 binding and DYN mRNA expression. As discussed above, the differences concentrated in the nucleus accumbens are related to higher accumbal dopaminergic function in RHA-I rats when compared to RLA-I rats. Some findings can be localized in cortical areas forming the motive circuit. Only the anterior

cingulate cortex shows similar concentration of findings, namely basal differences in ENK, CCK and DARPP-32 mRNA. However, as it can be appreciated in the respective papers, the differences between strains in ENK and CCK mRNA levels are localized in different subdivisions of the anterior cingulate cortex.

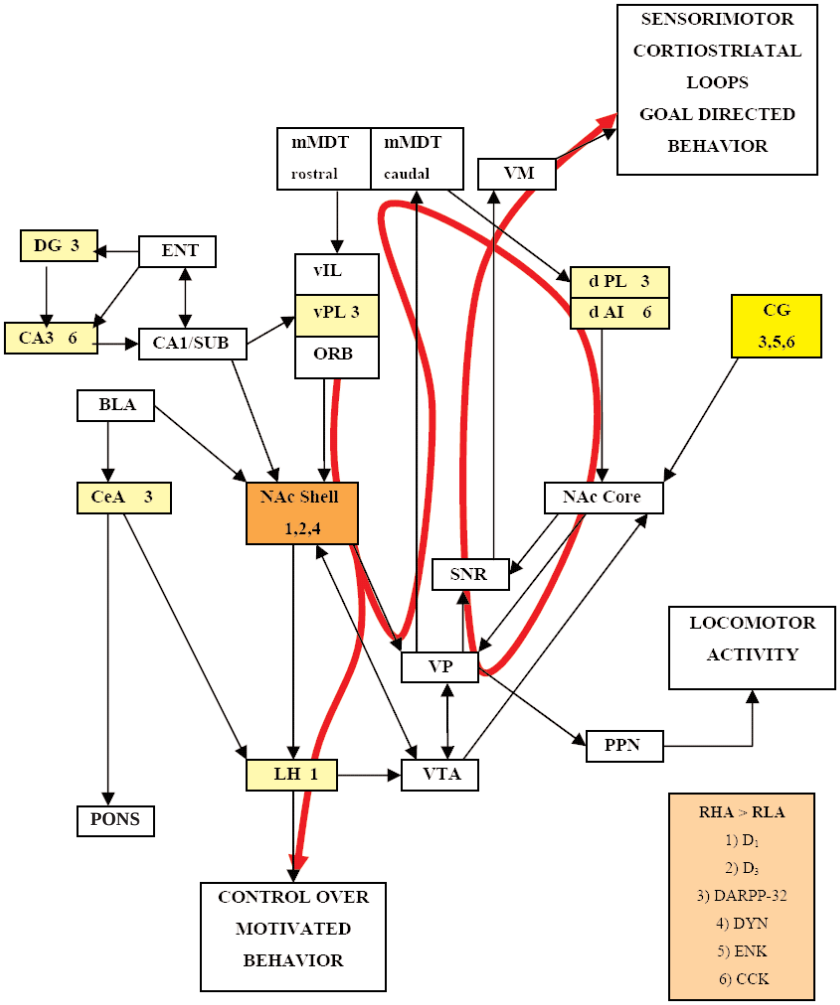

Figure 8: The motive circuit centered in the nucleus accumbens shell and spiral organization of the ventral corticostriatal loops.

The same figure previously used to illustrate the motive circuit has been modified to show the hypothetical control of the NAc shell over locomotor activity through its projection to the ventral pallidum and ultimately to the peduncolopontine nucleus. The areas where RHA and RLA differ in neurochemical measures are given a number whose legend can be read on the figure. Abbreviations: medial dorsal thalamus (mMDT); ventromedial thalamus (VM); ventral infralimbic cortex (vIL); ventral prelimbic cortex (vPL); orbitofrontal cortex (ORB); dorsal prelimbic cortex (dPL); dorsal agranular insular cortex (dAI); anterior cingulate cortex (CG); enthorhinal cortex (ENT); dentate gyrus (DG); subiculum (SUB); basolateral amygdala (BLA); central amygdala (CeA); nucleus accumbens (NAc); substantia nigra reticulata (SNR); ventral pallidum (VP); lateral hypothalamus (LH); ventral tegmental area (VTA); pedunculopontine nucleus (PPN).

As discussed in the introduction, higher dopaminergic function has been associated with the novelty-seeking profile of RHA rats as well as in HR rats when compared to RLA and LR rats, respectively. Higher novelty-seeking in RHA rats when compared with RLA rats is in the basis of the use of this strain to model novelty-seeking in humans (Driscoll *et al.*, 1998). As discussed previously, human personality theories postulate a connection between preference for novel situations and preference for rewarding substances (Zuckerman, 1996; Bardo *et al.*, 1996; reviewed by Dawe and Loxton, 2005). This notion is supported by the higher vulnerability to self-administer amphetamine shown by HR rats when compared to LR rats (Piazza *et al.*, 1989). The fact that RHA rats drink ethanol voluntarily whereas RLA rats do not (Driscoll *et al.*, 1990; Razafimanalina *et al.*, 1996; Fernández-Teruel *et al.*, 2002) is also in line with such an assumption. Matching the psychostimulant theory of addiction, it is known that RHA rats, compared to RLA rats, respond with higher DA release in the nucleus accumbens after cocaine, amphetamine and morphine administration (Giorgi *et al.*, 1997; Lecca *et al.*, 2004), as well as during voluntary ethanol consumption (Corda *et al.*, 2001). Similarly higher ethanol-induced dopamine release in the NAc has also been reported for the alcohol-preferring AA and P rats when compared to alcohol non-preferring ANA and NP rats (Katner, 2001 and Murphy *et al.*, 2002, respectivelly). HR rats also show higher cocaine induced DA levels at this site than LR rats (Hooks *et al.*, 1992b). Therefore, drug-induced dopamine release seems to be the common feature leading animals to higher drug preference (ethanol in RHA, P and AA rats and amphetamine in HR rats).

Dopaminergic tone in the NAc has been linked with the motor activity and exploration induced by novelty (Koob *et al.*, 1981; Jones and Robbins, 1992). When animals are placed in a novel environment, the cortex is activated and sends signals, among other areas, to the NAc shell. Considering the model of the basal ganglia presented in the introduction and figure 8, higher dopamine release in the NAc shell will be translated into higher flow of the cortical activity reaching it. Namely, spiny neurons are going to fire much more easily and increased neuronal activity is going to reach the ventral pallidum. A subset of neurons in the ventral pallidum projecting to the pedunculopontine nucleus becomes inhibited and exploratory locomotor activity is generated (Pennartz *et al.*, 1994). Higher dopamine release during initial self-administration session will also result in higher flow of information through the corticostriatal loops and rats may much more easily learn the contingencies between the unconditioned pharmacological stimulus inducing dopamine release preferentially in the shell (Pontieri *et al.*, 1995), and the lever press that requires a complex motor pattern depending on somatosensory corticostriatal loops (Yin *et al.*, 2005a; Yin *et al.*, 2005b). In agreement with this model, rats will acquire cocaine self-administration into the NAc shell but not into the NAc core (Rodd-Henricks *et al.*, 2002), although lesion of the NAc shell does not disrupt acquisition of intravenous cocaine self-administration (Ito *et al.*, 2004). Lever pressing for cocaine (Phillips *et al.*, 2003) or food (Roitman *et al.*, 2004) is preceded by a transient increase in dopamine in the NAc. Electrical stimulation of the VTA resulting in phasic dopamine release in the NAc is effective in initiating the goal directed behaviors that eventually end in pressing the lever (Phillips *et al.*, 2003). This latter evidence clearly shows

the role of dopamine to gate, initiate, goal directed behaviors such as lever pressing. It appears, thus, that novelty induced locomotor activity is predictive of higher drug self-administration because, as predicted by Wise and Bozarth (1987) in their psychostimulant theory of addiction, this two phenomena share neurobiological substrate. A prediction of this model would be that RHA rats will be more liable than RLA rats to learn a self-administration task for psychostimulant. However, as discussed in the introduction, the differences in dopamine function are stronger in HR/LR rats. This quantitative difference could make the differences in self-administration smaller in RHA/RLA rats in comparison to HR/LR. Moreover, NAc shell administration of amphetamine increases dopamine levels at this site and it also increases conditioned responses depending on the NAc core (Parkinson *et al.*, 1999; Wyvell and Berridge, 2000). Therefore, rats with higher dopamine function in the NAc shell, such as RHA rats, are more likely to show stronger drug seeking supported by conditioned stimuli.

In the present thesis, differences in D_3 binding in the Calleja islands have also been identified as a contributing neurobiological factor that makes the difference between RHA-I and RLA-I in terms of novelty induced locomotor activity. In one set of experiments using HR and LR rats, Pierre and Vezina (1997) studied the impact of a context dependent sensitization regime with amphetamine in the self-administration acquisition and maintenance. As expected, they found that all HR rats showed higher amphetamine self-administration than LR rats during the first 6 days. However, in the following days, only amphetamine pretreated HR rats maintained this higher self-administration behavior. According to them, the response to novelty predicted, at least in their experiment, the propensity to get sensitized and its facilitatory effects on subsequent drug self-administration rather than vulnerability to self-administration itself. The differences in D_3 receptors described between RHA-I and RLA-I rats support this notion: one of the factors that determine low novelty-seeking, namely high D_3 binding in the Calleja islands, may also dampen the effects of chronic drug administration preventing sensitization. As discussed in paper III, the novelty-seeking RHA-I rats show a sensitized-like D_3 receptor system. On the other hand, higher levels of D_3 receptors in the Calleja islands may dampen the impact of chronic treatments with drugs of abuse on the dopaminergic system in RLA-I rats and contribute to the lack of sensitization observed in these animals.

3. Behavioral sensitization in the Roman rats, a model of divergent vulnerability to behavioral and neurochemical drug induced plasticity

The present thesis revealed that although RLA-I showed higher amphetamine induced locomotor activity, they did not show behavioral sensitization whereas RHA-I rats did so. Moreover, RHA-I rats showed stronger behavioral sensitization when compared to SD-OFA rats. The differences in behavioral sensitization between RHA-I and RLA-I rats have been associated with differences in the neuronal activity maps as assessed with several different IEG. These results are extensively discussed in manuscripts IV and V. Here, a summary of the previous discussions is going to be

integrated with an attempt to understand the mechanisms of the observed differences in behavioral sensitization between the two Roman strains, the relationship with the basal differences, as well as the implications for the model of vulnerability to addiction.

Induction and expression of behavioral sensitization are two distinct neurological processes (Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000) and it is commonly accepted that behavioral sensitization is better observed after a withdrawal period, suggesting that it needs an incubation period (Pierce and Kalivas 1997). As presented in papers IV and V, both RHA-I and SD-OFA rats showed behavioral sensitization but two observations related to these neurological processes indicate that RHA-I rats are more vulnerable to behavioral sensitization than a standard strain like the SD-OFA: 1) RHA-I rats already showed behavioral sensitization the last 2 days of the induction phase which may be interpreted as greater sensitivity of neural systems underlying induction or alternatively the appearance of the phenomenon before withdrawal; 2) the sensitized response upon a challenge with amphetamine after 14 days withdrawal is longer-lasting in RHA-I rats than in SD rats.

RLA-I rats did not show behavioral sensitization either during the induction treatment or upon a challenge with amphetamine after 14 days of withdrawal. However, RLA-I rats showed higher amphetamine induced locomotor activity than RHA-I rats in all instances when amphetamine was administered in agreement with previous work performed with the inbred rats (Cañete *et al.*, 2003). As presented in the introduction, this result contrasts with the amphetamine response reported for the outbred rats that has been related to higher amphetamine induced dopamine release in the NAc shell (Giorgi *et al.*, 1997; Lecca *et al.*, 2004; Corda *et al.*, 2005). As extensively discussed in paper IV and V, experiments done with the inbred rats have been replicating the known differences in the dopaminergic system between RHA and RLA rats including reactivity to novelty. Some of these experiments, such as dopamine receptor studies, have been presented in the present thesis. Instead, as extensively discussed in paper IV, the noradrenergic system may be implicated in this high response to amphetamine. Pharmacological interventions that reduce the central noradrenergic tone also decrease the acute response to amphetamine (Drouin *et al.*, 2002; Vanderschuren *et al.*, 2003) without effect on expression of behavioral sensitization (Vanderschuren *et al.*, 2003). The fact that RLA-I rats do not show behavioral sensitization regardless of their higher response to acute amphetamine also supports the idea that the difference between inbred and outbred Roman rats does not lie on the dopaminergic system. Moreover, described differences in susceptibility to amphetamine sensitization between outbred Roman lines (Corda *et al.*, 2005; Giorgi *et al.*, 2005a) have been maintained with inbreeding.

It could be questioned whether RLA-I rats are constitutively sensitized to amphetamine effects. However, as discussed in paper V, pharmacological manipulations have evidenced that the acute response to amphetamine is dissociated from its sensitizing effects. As extensively shown in the introduction, behavioral sensitization may be seen as a model of behavioral and neuronal

plasticity induced by chronic drug treatments and underlying relapse (Robinson and Berridge, 1993; 2001). Moreover, animals showing behavioral sensitization also show increased liability to self-administer psychostimulants (Vezina, 2004). Although the use of a more robust or extended sensitization regime could have increased the motor response of RLA-I rats beyond their initial amphetamine response, it is a fact that they did not develop behavioral sensitization with the protocol used in the present thesis. Differences in behavioral sensitization observed between RHA-I and RLA-I rats may represent, thus, different vulnerability to develop such plastic events induced by chronic amphetamine administration. The study of the brain neuronal activity maps upon a challenge with amphetamine may shed light on the brain areas and possible cellular mechanisms involved in divergent vulnerability to behavioral sensitization. We performed neuronal activity maps with five different activity regualated genes: NGFI-A, DYN, ENK, secretogranin and PSD-95.

As extensively discussed in paper V, previous studies that assessed the response of c-*fos*, NGFI-A, DYN or ENK mRNA to an amphetamine challenge after a withdrawal did not detect an effect of chronic amphetamine treatment in most areas of the brain (Wang and McGinty, 1995; Hu *et al.*, 2002; Ostrander *et al.*, 2003). Similar results have been obtained when mapping neuronal activity in the Roman rats after the amphetamine challenge. However, two findings deserve special consideration: 1) RLA-I pre-treated with saline and receiving amphetamine for the first time the day of the challenge showed a massive activation of NGFI-A gene expression in the CeA. This response was not observed in amphetamine pretreated RLA-I rats. As argued in paper V, activation of c-*fos* in the CeA has already been found in rats not developing behavioral sensitization (Ostrander *et al.*, 2003). Moreover, induction of NGFI-A mRNA in the CeA is a neuronal correlate of unconditioned fear (Malkani and Rosen, 2001). Although much research must be done to understand the significance of this finding, CeA activation might be a correlate of unconditioned fear induced by the first amphetamine experience in RLA-I and may be a neuronal event that prevents sensitization to occur in that strain; 2) amphetamine pre-treated RHA-I rats showed an increased response of DYN and ENK to the challenge with amphetamine than saline pre-treated RHA-I. The coincident up-regulation of both opioid peptides in the ventral striatum may be a correlate of adaptations in cellular responsiveness underlying vulnerability to sensitization in RHA-I.

In RHA-I rats, behavioral sensitization was associated with higher secretogranin and PSD-95 expression in the NAc core. Yao *et al.* (2004) found that PSD-95 was constitutively down-regulated in genetic and pharmacological models of cocaine sensitization. However, they did not measure PSD-95 after a challenge with cocaine. It is known that extracellular glutamate is decreased after chronic cocaine treatment but a challenge restores glutamate levels (Baker *et al.*, 2003). As argued in paper IV, this finding is suggestive of increased amphetamine induced glutamatergic activity in the NAc core of RHA-I pretreated with amphetamine during induction. Some have argued that chronic cocaine depresses excitatory transmission (White *et al.*, 1995; Thomas *et al.*, 2001), but a close survey of these evidence demonstrates that decreased excitatory transmission may rather be related to a short cocaine abstinence period (3 and 1 day withdrawal respectively). In fact,

excitatory transmission in the NAc is enhanced by a chronic cocaine treatment after longer cocaine withdrawal (Kourrich *et al.*, 2006). PSD-95 is a scaffolding protein that binds to plasma membrane AMPA and NMDA glutamate receptors and proteins related to the postsynaptic transmission among other proteins (Kennedy, 2000). As presented in the introduction and discussed in paper V, increased glutamatergic activity at this site is a common finding associated with psychomotor sensitization (Vanderschuren and Kalivas, 2000). Moreover, an amphetamine sensitization regime enhances reinstatement of cocaine-seeking induced by AMPA agonist infusion into the NAc-core (Suto *et al.*, 2004).

However, glutamate is not always involved in expression of amphetamine sensitization (Vanderschuren and Kalivas, 2000). In the present thesis, the sensitization protocol used implied pairing the induction and the challenge environment in half of the days. Pairing the challenge environment with the environment where rats receive induction treatment is known to increase behavioral sensitization (Robinson *et al.*, 1998). The main factor enhancing sensitization is the fact that the test cage is different from the home cage (Badiani *et al.*, 1995; Browman *et al.*, 1998a, 1998b; Crombag *et al.*, 2001). Amphetamine experienced in a novel environment induces IEG in D_1-receptor containing and D_2-receptor containing neurons (Jaber *et al.*, 1995; Badiani *et al.*, 1999; Ferguson and Robinson, 2004) whereas amphetamine experienced in the home cage only induces IEG in D_1 neurons (Berretta *et al.*, 1992; Johansson *et al.*, 1994). The combined neuronal response in D_1 and D_2 containing cells is dependent on cortical glutamatergic activity (Fergusson and Robinson, 2004). As discussed in the introduction, dopamine preferentially facilitates changes at the active glutamatergic synapses. Therefore, the placement in the test cage every two days during induction may enhance sensitization by favoring glutamate release that interacts with dopamine in the striatum. Similarly, HR rats show stronger behavioral sensitization when sensitization is context dependent (Hooks *et al.*, 1992a) but sensitization may exclusively appear in LR rats when sensitization is context independent (Piazza *et al.*, 1989). This may be interpreted as higher dopamine-glutamate interaction, mainly because of increased dopamine, in RHA and HR rats when compared to RLA and LR rats, respectively.

In the previous sections, the observed differences in NAc shell dopamine function between RHA and RLA rats has been placed on the basis of the observed differences in novelty induced locomotor activity, the main symptom of novelty-seeking in rodents. The same aggregate of results is the base for a predicted stronger psychostimulant self-administration. In the present section, I will argue that higher dopamine function in the NAc shell plays a role in the enhanced vulnerability to develop sensitization in RHA-I rats. Robbins and Everitt (2002) hypothesized that as a consequence of extended self-administration training a shift in the brain systems controlling behavior takes place: ventral corticostriatal loops initially underlying goal directed drug seeking behavior may eventually consolidate dorsal corticostriatal loops underlying stimulus-response drug seeking. The anatomical fundaments enabling this shift have been presented in the introduction. Dorsalization of the striatal portion involved in controlling behavior can be seen as

the self-administration training progresses: the NAc shell is involved in acquisition of self-administration (Rodd-Henricks *et al.*, 2002), although it is not necessary for rats to learn the task (Ito *et al.*, 2004); the NAc core is necessary for conditioned stimuli to guide lever pressing behavior (Corbit *et al.*, 2001; Di Ciano and Everitt, 2001, , 2004; Ito *et al.*, 2004). Moreover, as previously discussed in this thesis, glutamate rather than dopamine in the NAc core is involved in cocaine-seeking mediated by cues (Di Ciano and Everitt, 2001; Park *et al.*, 2002; Di Ciano and Everitt, 2004), or by cocaine itself (Kalivas and Volkow, 2005). The ability of glutamate to cause behavioral activation (drug seeking) independently of dopamine when applied in the NAc core may be the heart of an addicted state. As discussed in the introduction, dopamine gates cortical activity in the striatum. However, after chronic drug exposure, conditioned stimuli or drug administration induces glutamate release in the core which is necessary and sufficient for drug seeking in reinstatement and second order schedule paradigms. This suggests that effectiveness of glutamate transmission must be enhanced once the stimulus has become conditioned. When a cocaine paired stimulus is presented non-contingently to a rat, dopamine is released in the NAc core (Ito *et al.*, 2000; Weiss *et al.*, 2000; Phillips *et al.*, 2003). However, when this stimulus supports lever pressing as a conditioned stimulus (cocaine-seeking) in a second order schedule, dopamine release occurs in the dorsal striatum (Ito *et al.*, 2002), and this task is disrupted by dopamine and AMPA glutamate antagonist infusion in the dorsal striatum (Vanderschuren *et al.*, 2005). Acquisition of responding for a conditioned reinforcement is not dependent on dorsal striatal dopamine (Taylor and Robbins, 1986; Kelley and Delfs, 1991). This cue-induced dopamine release in the dorsal striatum observed in long trained animals may be gating, and consolidating, the formation of habits. One may hypothesize that dorsal striatal dopamine response disappears in longer-trained animals.

A progressive involvement of more dorsal striatal portions may also account for sensitization: dopamine release is circumscribed in the NAc shell after acute psychostimulant administration (Pontieri *et al.*, 1995; Lecca *et al.*, 2004) but the sensitized dopamine response is found in the NAc core (Cadoni *et al.*, 2000; Giorgi *et al.*, 2005a; Di Chiara *et al.*, 2006). In the model presented in figure 9, the development of behavioral sensitization in RHA-I rats is explained in these terms. The differences in dopamine function described in the previous section could account for the elevated vulnerability to develop behavioral sensitization observed in RHA-I rats. If dorsalization played a role in sensitization as suggested by these evidence, increased vulnerability to develop sensitization in RHA rats could be explained by higher dopaminergic function in the NAc shell. Exposure to amphetamine in the RHA lines/strains induces a more pronounced DA release in the NAc shell (Lecca *et al.*, 2004), which may lead to higher gating of cortical activity through the NAc shell and increased cortical activity to the NAc core (Zahm, 1999). Increased DA release in the NAc core occurs in outbred RHA rats sensitized to amphetamine (Giorgi *et al.*, 2005a). In a protocol where the context is involved, e.g. the protocol used in the present thesis, increased glutamate in the NAc during induction can be expected. Therefore, plasticity at the striatal glutamatergic synapses dependent on the local concurrence of DA and glutamate (White, 1996; Berke and Hyman, 2000) could occur. Psychostimulant sensitization increases the density of dendritic spines

in the NAc which is understood as a rearrangement of the synaptic convergence of dopamine and glutamate in the spiny neurons (Robinson and Kolb, 1997, 1999). The increased secretogranin and PSD-95 mRNA expression in the NAc-core in sensitized RHA-I rats lend support to the dorsalization hypothesis of sensitization. A prediction that could be tested emerges from this model: longer sensitization regimes will sensitize dopamine response in the dorsal striatum. In this context, a chronic psychostimulant treatment would enable certain neurochemical phenomena to happen. When these phenomena took place in the context of drug self-administration, they became the basis of phenomena leading to habit formation as well as the persistent hypersensitivity to stimuli that engage the NAc core glutamate system such as drugs and drug associated stimuli (Robinson and Berridge, 1993; Kalivas *et al.*, 1998; Vezina, 2004).

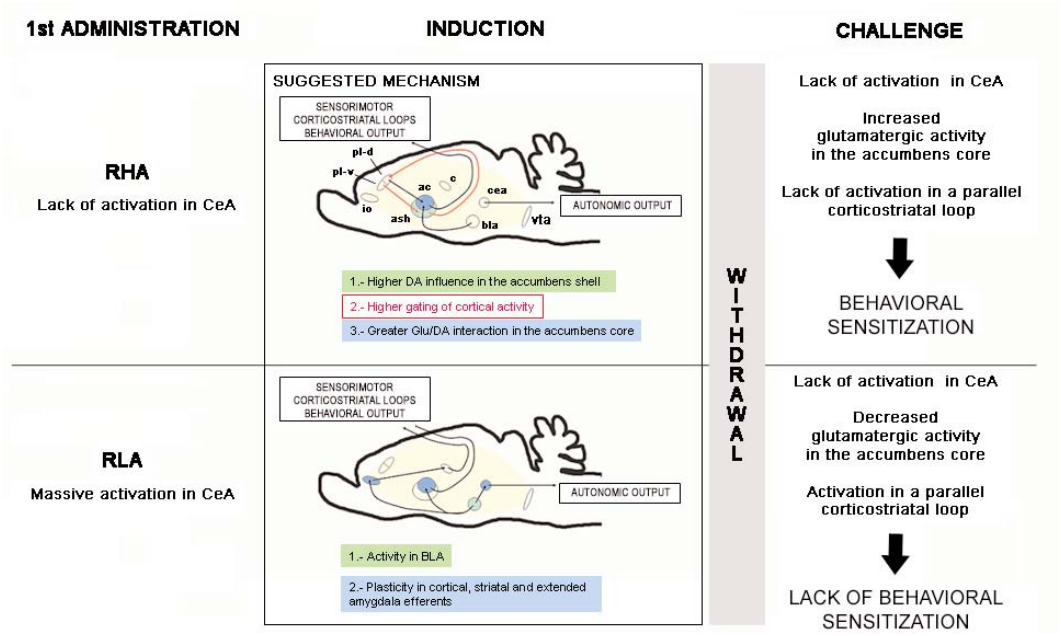

Figure 9: A neuronal model to explain the different vulnerability to behavioral sensitization between RHA and RLA rats

This figure summarizes the findings regarding regional neuronal activity upon a challenge with amphetamine in saline or amphetamine pretreated RHA and RLA rats. The results described in this thesis are integrated in a model of suggested mechanisms that explain how the different vulnerability to behavioral sensitization between the Roman rats may emerge. The neurochemical results regarding the first amphetamine administration were obtained in animals that received saline as induction treatment and were challenged with amphetamine on the challenge day. The anatomical structures are identified by the following abbreviations: *pl-d* for prelimbic dorsal cortex; *pl-v* for prelimbic ventral cortex; *io* for infraorbital cortex; *ash* for accumbens shell; *ac* for accumbens core; *c* for central subdivision of the caudal striatum; *cea* for central amygdala; *bla* for basolateral amygdala; and *vta* for ventral tegmental area. In each schema only the relevant connections are depicted. The yellow area represents the dopaminergic projection from VTA. The suggested mechanisms are depicted in a color code that allows localization in the corresponding schema.

On the other hand, in RLA-I rats, the lack of behavioral sensitization in amphetamine pre-treated animals was associated with an increase in secretogranin expression in the infraorbital cortex and decreased PSD-95 in the NAc. The lack of sensitized behavior in RLA-I rats is less understood than the increased vulnerability in RHA-I rats. However, as shown in figure 9, a constellation of neuronal adaptations in RLA-I rats chronically treated with amphetamine were found despite the lack of behavioral sensitization. First, decreased PSD-95 mRNA in the NAc core, which is the opposite of what it is observed in the sensitized RHA-I rats, may be related to a dampened response in accumbal glutamatergic synapses. Second, amphetamine-pretreated RLA-I rats show increased secretogranin mRNA in the infraorbital cortex and increased ENK mRNA in the central caudal striatum. The orbitofrontal cortex projects to the central subdivision of the caudal striatum (Berendse *et al.*, 1992). This multi-axon corticostriatal loop may be involved in dampening the effect of chronic amphetamine treatment. Rats that self-administer amphetamine for 14-20 days show, 1 month after withdrawal, a decrease in spine density in pyramidal neurons of the orbitofrontal cortex. This result was interpreted as a correlate of the cognitive deficits observed in animals chronically treated with psychostimulants (Crombag *et al.*, 2005). Although speculative, increased secretogranin is more suggestive of increased synaptic contacts. If these were the case, amphetamine pre-treated RLA-I rats would show, again, a neuronal finding opposed as the one found in standard animals after chronically experiencing psychostimulants. Finally, the amphetamine challenge caused high induction of NGFI-A mRNA in the CeA of RLA-I rats receiving amphetamine for the first time, and this response was not seen in RLA-I rats chronically treated with amphetamine. All these structures (CeA, infraorbital cortex and NAc-core) receive afferents from the BLA (Gray and McNaughton, 2000; De Olmos *et al.*, 2004). Therefore, as a hypothesis, repeated exposure to amphetamine triggers activity in the BLA in RLA-I rats, which organizes a differential response in its efferents and eventually prevents behavioral sensitization from occurring in these animals. Thus, a lack of sensitization may be explained by active homeostatic mechanisms rather than a lack of neurochemical responses to the drug.

4. Converging evidence point to the central nucleus of the amygdala as a possible candidate for limiting addiction

In parallel experiments performed in NMRI mice, it was found that the mixed opioid antagonist naltrexone, a drug used to prevent relapse in alcoholics, interacts synergically with ethanol to induce a massive activation of NGFI-A in the CeA. It was already known that ethanol induces *c-fos* in the CeA (Chang *et al.*, 1995; Hitzemann and Hitzemann, 1997), that this structure is necessary for rats to keep on drinking (Möller *et al.*, 1997), and that naltrexone infused in the CeA reduces ethanol drinking in rats (Foster *et al.*, 2004). However, it was unexpected that naltrexone would add to the effects of ethanol in the CeA. A discussion of the possible implications of this finding can be found in paper VI. In summary, naltrexone is known to devaluate the reinforcing effect of ethanol leading to extinction (Sinclair, 2001). On the other hand, the opioid system within the CeA contributes to the assignment of hedonic impact of ingested foods or liquids (Glass *et al.*,

1999), whereas NGFI-A is known to trigger cellular events that are necessary for reconsolidation of memory traces in the hippocampus and the BLA (Lee *et al.*, 2004; Lee *et al.*, 2005). Activation of this IEG in the CeA could represent the neurological correlate of reassigning, in the brain, a new hedonic value to ethanol when experienced under the effects of naltrexone. This devaluated hedonic representation of ethanol would leave ethanol without reinforcing value. However, if ethanol is experienced again in a naltrexone-free state, ethanol might recover its hedonic value and relapse could happen again.

This massive activation of the CeA when ethanol was given together with naltrexone resembles the massive activation of NGFI-A in the CeA observed in RLA-I rats experiencing amphetamine for the first time. However, this response underwent tolerance since it was not observed anymore in RLA-I rats chronically treated with amphetamine. As extensively discussed previously, RLA-I rats seem to model those individuals that do not become addicted even though they have access to drugs. Until now, experimental evidence has only shown that RLA-I does not drink ethanol when it is available. Future experiments must demonstrate wheter the hypothesized lack of addiction is still observed when these animals have free access to psychostimulants. The sensitization experiments show that after a chronic amphetamine treatment these rats did not show behavioral and neuronal plasticity that seems to underlie relapse after withdrawal. Activation of the ERK signaling pathway (ERK phosphorylation) in the CeA parallel incubation (e.g. time-dependent increase) of cue-induced reinstatement during drug withdrawal: cue-induced reinstatement and ERK activation in the CeA is only seen after 30 days of withdrawal. Moreover, inhibition of ERK phosphorylation decreases cue-induced drug seeking (Lu *et al.*, 2005). These results show that time-dependent increases in the responsiveness of CeA ERK pathway to cues mediate the incubation of cocaine-seeking. The activation of the ERK pathway may contribute to drug induced expression of IEG like c-*fos* and NGFI-A (Sgambato *et al.*, 1998; Valjent *et al.*, 2005). However, this is not always the case and sensitivity to ERK inhibition depends on IEG, brain area and whether drug treatment is acute or chronic: in the CeA, NGFI-A is induced by acute cocaine in an ERK dependent manner. This response shows tolerance after chronic treatment without withdrawal, although it is still induced when compared to saline treated animals (Radwanska *et al.*, 2005). The lack of withdrawal period hinders comparison with our study or the study by Lu *et al.* However, amphetamine pre-treated RLA-I rats showed tolerance to CeA activation (measured with NGFI-A mRNA) when challenged with amphetamine, whereas activation of the CeA (measured with ERK phosphorylation) underlies cue mediate craving. Again, RLA-I rats show the opposite neuronal correlate of the expected in addicted subjects.

The coincidence of CeA activation in two rodent models that simulate lack of effect of a chronic drug treatment suggests that CeA could make the difference between those that have restricted experience with drugs and those that go on taking them and eventually develop addiction. Moreover, the CeA has a heavy projection to the lateral hypothalamus, a brain area recently related to escalation of drug intake in an animal model (Ahmed *et al.*, 2005). It is tempting to

speculate that massive activation of the CeA may block the neuronal adaptations that normally take place in the lateral hypothalamus and lead animals to escalate their drug intake. The CeA might be a crucial brain area in determining which individuals develop behavioral and neuronal plasticity upon a chronic drug treatment. A first experiment to test this hypothesis would be to test whether inactivation of NGFI-A in the CeA during induction makes RLA-I rats prone to amphetamine sensitization.

Conclusions

This thesis has shown that RHA-I and RLA-I rats, a rodent model of divergent novelty- and sensation-seeking, differ in behavioral and neurochemical parameters that may explain their different sensitivity to drugs of abuse. RHA-I rats showed lower behavioral response when they were injected with a low dose of ethanol and this difference adds to the already known alcohol preference and makes RHA-I a unique rodent model of predisposition to alcoholism in humans. This thesis also revealed that RHA-I rats show higher expression levels of D_1 and D_3 dopamine receptor binding and DYN mRNA in the NAc shell. These differences are related to higher dopaminergic tone at this site and it may be a key neurobiological feature that determines increased novelty-seeking and drug preference in RHA-I rats when compared to RLA-I rats. Furthermore, RHA-I rats showed lower levels of D_3 dopamine receptors in the Calleja islands that may explain the differences in locomotor activity developed by RHA-I and RLA-I rats when they are placed in a novel environment.

RHA-I rats showed increased vulnerability to behavioral sensitization to amphetamine than SD-OFA rats, whereas RLA-I rats did not show behavioral sensitization despite their hyperresponse to acute amphetamine administration. The study of the neuronal activity maps with several IEG has allowed the identification of candidate anatomical structures and molecular mechanisms that may underlie vulnerability to addiction. As expected, increased glutamatergic transmission in the NAc core has been linked to the expression of behavioral sensitization in RHA-I rats. On the other hand, dampened glutamatergic transmission in the NAc core and changes in a parallel corticostriatal loop running from the orbitofrontal cortex to the central caudal striatum has been linked to the lack of behavioral sensitization in RLA-I rats that were chronically treated with amphetamine. Finally, activation of the CeA has been identified in two different models or situations, in animals, which simulate the blockade of consequences of chronic drug use, namely RLA-I rats receiving amphetamine for the first time and mice treated with ethanol and naltrexone, a drug used to prevent relapse in alcoholism. This latter finding suggests that activation of the CeA could be the neurological mechanism that makes the brain's difference between people who have a brief affair with drugs and people who go on taking them compulsively and develop addiction.

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Paper I

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Reduced ethanol response in the alcohol-preferring RHA rats and neuropeptide mRNAs in relevant structures

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Abstract

Roman rat strains, genetically selected for high (RHA) or low (RLA) active avoidance acquisition in the two-way shuttle box, differ in dopaminergic activity. These two strains appear to be a valid laboratory model of divergent sensation/novelty and substance-seeking profiles. RHA rats show higher ethanol intake and preference than do RLA rats, and it was suggested that RHA rats are more tolerant than RLA to the effects of alcohol. In the hole-board test, we found that the non-alcohol-preferring RLA rats showed enhanced responsiveness to the stimulatory effects of intraperitoneal administration of 0.25 g/kg ethanol when compared with RHA rats. *In situ* hybridization analysis showed higher levels of preprodynorphin in the accumbens shell and higher levels of preproenkephalin in the cingulate cortex in RHA rats. RLA rats showed higher levels of enkephalin gene transcripts in restricted areas of the dorsal striatum. Finally, differences in cholecystokinin gene transcript, suggestive of a different arrangement of certain interneurons, were found in different cortical areas. The differences in peptide gene expression found between the two strains might reflect the differences in alcohol preference and sensitivity. RHA rats may have more predictive value than other rodent alcoholism models, as high initial tolerance to ethanol is a risk factor for alcoholism in humans.

Introduction

As in other addictive behaviours, ethanol (EtOH) consumption has been linked with the mesolimbic dopaminergic system that marks incentive salience or predicts reward (reviewed by Gonzales *et al*., 2004). EtOH, like most abused drugs, increases dopamine (DA) neurotransmission in the nucleus accumbens and other areas of the mesolimbic system (Imperato & Di Chiara, 1986). However, EtOH interacts with many other neurotransmission systems. The enhanced DA neurotransmission seems to be mediated by the opioid system (reviewed by Oswald & Wand, 2004). Activation of μ and δ receptors on γ-aminobutyric acid (GABA) interneurons in the ventral tegmental area (VTA) enhances dopaminergic neuron firing activity (Johnson & North, 1992). Simultaneously, a large body of evidence has shown that DA regulates the expression of opioid peptides in striatal projecting neurons (Nylander & Terenius, 1987; reviewed by Angulo & McEwen, 1994). Another neuropeptide that interacts with DA and has been related to addictive behaviours is cholecystokinin (reviewed by Rotzinger & Vaccarino, 2003). Cholecystokinin reaches the striatum from pyramidal neurons and dopaminergic neurons (reviewed by Hökfelt *et al*., 2002). An antagonistic effect of cholecystokinin on the D_2 receptor through preprocholecystokinin $(CCK)_b$ receptor activation has been demonstrated both at the behavioural and cellular levels (Fuxe *et al*., 1995).

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Current theories and experimental evidence consistently suggest a relationship among enhanced mesolimbic dopaminergic transmission and higher novelty/incentive-seeking and drug-seeking behaviour (e.g. Bardo *et al*., 1996; Zuckerman, 1996). In this regard, the sublines of Roman high- (RHA) and low-avoidance (RLA) rats, psychogenetically selected for, respectively, rapid vs. extremely poor two-way active avoidance acquisition (Driscoll & Bättig, 1982), appear to be a valid laboratory model of divergent novelty- and substance-seeking profiles, as well as of differential central DAergic activity in a wide range of experimental situations (reviewed by Driscoll *et al*., 1998). Thus, compared with RLA/Verh rats, RHA/Verh rats show: (i) higher levels of exploratory behaviour in tests of novelty seeking (Fernández-Teruel *et al*., 1992, 1997a, 2002; Escorihuela *et al*., 1999); (ii) higher preference for alcohol (Driscoll *et al*., 1990; Razafimanalina *et al*., 1996; Corda *et al*., 2001), as well as saccharin and quinine solutions (Razafimanalina *et al*., 1996; Fernández-Teruel *et al*., 2002); (iii) stronger mesocortical and mesolimbic dopaminergic responses to, respectively, stress (D'Angio *et al*., 1988; Giorgi *et al*., 2003) and addictive substances, including EtOH (Giorgi *et al*., 1997, 2005; Corda *et al*., 2001). Moreover, RHA/Verh rats are less sensitive than RLA/Verh rats to the hypnotic effects of alcohol (Fernández-Teruel *et al*., 1997b).

On the premise that a low sensitivity to EtOH is associated with a higher risk for alcoholism in humans (Schuckit, 1994), we investigated whether the stronger EtOH preference of RHA vs. RLA rats may be related to a lower sensitivity of the RHA strain to the acute effects of EtOH. The hole-board test of novelty seeking was used, as this test

allows independent measurement of both psychomotor-stimulant and novelty seeking-enhancing effects of treatments (Fernández-Teruel *et al.*, 1992; Escorihuela *et al.*, 1999). Thereafter, we characterized preproenkephalin (ENK), preprodynorphin (DYN) and CCK gene expression by *in situ* hybridization in brain regions of drug-naïve RHA and RLA rats.

Materials and methods

Animals

The Roman/Verh rat lines originate from Wistar rats. They have been psychogenetically selected for many decades by mating the animals based on their acquisition of two-way active avoidance behaviour in the shuttle box (Driscoll & Bättig, 1982). The two rat lines were originally outbreds, but an inbreeding programme was started in 1993 (Driscoll *et al.*, 1998; Escorihuela *et al.*, 1999). Periodically, animals representative of the whole colony are tested in the shuttle box in order to assess the stability of the selected phenotypes (Escorihuela *et al.*, 1999; and unpublished results).

The rats used in the present work were 4-month-old males belonging to the 24th inbreeding generation. Two different sets of animals were used for the behavioural and neurochemical experiments. All animals were randomly assigned to the different groups and maintained two per cage (Macrolon, 21.5 × 46.5 × 14.5 cm), under standard laboratory conditions (food and water *ad libitum*, 22 ± 2 °C and 12 : 12 h light : dark cycles, lights on at 07.00 h).

Behavioural studies

On the day of the test, animals from the two strains were weighed and carefully moved (two per cage) from the animal room to a soundproof and dimly illuminated room that was adjacent and identical to the experimental room. Once there, they were left undisturbed for 60 min before the start of the behavioural test. The animals (six–eight per group) were given EtOH (0.25 g/kg i.p.; Panreac, Castellar del Vallès, Spain) or saline and evaluated, 15 min later, in the hole-board test. The hole-board apparatus was a white 66 × 66 × 47 cm wooden box divided into 16 equal squares, containing four holes (diameter: 3.7 cm) in the floor. Four identical objects (plastic balls partially hidden in metal containers) were placed under the holes, because it has been reported that specific novelty-seeking rather than non-specific exploratory behaviour or locomotor activity is measured with that procedure (Escorihuela *et al.*, 1999). The animal was placed in the centre of the hole-board. Horizontal crossings and vertical rearing activities, head-dipping behaviour (the number of head-dips, the number of different explored holes and the time spent head-dipping at each hole) and self-grooming were recorded for 5 min.

All observations were performed by two trained independent observers, in the early part of the light cycle between 10.00 and 13.00 h to reduce the possible influence of diurnal variation in activity. Animals were used only once. Strains and treatments were distributed in a counterbalanced manner so that separated units conformed by one animal of each group progressively entered the experiment and the order within each unit was changed each time. The research was conducted in accordance with guidelines and protocols approved by the European Economic Community (86/609/EEC Council) regarding the care and use of animals for experimental procedures, and by the Ethical Commission of the Autonomous University of Barcelona.

Sections for in situ *hybridization histochemistry*

Drug-naïve male RHA and RLA rats (seven per group), which did not take part in the behavioural experiments, were killed by decapitation and brains were rapidly dissected, frozen on dry ice and stored at −80 °C until processed. Coronal sections (14 μm thick) were cut in a cryostat (Johansson *et al.*, 1994). Sections were thaw-mounted onto SuperFrost Plus (Menzel-Gläser, Braunschweig, Germany) slides, dried briefly at 30 °C, and stored at −20 °C until used. For the identification of the different brain structures, adjacent sections to those used for *in situ* hybridization were stained with Cresyl violet (Johansson *et al.*, 1994).

Equivalent sections for all brains were collected at five different levels, which allowed us to map different brain areas along the rostrocaudal axis (Fig. 1). The section levels were: level 1, bregma 3.7–3.2 (prefrontal cortex); level 2, bregma 1.6–1.2 (nucleus accumbens, rostral caudate putamen, rostral cingulate cortex); level 3, bregma −0.8 to −0.92 (caudal caudate putamen, bed nucleus of stria terminalis, caudal cingulate cortex); level 4, bregma −3.60 to −3.8 (dorsal hippocampus); level 5, bregma −4.8 to −5.2 (ventral hippocampus, VTA and substantia nigra compacta), according to Paxinos & Watson (1998) atlas. The selected areas, prefrontal cortex, nucleus accumbens and VTA, are the main structures of the reward incentive salience circuitry (reviewed by Robinson & Berridge, 1993; Pierce & Kalivas, 1997; Schultz *et al.*, 1998). Dorsal striatum and substantia nigra pars compacta were added, although these latter areas have not been strongly linked with reward systems. However, they basically share the same DA/peptide interactions (De Olmos & Heimer, 1999; Zahm, 2000). Finally, the hippocampus was added because it is rich in dopaminergic innervation and opioid peptides in the hippocampus have been linked to learning abilities (Gallagher *et al.*, 1983; Sandin *et al.*, 1997), which differ between RHA and RLA rats (reviewed by Fernández-Teruel *et al.*, 1997a).

In situ *hybridization histochemistry*

The analysis of mRNA levels was carried out by a procedure for *in situ* hybridization using oligodeoxyribonucleotide probes complementary to rat mRNAs coding for CCK (nucleotides 298–341; Deschenes *et al.*, 1984) and ENK (nucleotides 255–299; Zurawski *et al.*, 1986), synthesized and purified through high-performance liquid chromatography (MedProbe, Oslo, Norway). An oligonucleotide probe complementary to the mRNA coding for nucleotides 871–918 of rat DYN (Civelli *et al.*, 1985) was synthesized on an Applied Biosystems DNA synthesizer 381A (Foster City, CA, USA) and purified on an OPC-column (Applied Biosystems). The specificity was checked by the addition of a 225 × excess of unlabelled probe, which blocked the signal, whereas the signal was not influenced by a 225 × excess of a non-related oligo (data not shown). Oligonucleotide probes were 3′-end-labelled with [^{33}P]-dATP (300 Ci/mmol; NEN, Perkin Elmer) using terminal deoxynucleotidyl-transferase (Amersham). Slide-mounted sections were incubated for 16–20 h at 42 °C with the labelled oligonucleotide probe. Following hybridization, the sections were washed four times in 1 × SSC (1 × SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) at 55 °C for 15 min each time, rinsed in water at room temperature for 1 min, dehydrated through EtOH (60%, 95% and 100%, 1 min each) and air-dried. Brain sections of both RHA and RLA rats were apposed to the same Kodak Biomax film (Amersham) for 2–12 days.

Analysis of autoradiograms

Autoradiograms were analysed with a Macintosh computer using the public domain NIH Image program (US National Institutes of Health;

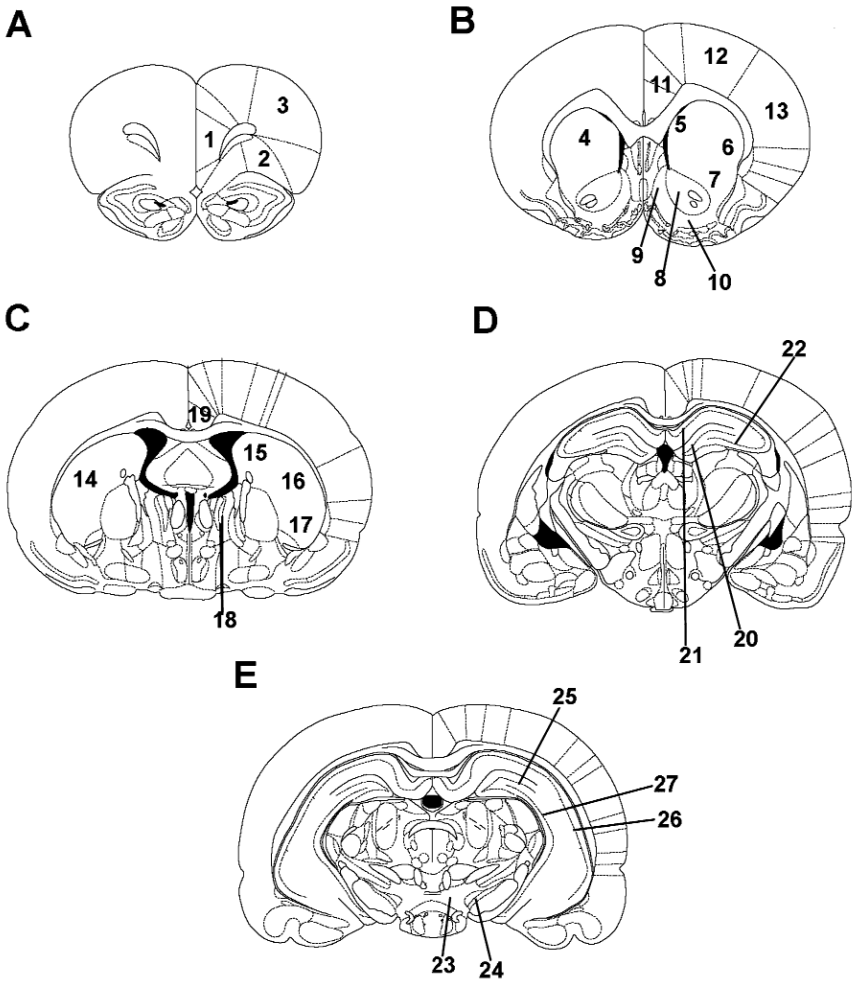

FIG. 1. In this diagram the approximate locations of measured brain areas for the *in situ* hybridization experiments are depicted. (A) Level I (bregma 3.7 mm): 1, prelimbic cortex; 2, infraorbital cortex; 3, agranular insular cortex. (B) Level II (bregma 1.6 mm): 4, rostral caudate putamen (for CCK probe); 5, rostral dorsomedial caudate putamen; 6, rostral dorsolateral caudate putamen; 7, rostral ventral caudate putamen; 8, nucleus accumbens core; 9, nucleus accumbens shell medial portion; 10, nucleus accumbens shell ventral portion; 11, cingulate cortex (Cg1, Cg2 rostral); 12, motor cortex; 13, somatosensory cortex. (C) Level III (bregma −0.8 mm): 14, caudal caudate putamen (for CCK probe); 15, caudal dorsomedial caudate putamen; 16, caudal dorsolateral caudate putamen; 17, ventrocaudal caudate putamen; 18, bed nucleus of stria terminalis; 19, cingulate cortex (Cg1, Cg2 caudal). (D) Level IV (bregma −3.6 mm): 20, dentate gyrus of dorsal hippocampus; 21, CA1 of dorsal hippocampus; 22, CA3 of dorsal hippocampus. (E) Level V (bregma −4.8 mm): 23, VTA; 24, substantia nigra pars compacta; 25, dentate gyrus of ventral hippocampus; 26, CA1 of ventral hippocampus; 27, CA3 of ventral hippocampus. Because CCK presents a different expression pattern, some brain areas were not measured for all three gene transcripts. Certain areas were measured at different layers for CCK (four layers on cingulate cortex, six layers on motor cortex and three layers for somatosensory cortex), and as a whole for ENK and DYN. Similarly, the caudate putamen was measured at different subareas for the opioid peptide gene transcripts and as a whole for CCK.

see *http://rsb.info.nih.gov/nih-image*). Optical densities were calculated from the uncalibrated mode by subtracting from each measurement its corresponding background and expressed in grey levels. All measurements were done on both cerebral hemispheres (from six or seven animals per group), and data were pooled for each animal. During the whole analytical procedure, analysis in individual batches and measurements by researchers blinded to the experimental conditions were used in order to avoid methodological bias. For DYN and ENK the same areas were measured because both gene transcripts present similar expression patterns in the rat brain. Because CCK presents a different expression pattern, some brain areas were not measured for all three gene transcripts. Some cortical areas were

measured at different layers for CCK (four layers on cingulate cortex, six layers on motor cortex and three layers for sensorial cortex), and as a whole for ENK and DYN. Similarly, the caudate putamen was measured at different subareas for the opioid peptide gene transcripts and as a whole for CCK.

Statistical analysis

The means ± SEM of different behavioural variables were calculated for each strain after treatment with EtOH or saline. According to the experimental design, results were analysed with two-way ANOVA, with 'strain' and 'treatment' as main factors. Multiple group comparisons were performed using the *post-hoc* Duncan's test. In the molecular studies, we performed a Student's *t*-test comparing optical densities measured on each specific brain region, considering each Roman strain as a group.

Results

Behavioural studies

As shown in Table 1, the two strains differed in most of the studied variables (strain effect, all $F_{1,27} > 11.74$, $P < 0.011$), except for latency to head-dipping behaviour and vertical activity where both strains showed similar activities. Higher number of head-dips (from five- to 10-fold higher) and horizontal activity (threefold higher) was exhibited by RHA rats as compared with the RLA rats which, accordingly, showed lower self-grooming behaviour (threefold lower; two-way ANOVA, in all cases 'strain' effects). The 'strain–treatment' interaction showed genetic differences in the response, with RLA but not RHA rats being sensitive to EtOH. Thus, in the RLA strain, EtOH increased head-dipping behaviour ('strain × treatment' effect, number of head-dips $F_{1,27} = 7.208$, $P < 0.013$ and the number of different explored holes, $F_{1,27} = 5.498$, $P < 0.028$; also Student's *t*-test only between RLA–EtOH and RLA–saline groups), and this effect was specific for novelty-seeking behaviour (head-dipping) and independent of locomotor activity (horizontal and vertical) and self-grooming behaviour.

In situ *hybridization histochemistry*

As shown in Table 2, there were some areas (for localization see Fig. 1) in which the two Roman strains differ in peptide mRNA expression. RHA rats showed higher expression of CCK mRNA than RLA rats in the agranular insular cortex ($t_{2,11} = 2.42$, $P = 0.034$), in layer 2 of cingulate cortex ($t_{2.8.2} = 3.44$, $P = 0.009$), in layers 1 and 2 of motor cortex ($t_{2,12} = 5.13$, $P < 0.001$ and $t_{1,12} = 3.2$, $P = 0.008$, respectively), and in CA3 of dorsal hippocampus ($t_{2,12} = 4.93$, $P < 0.001$). When measuring CCK in the hippocampus, we detected a signal outside the pyramidal layer appearing as spots. The typical distribution obtained at this hippocampus level for CCK *in situ* hybridization is shown in Fig. 2A and B. As shown in Fig. 2C, RLA rats showed higher number of spots ($t_{2,12} = 3.58$, $P = 0.004$) at the same level where they express less mRNA in the pyramidal layer of CA3 field, namely the dorsal hippocampus.

RHA rats showed higher expression of DYN mRNA than RLA rats in both medial and ventral portions of the nucleus accumbens shell ($t_{2,12} = 2.31$, $P = 0.04$ and $t_{2,12} = 3.61$, $P = 0.004$, respectively). RHA rats also showed higher expression of ENK than RLA rats in the caudal portion of the anterior cingulate cortex ($t_{2.12} = 8.47$, $P < 0.001$), although RLA rats showed higher expression of ENK in the rostral dorsolateral caudate putamen ($t_{2,12} = 3.18$, $P = 0.008$).

Representative pictures for DYN and ENK *in situ* hybridization are shown in Fig. 3.

Discussion

The results of the present study extend the behavioural characterization of the alcohol-preferring RHA rats and reveal a particular neurochemical profile that could in part account for the intrinsic predisposition of RHA rats to alcohol drinking behaviour as well as consumption of other addictive drugs.

The significance of differences in locomotor and head-dipping behaviour between the RHA and RLA strains

The RHA and RLA rats, with divergent profiles for novelty-seeking and EtOH preference, differed in their response to the stimulating effects of systemic administration of EtOH in a novelty-seeking test. The control rats from the two strains exhibited the previously described higher exploratory (i.e. locomotion) and novelty-seeking (i.e. head-dipping) behaviour of RHA as compared with RLA rats (Escorihuela *et al.*, 1999; Fernández-Teruel *et al.*, 2002), as measured by enhanced head-dipping and locomotor activity and, conversely, reduced self-grooming behaviour. Several studies with humans have

TABLE 1. Stimulatory effects of EtOH in RHA and RLA rats measured in the hole-board test

Measurement	RHA (saline) ($n = 6$)	RHA (EtOH) ($n = 6$)	RLA (saline) ($n = 7$)	RLA ethanol (EtOH) ($n = 8$)	Two-way ANOVA effects
Head-dipping					
Latency (s)	47.83 ± 15.53	22.83 ± 8.14	88.71 ± 38.33	29.62 ± 7.33	Not significant
Number of head-dips	16.5 ± 1.84	14.17 ± 1.51	3.29 ± 1.02*	7.75 ± 0.75*·†	Strain; strain × EtOH
Number of different holes	4 ± 0	3.5 ± 0.22	2 ± 0.53*	3.13 ± 0.3†	Strain; strain × EtOH
Time spent (s)	49.01 ± 8.1	44.76 ± 8.94	5.83 ± 2.09*	15.76 ± 1.88*·‡	Strain
Self-grooming					
Latency (s)	290.17 ± 9.83	284.17 ± 15.83	104.29 ± 21.25*	112.75 ± 11.86*	Strain
Number of groomings	0.17 ± 0.17	0.17 ± 0.17	3.14 ± 0.59*	2.88 ± 0.48*	Strain
Time spent (s)	0.83 ± 0.83	1.5 ± 1.5	36.86 ± 9.57*	32.38 ± 6.39*	Strain
Locomotor activity					
Horizontal (number of crossings)	103.83 ± 6.87	104 ± 16.2	33.86 ± 3.47*	48.38 ± 9.24*	Strain
Vertical (number of rearings)	8.83 ± 1.08	11.33 ± 2.06	10.14 ± 1.68	10.88 ± 1.6	Not significant

Duncan's post-hoc comparisons: *$P < 0.05$ vs. respective RHA control group (same treatment); †$P < 0.05$ vs. respective non-EtOH-treated group (same strain); ‡$P < 0.05$ vs. all the other groups.

TABLE 2. Results of *in situ* hybridization for CCK, DYN and ENK gene transcripts in the RHA ($n = 7$) and RLA ($n = 7$) rats

Area (and identification number in Fig. 1)	ID no. in Fig. 1	CCK optical density†		DYN optical density†		ENK optical density†	
		RHA rats	RLA rats	RHA rats	RLA rats	RHA rats	RLA rats
Prelimbic/ infralimbic	(1)	90.3 ± 2.9	88.1 ± 3.8	30.6 ± 2.2	29.7 ± 2.3	14.3 ± 2.4	13.9 ± 1.5
Infraorbital	(2)	70.1 ± 5	77 ± 2.9	35.7 ± 1.8	31.8 ± 2.3	24.3 ± 1.3	25.4 ± 2
Agranular insular	(3)	69 ± 2.5	59.2 ± 3*	37.9 ± 3.2	30.9 ± 3.1	15.5 ± 1.7	19.8 ± 2.7
Caudate putamen	(4)	4.6 ± 1	5.7 ± 1.22	–	–	–	–
Dorsomedial	(5)	–	–	52.7 ± 2.1	47.6 ± 3.1	119 ± 3.2	127.5 ± 2.8
Dorsolateral	(6)	–	–	44.5 ± 3.1	40.4 ± 2.4	128.9 ± 3	142.9 ± 3.2**
Ventral	(7)	–	–	76.3 ± 4.5	71.4 ± 2.2	134.6 ± 3.2	139 ± 3.8
Accumbens core	(8)	–	–	72 ± 2.3	60.2 ± 5.1	121 ± 3.6	117.7 ± 5.6
Accumbens shell, medial	(9)	–	–	90.1 ± 3.3	79.4 ± 3.3*	99.9 ± 3.8	94.5 ± 11.5
Accumbens shell, ventral	(10)	–	–	75.4 ± 1.7	66 ± 2**	103.1 ± 2.4	111.9 ± 4.4
Cingulate cortex	(11)	114.8 ± 2.3	110.3 ± 3.6	21.5 ± 1.2	21.1 ± 0.9	12.4 ± 0.4	10.9 ± 0.7
(rostral: Cg1, Cg2)		53.4 ± 1.3	41.9 ± 3.1**	–	–	–	–
		92.1 ± 2.2	88.5 ± 2.72	–	–	–	–
		27.3 ± 2.4	26.6 ± 1.82	–	–	–	–
Motor cortex	(12)	83.1 ± 1.8	71.2 ± 1.4***	23.2 ± 1.3	21.6 ± 1.4	8.8 ± 0.7	9.2 ± 1
		48.6 ± 1.3	39.1 ± 2.6 **	–	–	–	–
		68.8 ± 2.4	62 ± 3.42	–	–	–	–
		55.1 ± 2.9	54.9 ± 22	–	–	–	–
		60.2 ± 2.5	59.4 ± 22	–	–	–	–
		29 ± 2	24.1 ± 1.92	–	–	–	–
Sensory cortex	(13)	62.3 ± 4	58.3 ± 2	22.5 ± 0.9	21.7 ± 1.4	9.7 ± 0.5	9.6 ± 0.6
		14.9 ± 1.3	11.1 ± 1.72	–	–	–	–
		63.1 ± 4	54.9 ± 2.62	–	–	–	–
Caudate putamen	(14)	2.5 ± 1.7	2.7 ± 1.32	–	–	–	–
Dorsomedial	(15)	–	–	67.1 ± 2.5	62.8 ± 1.4	134.5 ± 3.4	136.8 ± 3.6
Dorsolateral	(16)	–	–	40.1 ± 1.9	42.9 ± 3.5	110.5 ± 3.7	115.1 ± 3.6
Ventral	(17)	–	–	54.2 ± 3	58.5 ± 4.6	149.5 ± 1.8	147.6 ± 3.3
Bed nucleus of stria terminalis	(18)	–	–	33.5 ± 1.2	28.8 ± 3.2	24.7 ± 2.9	28.9 ± 3.7
Cingulate cortex	(19)	90.9 ± 6.2	99.1 ± 6.2	17.9 ± 1.6	17.7 ± 1.5	29.5 ± 1.5	15.6 ± 0.7***
(caudal: Cg1, Cg2)		34.1 ± 3.2	35.8 ± 22	–	–	–	–
		82.5 ± 5.2	87.2 ± 4.72	–	–	–	–
		20.6 ± 3.6	22.5 ± 1.52	–	–	–	–
Dentate gyrus	(20)	–	–	89.2 ± 3.1	88.4 ± 4.1	23.4 ± 3	26.3 ± 5.6
CA1	(21)	81.4 ± 2	76 ± 3.52	–	–	–	–
CA3	(22)	64.5 ± 4	40.3 ± 2.5***	–	–	–	–
VTA	(23)	43.7 ± 3.6	37.6 ± 4.42	–	–	–	–
SNC	(24)	76 ± 8.2	88.5 ± 7.62	–	–	–	–
Dentate gyrus	(25)	–	–	96 ± 6.9	112.7 ± 6.6	21.9 ± 2.1	20 ± 2.7
CA1	(26)	102.8 ± 4.4	108.6 ± 3.5	–	–	–	–
CA3	(27)	97.9 ± 3.7	90.5 ± 4.2	–	–	–	–

Results are expressed as mean optical density in the specific areas ± SEM. The number next to each area corresponds to the identification number in Fig. 1. For cortical areas, CCK optical density was measured in different layers shown in the table in order of increasing depth. Each measured layer consisted of an area of homogenous labelling. The statistical analysis was performed using *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. †In arbitrary units.

revealed associations among exploratory behaviour, impulsivity, alcohol consumption and substance abuse (e.g, Nagoshi *et al*., 1991; Moss *et al*., 1992). The association between head-dipping (i.e. novelty-seeking) in the hole-board and EtOH consumption (Fernández-Teruel *et al*., 2002) is in line with the hypothesis that there is a connection between a behaviour reflecting preference for novelty (or new stimulation) and the preference for rewarding substances (i.e. positive reinforcing), as proposed in human personality theories of sensation/novelty seeking (e.g. Bardo *et al*., 1996; Zuckerman, 1996). As reviewed by Driscoll *et al*. (1998; see also Giorgi *et al*., 2005), the system of most interest in this regard appears to be the mesoaccumbens dopaminergic projection, which has been proven to be of particular relevance in relation to the dependence-producing effects of amphetamine, cocaine, morphine and alcohol (Giorgi *et al*., 1997, 2005; Corda *et al*., 2001; Lecca *et al*., 2004).

In line with the well-known locomotor stimulant effects of most drugs of abuse, systemic injection of 0.25 g/kg of EtOH has been shown to markedly increase both exploration and locomotion in low exploratory Sprague–Dawley rats in a hole-board test (June & Lewis, 1994). In the present study, the low exploratory RLA rats also significantly increased exploration (i.e. head-dipping) after EtOH administration when compared with the high exploratory RHA rats. General locomotor activity of RLA rats was not modified by EtOH, suggesting that enhanced drug-induced novelty seeking is the most likely interpretation of the increase in head-dipping (Fernández-Teruel *et al*., 1992; Escorihuela *et al*., 1999), although disinhibitory (or anxiolytic-like) effects of EtOH can not be completely ruled out, as some degree of anxiety is involved in the hole-board testing situation. Animals with low basal exploratory rates are preferred when studying the stimulatory effects of EtOH to increase the likelihood that the drug will increase these behaviours (i.e. June & Lewis, 1994). In this sense, the lack of effect of EtOH in RHA rats could also be understood as a ceiling effect, as RHA rats treated with saline had a high head-dipping and locomotor activity during the 5-min recording period. However, this exploratory activity in the hole-board was still far from a maximum as, for instance, it was just half of that reported in RHA rats

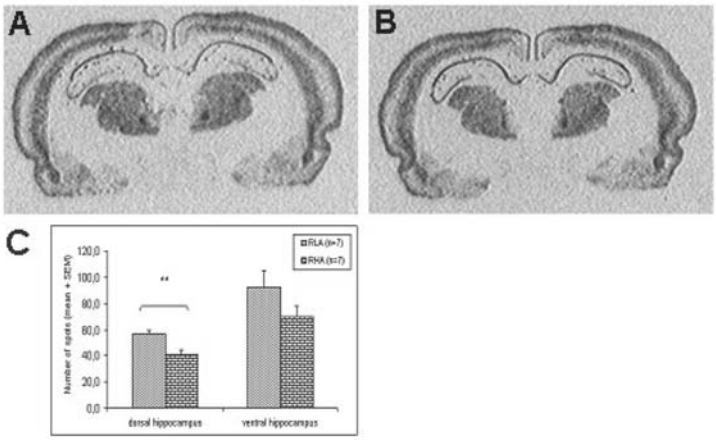

FIG. 2. *In situ* identification of CCK gene transcript in the dorsal hippocampus. (A) Typical autoradiograms obtained in Roman low-avoidance (RLA) rats after *in situ* hybridization in rat brain coronal sections. The images correspond to the fourth level of study (bregma −3.6 mm) and the hippocampus can be identified. See Fig. 1 for orientation. (B) Corresponding picture for a Roman high-avoidance (RHA) rat. A difference in optical density in the CA3 pyramidal layer as well as a difference in the number of stained spots in the other layers of the hippocampus can be appreciated. (C) Total number of spots outside the pyramidal layer of the hippocampus. $^{**}P < 0.01$.

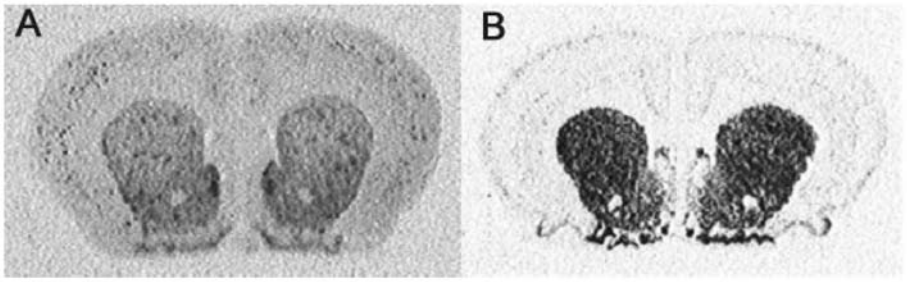

FIG. 3. (A) *In situ* identification of DYN gene transcript in the rostral striatum of a RLA rat. (B) *In situ* identification of ENK gene transcript in the rostral striatum of a RLA rat.

raised in an enriched environment (Fernández-Teruel *et al*., 2002). Therefore, we can conclude that the alcohol-preferring RHA rats were less sensitive to EtOH administered intraperitoneally than the alcohol non-preferring RLA rats.

The response to ethanol in humans and rodent strains

In humans, family history of alcoholism and a low level of response to ethanol (EtOH) are factors known to predict future alcoholism (Schuckit, 1984, 1985, 1994). Thus, it seems that a lower sensitivity to EtOH is associated with a significant increase in the risk of future alcoholism, perhaps through increasing the chances of drinking more heavily and more often (Schuckit, 1994). The psychomotor stimulant theory of addiction (Wise & Bozarth, 1987) argues that low-dose locomotor stimulant effects, related to activation of mesolimbic dopaminergic fibres, may represent a model of the euphoric effects and rewarding properties of EtOH. This assumption has been supported by some animal strains selectively bred for their high-EtOH-drinking properties (Murphy *et al*., 2002), but is contradictory to a preventive role attributed to high sensitivity to EtOH. Matching the psychostimulant theory of addiction, it is known that RHA rats, compared with RLA rats, respond with higher DA release in the nucleus accumbens after cocaine, amphetamine and morphine administration (Giorgi *et al*., 1997; Lecca *et al*., 2004), as well as during voluntary EtOH consumption (Corda *et al*., 2001).

EtOH is known to act upon other neurotransmitters (e.g. GABA and neuropeptides) besides DA. This may explain why different responses to EtOH do not always co-vary, and why peripherally administered low doses of EtOH do not always induce locomotor activity in commonly studied rat strains (Masur *et al*., 1986; Correa *et al*., 2003). Thus, for instance, Sprague–Dawley rats selected for low activity, LA rats, show an increase of motor activity during the first 10 min after low-dose EtOH administration, while Sprague–Dawley rats selected for high activity, HA rats, show suppression of motor activity at the same doses (Moore *et al*., 1993). The EtOH-preferring AA (Alko,

Alcohol) rats and their psychogenetic counterpart, the EtOH non-preferring ANA (Alko, Non-Alcohol) rats, show the same extent of motor response to low doses of EtOH administered intraperitoneally (Päivärinta & Korpi, 1993). The EtOH-preferring P rats have been shown to be more sensitive than the non-preferring NP rats to intraperitoneally injected low doses of EtOH in some studies (reviewed by Murphy *et al*., 2002), but a lack of locomotor stimulation in both strains has also been reported (Criswell *et al*., 1994). Furthermore, Fawn Hooded (FH) rats, which voluntarily consume EtOH at levels similar to P rats, do not show locomotor stimulation after low EtOH doses (including the 0.25 g/kg dose) (Criswell *et al*., 1994). When mice were instead bred for high or low locomotor stimulation by EtOH, no difference in voluntary EtOH consumption was found (Sanchez *et al*., 1996). Together with the present results, these data should be interpreted as a lack of consistent association between the preference of EtOH and the sensitivity to low psychostimulant doses of the drug in rodent models.

It is worth pointing out that the Roman lines/strains originated differently than the other rodent models of vulnerability to alcoholism discussed above. P/NP and AA/ANA rats were originally selected for the amount of EtOH ingested in a free-choice paradigm (Eriksson, 1968; Lumeng *et al*., 1977). As mentioned in the Introduction, the Roman lines/strains were selected for their performance in the shuttle box, which led to a hyperemotional line/strain (RLA) and a novelty/sensation-seeking line/strain (RHA) (Driscoll & Battig, 1982; reviewed by Driscoll *et al*., 1998). Therefore, different aggregates of traits are selected in each pair of strains. Among the traits that three EtOH-preferring strains (RHA, P and AA) share when compared with their non-EtOH-preferring counterparts is a greater preference for sweet solutions and a greater EtOH-induced release of DA in the nucleus accumbens (Murphy *et al*., 2002 for P/NP; Katner & Weiss, 2001 for AA/ANA; Corda *et al*., 2001 for RHA/RLA). In this context, the lowest sensitivity to the psychomotor-activating doses of EtOH showed in the present experiments is another trait to be added to the novelty- and incentive-seeking profile that defines RHA rats. In the Roman strains the anxiety trait is completely segregated (higher in RLA than RHA rats; reviewed by Fernández-Teruel *et al*., 1997a and Steimer & Driscoll, 2003), and there is apparently no direct relationship between anxiety trait and EtOH consumption. Anxiety traits are not completely segregated in the other two pairs of strains, as P rats show more anxiety-like behaviours than NP in some paradigms and less in others (Murphy *et al*., 2002). The same is true for the AA/ANA strains (Fahlke *et al*., 1993; Knapp *et al*., 1997; Möller *et al*., 1997). These observations give further support to the contention that novelty seeking as a trait probably has a more important role than anxiety in incentive- or substance-seeking behaviour (e.g. Zuckerman, 1996).

Neurochemical analysis

In the striatum, we found differences restricted to specific regions in the mRNA levels of opioid peptides that have a reciprocal interaction with DA: DYN mRNA levels were higher in the nucleus accumbens and ENK mRNA levels were lower in the rostral dorsolateral caudate putamen of RHA rats. We hypothesize that these differences are a neurochemical correlate of the DAergic tone. It has been suggested that dynorphin levels upregulate as a result of elevated DAergic activity, and that this upregulation is related to a decrease in cellular responsivity of striatal neurons to activation signals. An opposite relationship has been proposed for enkephalin–DA interaction (reviewed by Steiner & Gerfen, 1998). Pharmacological, lesion (Li *et al*., 1990) and gene knock-out studies (Giros *et al*., 1996) that modify dopaminergic tone corroborate this compensatory role for opioid peptides in the striatum.

According to a compensatory hypothesis, higher ENK mRNA levels in the rostral dorsolateral caudate putamen in the RLA would be a correlate of a lower dopaminergic input. This area of the striatum is predominantly sensorimotor in terms of connectivity (reviewed by Voorn *et al*., 2004), which fits with the already-mentioned protection of RLA rats to the motor stereotypes induced by high doses of apomorphine (Durcan *et al*., 1984; Giménez-Llort *et al*., 2005) and amphetamine (Driscoll *et al*., 1986).

Rodent strains that are alcohol-preferring or non-preferring have been used to study the relationship between susceptibility to alcohol dependence and opioid–DA interactions. Higher levels of different DYN-derived peptides in the nucleus accumbens of ANA rats when compared with the AA rats were described in experiments using radioimmunoassay on homogenized samples (Nylander *et al*., 1994), but were not replicated in the same strains using *in situ* hybridization (Marinelli *et al*., 2000).

It is especially relevant that mRNA for DYN was only higher in the nucleus accumbens of RHA rats compared with RLA rats. Dopaminergic tone in this area has been linked with the motor activity and exploration induced by novelty (Koob *et al*., 1981), which is in accordance with behavioural results presented by controls in the present experiments. Moreover, RHA rats have shown higher DA release in the nucleus accumbens after administration of several abused drugs being due to differences in the shell compartment (Lecca *et al*., 2004). HR (high responders to novelty) rats are another rodent model of vulnerability to addiction (Piazza *et al*., 1989), which also show higher DYN mRNA levels than LR (low responders to novelty) rats in the nucleus accumbens (Lucas *et al*., 1998) and induced DA levels at this site (Hooks *et al*., 1991, 1992). Our results are in line with the hypothesis outlined above, namely that changes in opioid peptide gene transcripts in the striatum are more likely to be compensatory: an effect rather than a cause. Increase in DA release at the shell of the nucleus accumbens is thought to be the common feature of all abused drugs (reviewed by Wise & Rompré, 1989; Bardo, 1998; Wise, 1998). Our results are therefore consistent with the previously proposed hypothesis that DAergic hyperactivity in the nucleus accumbens of RHA rats relative to their RLA counterparts is related to their higher EtOH consumption/preference (Corda *et al*., 2001), as well as in their increased responsiveness to the acute and repeated administration of psychostimulants and opiates (Giorgi *et al*., 1997, 2005; Lecca *et al*., 2004; Corda *et al*., 2005) In this context, the lower sensitivity to EtOH shown by RHA rats joins the novelty-seeking profile as an independent trait and does not seem to be related to the dopaminergic function.

We also found higher ENK mRNA in the caudal portion of anterior cingulate areas (Cg1, Cg2) of RHA rats. The cingulate cortex projects to the accumbens core (Zahm & Brog, 1992). It has been suggested that the accumbal opioid system can mediate the hedonic impact of rewarded stimuli (Kelley, 2004). Therefore, one part of the network presumably related to hedonic impact processing is richer in ENK-derived peptides in RHA rats compared with RLA rats. Much more research is needed to clarify which are the neurons that express ENK mRNA at this level and their physiological role. Anyway, this seems a relevant finding as AA/ANA rats differ in ENK mRNA expression in the cingulate cortex in the same way Roman strains do (Marinelli *et al*., 2000). Moreover, in experiments that we are currently running with Roman strains as well as with Sprague–Dawley rats, a lower level of ENK in the cingulate cortex seems to be a characteristic of RLA rats (unpublished data).

Striking between-strain differences were found in CCK mRNA expression in superficial layers of some cortical areas, namely

prefrontal agranular insular cortex, anterior cingulate cortex, motor cortex and dorsal hippocampus. In the CA3 field of the dorsal hippocampus we found a very interesting result: RHA rats showed higher expression of CCK mRNA in the pyramidal layer while RLA rats had more spots of CCK staining in the other two layers. In cortical structures, CCK is expressed by both pyramidal projecting neurons (Burgunder & Young, 1990) as well as by interneurons (Vallebuona *et al*., 1993; Nunzi *et al*., 1997). CKK-interneuron activity may be superimposed to the synchronized firing pattern of pyramidal and parvalbumin cells, and drive mood and emotional influences both in the hippocampus and in the isocortex (Freund, 2003). In the CA1 field of the dorsal hippocampus, the different arrangement of CCK staining is suggestive of different anatomical distribution of the CCK neuronal networks. Consequently, different functionality of the CA3 field and the whole hippocampus may be expected. It is a possibility that a richer CCK interneuron network in the prefrontal cortex could account for the proactive behaviour (i.e. active coping strategies) described for RHA rats, while a richer CCK network in the hippocampus could account for at least some of the reactive behaviour (i.e. passive coping strategies) described for RLA rats (reviewed by Steimer & Driscoll, 2003).

Concluding remarks

In conclusion, the results of these experiments reveal that RHA rats may represent a unique rodent model for alcoholism predisposition in humans, as these rats show reduced sensitivity to the stimulatory effects of a low dose of EtOH (and have a reduced sensitivity to its hypnotic effects; Fernández-Teruel *et al*., 1997b) when compared with the alcohol-avoiding RLA rats. In addition, the present experiments extend our knowledge about the neurochemical traits that drive the alcohol-preferring phenotype in the RHA rats. The mesoaccumbens dopaminergic pathway has been implicated in the differentiation between the two Roman strains in novelty/sensation- and substance-seeking behaviour. Differences in the expression levels of ENK in the cingulate cortex could account for the difference in alcohol preference in the Roman strains. Finally, striking differences in the distribution of CCK staining between the Roman strains suggest a differential organization of some cortical networks that could account for part of the known differences in coping strategies and anxiety-related behaviour of these strains.

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Abbreviations

CCK, preprocholecystokinin; DA, dopamine; DYN, preprodynorphin; ENK, preproenkephalin; EtOH, ethanol; GABA, γ-aminobutyric acid; RHA, Roman high avoidance; RLA, Roman low avoidance; VTA, ventral tegmental area.

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Paper II

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DIVERGENT ANATOMICAL PATTERN OF D_1 AND D_3 BINDING AND DOPAMINE- AND CYCLIC AMP-REGULATED PHOSPHOPROTEIN OF 32 kDa mRNA EXPRESSION IN THE ROMAN RAT STRAINS: IMPLICATIONS FOR DRUG ADDICTION

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Abstract—Autoradiography analysis of D_1, D_2 and D_3 dopamine receptors and *in situ* hybridization analysis of mRNA for dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32) were performed in brains of naïve Roman high avoidance (RHA) and Roman low avoidance (RLA) inbred rats. These strains, genetically selected for high (RHA) or extremely low (RLA) active avoidance acquisition in the two-way shuttle box, differ in indices of dopaminergic activity along with sensation/novelty and substance-seeking behavioral profiles. The present study shows no differences in D_2 receptor binding between the two strains. In contrast, the D_1 and D_3 receptor binding in the nucleus accumbens was higher in RHA-I rats, whereas RLA-I rats show higher D_3 binding in the Calleja islands. Together with previous evidence showing behavioral and presynaptic differences related to the dopamine system, the present results suggest a higher dopaminergic tone at the nucleus accumbens shell in RHA-I rats. Besides, the comparison of the expression pattern of DARPP-32 mRNA with that of dopamine receptor binding revealed a mismatch in some amygdala nuclei. In some cortical structures (prelimbic and cingulate cortices, the dentate gyrus) as well as in the central amygdala, RHA-I rats showed higher DARPP-32 mRNA expression than RLA-I rats. Hence, RHA-I and RLA-I rats may be a useful tool to identify dopamine-related mechanisms that predispose to drug and alcohol dependence.

Key words: dopamine receptors subtypes, addiction, rodent models, amygdala, nucleus accumbens.

Rats selected for a particular behavioral trait that differ in the dopaminergic function are a valuable tool to disentangle the role of dopamine in the vulnerability to drug addiction. There are several examples of such animals including the well-characterized high reactive to novelty (HR) versus their counterparts, the low reactive to novelty (LR) rats (Piazza et al., 1989), differing in liability to self-administer psychostimulants. The higher propensity of the HR rats to acquire self-administration behavior is associated with higher novelty seeking behavior as well as higher dopaminergic tone in the striatum (Hooks et al., 1992).

The Swiss sublines of Roman high-avoidance (RHA/Verh) and low-avoidance (RLA/Verh) rats were psychogenetically selected for rapid vs. extremely poor two-way avoidance acquisition in the shuttle box respectively (Driscoll and Bättig, 1982). RHA/Verh rats are characterized by their ability to get engaged in active coping strategies (proactive behavior), whereas RLA/Verh rats are more often engaged in passive coping strategies (passive behavior) (Steimer and Driscoll, 2003). An inbreeding program was started in 1993 leading to the RHA-I and RLA-I inbred strains (Driscoll et al., 1998; Escorihuela et al., 1999). An important body of concordant behavioral and neurobiological evidence indicates that these rat lines/strains are a valid laboratory model of divergent sensation/novelty and substance-seeking profiles rooted in differences in dopaminergic function. Compared with RLA line/strain, the RHA line/strain show: (i) greater stereotypy response to high doses of the dopamine agonists apomorphine (Durcan et al., 1984; Giménez-Llort et al., 2005) and amphetamine (Driscoll et al., 1986); (ii) higher levels of exploratory behavior in tests of novelty seeking (Escorihuela et al., 1999; Fernández-Teruel et al., 1997, 2002; Guitart-Masip et al., 2006) (iii) stronger mesolimbic dopaminergic responses to drugs of abuse (Giorgi et al., 1997; Lecca et al., 2004) including ethanol (Corda et al., 2001); (iv) enhanced mesocortical dopamine release evoked by stressors (Giorgi et al., 2003); (v) enhanced sensitization to morphine (Piras et al., 2003), cocaine (Giorgi et al., 2005b) and amphetamine (Corda et al., 2005) as well as changes in the dopaminergic outflow occurring only in RHA rats sensitized to amphetamine (Giorgi et al., 2005a); and (vi) higher levels of preprodynorphin mRNA in the accumbens shell and lower levels of preproenkephalin mRNA in the dorsolateral striatum that also can be related to dopaminergic activity (Guitart-Masip et al., 2006). Dopamine receptors, including D_3 receptors, are important in the actions of drugs of abuse (Robinson and Berridge, 1993; Schwartz et al., 1994; Zahm, 1999; Everitt et al., 1999). Considering the body of behavioral and presynaptic evidence for a more active dopamine system in RHA than

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Abbreviations: DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; HR, high reactive to novelty; PKA, protein kinase A; RHA, Roman high avoidance; RLA, Roman low avoidance.

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RLA rats, we wanted to know if these strains also differ in their sensitivity to released dopamine. One may expect dopamine receptors to be reduced in RHA-I rats in response to strong dopamine release. Alternatively, if an active dopamine system is important to the behavioral phenotype of RHA-I rats, dopamine receptors may be as high as, or higher than, in RLA-I rats. In a study with tissue homogenates, Corda et al. (1997) provided evidence for higher density of D_1 receptors in the nucleus accumbens of RHA rats.

The aim of these experiments was therefore to characterize the density and tissue distribution of different dopamine receptor subtypes (D_1, D_2 and D_3) and dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) to add more detail to the described functional differences regarding dopamine system between RHA-I and RLA-I rats. To achieve these objectives, receptor autoradiography for D_1, D_2 and D_3 receptors and *in situ* hybridization for DARPP-32 mRNA were performed in brain regions of drug-naïve RHA-I and RLA-I rats. Although D_3 receptors are expressed in ventral striatal areas (Landwehrmeyer et al., 1993) and are thought to play a relevant role in drug abuse (Richtand et al., 2001), this dopamine receptor subtype has never been studied in the Roman rats, nor has DARPP-32 a key mediator of dopamine's cellular and behavioral effects (Fienberg et al., 1998). In the striatum, D_1 and D_2 receptors are generally expressed by different spiny cells, the direct and indirect pathways respectively (Ferré et al., 1997). However, virtually all these medium spiny neurons express DARPP-32 (Svenningsson et al., 2004), a protein with several phosphorylation sites controlling its activity. D_1 receptors activate and D_2 and D_3 receptors inhibit adenylyl cyclase and cAMP dependent protein kinase A (PKA). When activated by PKA phosphorylation, DARPP-32 becomes an inhibitor of protein phosphatase one and prevents dephosphorylation of PKA substrates. So, through DARPP-32 phosphorylation, dopamine achieves amplification of its cellular signaling effects (Nishi et al., 1997, 2000), evidenced e.g. by reduced cellular and behavioral responses to D_1 receptor stimulation in DARPP-32 knockout mice (see Svenningsson et al., 2004). Previous comparisons between DARPP-32 and D_2 mRNA expression patterns in rats did not include the amygdala (Schalling et al., 1990a).

EXPERIMENTAL PROCEDURES

Animals

Four months old male inbred RHA (RHA-I) and RLA (RLA-I) rats were used. Animals were bred and maintained in the animal facilities in the medical psychology unit at the Autonomous University of Barcelona (Bellaterra, Spain). The animals were maintained two per cage (Macrolon, 21.5×46.5×14.5 cm), under standard laboratory conditions (food and water *ad libitum*, 22±2 °C and 12-h light/dark cycles beginning at 07:00 h). Each of the two groups comprised animals from five different litters of each strain.

Tissue extraction and samples processing

Drug-naïve male RHA-I and RLA-I (seven per group) were killed by decapitation and brains were rapidly dissected, frozen on dry ice and stored at −80 °C until processed. Coronal sections (14 μm-thick) were cut in a cryostat (Johansson et al., 1994). Sections were thaw-mounted onto SuperFrost Plus (Menzel-Gläser) slides, dried briefly at 30 °C, and stored at −20 °C until used. For the identification of the different brain structures, adjacent sections to those used for *in situ* hybridization were stained with Cresyl Violet (Johansson et al., 1994).

Equivalent sections for all brains were collected at six different levels which allowed us to map different brain areas along the rostrocaudal axis. The section levels were: level 1. bregma: 3.7–3.2 (prefrontal cortex); level 2. bregma 1.6–1.2 (nucleus accumbens, rostral caudate putamen, rostral cingulate cortex); level 3. bregma −0.8 to −0.92 (caudal caudate putamen, caudal cingulate cortex); level 4. bregma −2.1 to −2.3 (rostral hippocampus, tail of the striatum and amygdala) level 5. bregma −3.60 to −3.8 (dorsal hippocampus); level 6. bregma −4.8 to −5.2 (ventral hippocampus, ventral tegmental area, substantia nigra reticulata, and substantia nigra compacta) according to Paxinos and Watson (1998) atlas.

Receptor autoradiography with [^{3}H] SCH 23390 (D_1 receptor)

Slides were dried for 60 min at room temperature and then incubated with 1 nM [^{3}H] SCH 23390 (N-methyl-^{3}H; 85.0 Ci/nmol; PerkinElmer Life Science, Boston, MA, USA) in a buffer containing 25 mM Tris, pH 7.5 with HCl, 100 mM NaCl, 1 mM $MgCl_2$, 1 μM pargyline and 0.001% ascorbic acid and 20 nM mianserin (Sigma, St. Louis, MO, USA) to block binding of SCH 23390 to 5-HT_2 receptors for which SCH 23390 has some affinity. Sections were incubated for 150 min at room temperature. Slides were then washed twice for 5 min each in ice-cold buffer, and rinsed briefly in ice-cold distilled water before drying at 4 °C over a strong fan.

Receptor autoradiography with [^{3}H]raclopride (D_2 receptor)

Slides were dried for 60 min at room temperature and then incubated with 2 nM [^{3}H]raclopride (methoxy-^{3}H; 87.0 Ci/nmol; PerkinElmer Life Science) in a buffer containing 170 mM Tris, pH 7.6 with HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $ClMg_2$ and 0.001% ascorbic acid for 60 min. Slides were then washed six times for 20 s each in ice-cold buffer and rinsed briefly in ice-cold distilled water before drying over a strong fan at 4 °C.

Receptor autoradiography with [^{3}H]PD 128907 (D_3 receptor)

Slides were dried for 60 min at room temperature and then incubated with 3 nM [^{3}H]PD 128907 (N-propyl-2,3-3H; 103 Ci/nmol; Amersham, Piscataway, NJ, USA) in a buffer containing 50 mM Tris, pH 7.4 with HCl, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.001% ascorbic acid. Slides were then washed three times for 5 min each in ice-cold buffer and rinsed briefly in ice-cold distilled water before drying over a strong fan at 4 °C.

In order to ensure the correctness of the concentration of [^{3}H]PD 128907 used in the experiments, we performed a saturation binding experiment using six different concentrations of radioligand. Sections through the Calleja magna in RHA rats were used. The films were exposed for 12 weeks. Readings from the Calleja magna, converted to fmol/mg tissue as below, were analyzed using non-linear regression with GraphPad Prism version 4 (Graphpad Software, San Diego, CA, USA).

Film exposure and unspecific binding for D_1, D_2 and D_3 receptor autoradiography

Slides were apposed to Hyperfilm-^{3}H (Amersham) together with plastic standards (Amersham) at 4 °C. Those slides incubated with [^{3}H] SCH 23390 were apposed for 6 weeks and those slides incubated with [^{3}H]raclopride or [^{3}H]PD 128907 were apposed for

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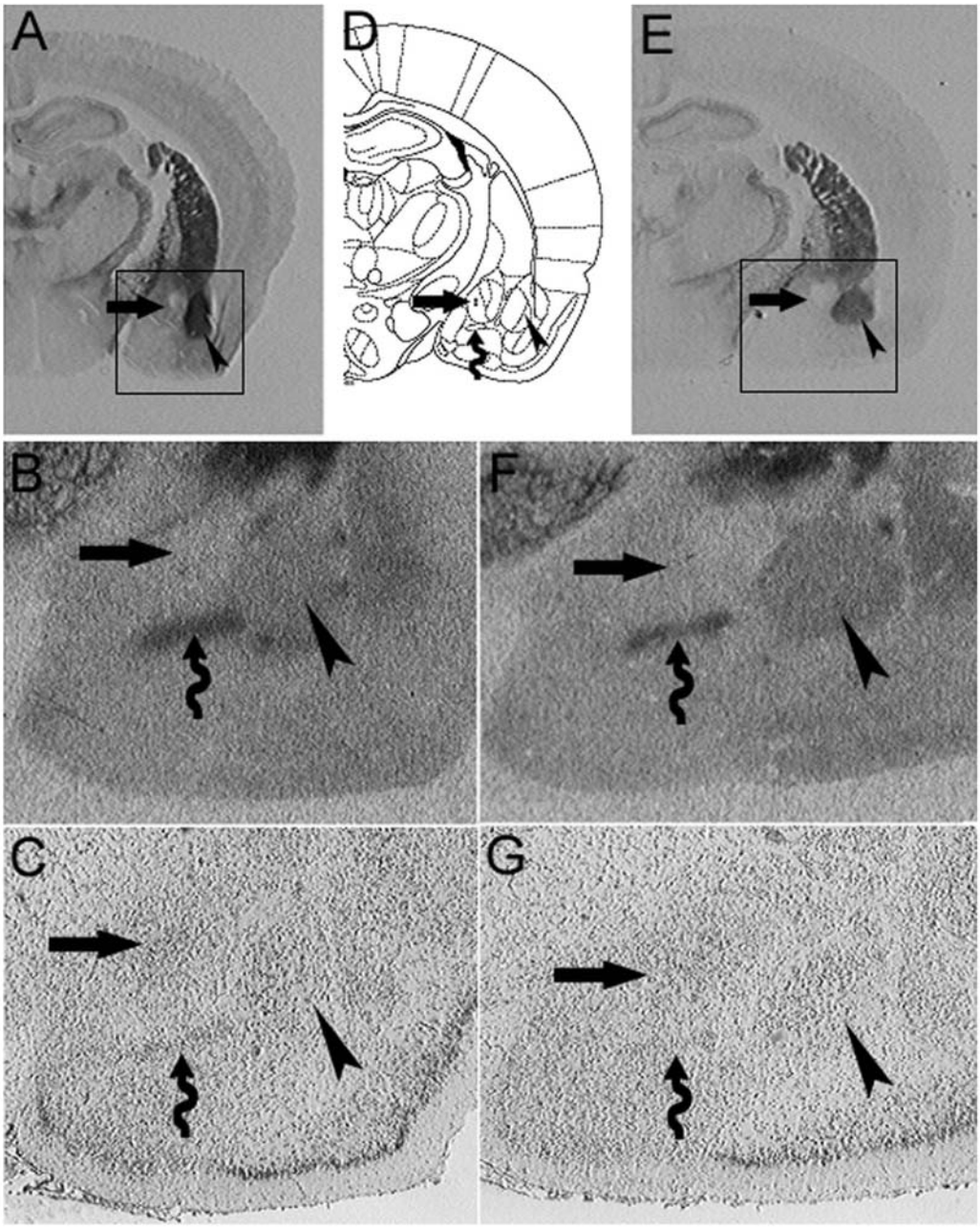

Fig. 1. Anatomical identification of D_1 binding in the amygdala region. In this figure pictures representative of RHA rats are shown in A–C, whereas in E–G are shown pictures representative of RLA rats. D_1 binding autoradiography (B and F) in the 4th level of study (bregma −2.3 mm) is depicted together with Cresyl Violet staining (C and G) on adjacent section, acetylcholinesterase staining (A and E) in the same section and the corresponding diagram of the Paxinos and Watson (1998) atlas (D). This comparison allows us to identify different amygdalar nuclei. The central amygdala is pointed with an arrow; the basolateral amygdala is pointed with an arrowhead; the intercalated nucleus is pointed with a curved arrow. As seen in the first picture, the central amygdala is an area in which no D_1 binding is detected, other nuclei of the complex like the basolateral and the intercalated nucleus show moderate to high binding.

8 weeks. Non-specific binding was defined by adding 10 μM (for D_1 receptors) or 1 μM (for D_2 and D_3 receptors) (+)butaclamol (Sigma) in order to use a ligand structurally unrelated to the radioligands used. This concentration of (+)butaclamol is about 1000 times the dissociation constant at D_1, D_2 and D_3 receptors respectively.

In situ hybridization histochemistry

The analysis of mRNA levels was carried out by a procedure of *in situ* hybridization using oligodeoxyribonucleotide probes complementary to rat mRNAs coding for DARPP-32 (nucleotides 691–740 (Ehrlich et al., 1990)) synthesized on a DNA synthesizer (KabiGen, Stockholm, Sweden) and subsequently HPLC purified. The specificity was checked by the addition of a 225× excess of unlabeled probe which blocked the signal, whereas the signal was not influenced by a 225× excess of a nonrelated oligo (data not shown). Oligonucleotide probes were 3′-end labeled with [^{33}P]-dATP (300 Ci/mmol; NEN, Perkin Elmer) using terminal deoxynucleotidyl-transferase (Amersham). Slide-mounted sections were incubated for 16–20 h at 42 °C with the labeled oligonucleotide probe. Following hybridization, the sections were washed four times in SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) at 55 °C for 15 min each time, rinsed in water at room temperature for 1 min, dehydrated through ethanol (60%, 95% and 100% 1 min each), and air-dried. Sections were apposed to Kodak Biomax (Amersham) for 5 days.

Analysis of autoradiograms

Autoradiograms were analyzed with a Macintosh computer using the public domain NIH Image program (US National Institutes of Health; see http://rsb.info.nih.gov/nih-image). For receptor autoradiography, optical densities were converted to density of bound ligand (fmol/mg gray matter) using the plastic standards and the specific activity of the radioligands. Unspecific binding from adjacent sections was subtracted. For *in situ* hybridization, optical densities (expressed in gray levels) were calculated from the uncalibrated mode by subtracting from each measurement its corresponding background. All measurements were done on both

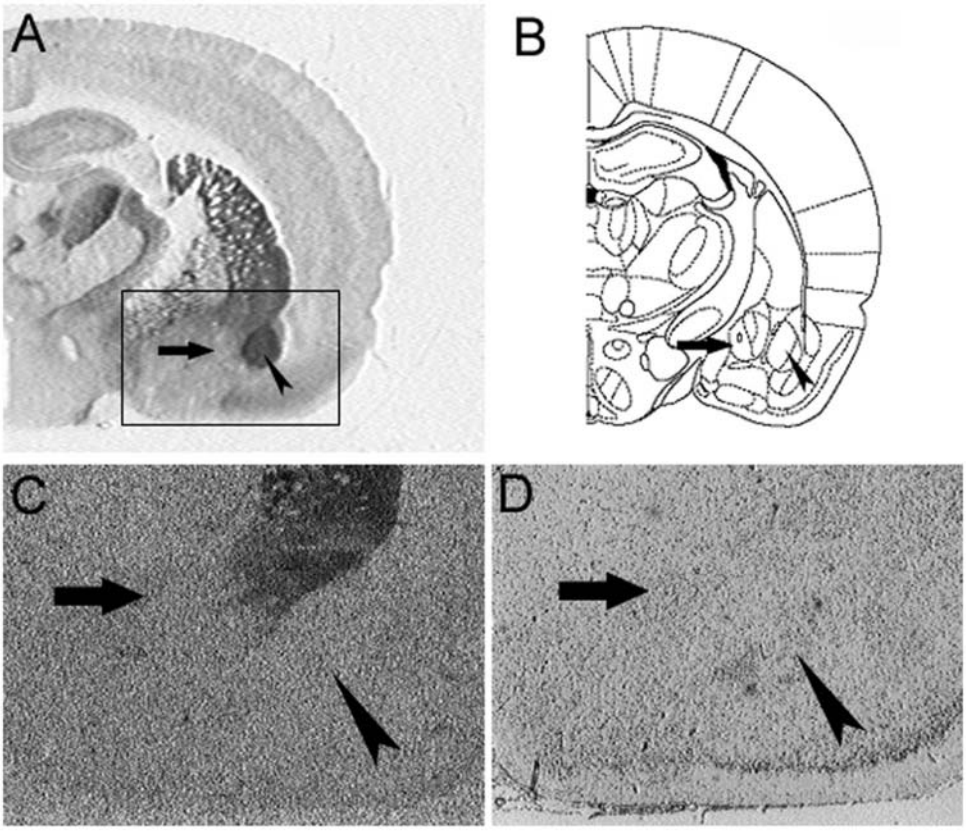

Fig. 2. Anatomical identification of D_2 binding in the amygdala region. In this figure D_2 binding autoradiography (C) in the 4th level of study (bregma −2.3 mm) is depicted together with Cresyl Violet staining (D) on adjacent section, acetylcholinesterase staining (A) in the same section and the corresponding diagram of the Paxinos and Watson (1998) atlas (B). This comparison allows us to identify different amygdalar nuclei. The central amygdala is pointed with an arrow; the basolateral amygdala is pointed with an arrowhead. As seen in the first picture, the central amygdala is an area in which weak but detectable levels of D_2 binding are detected. The basolateral complex appears free of D_2 binding.

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cerebral hemispheres (from seven animals per group) and data were pooled. During the whole procedure the analysis was blind to the experimental conditions. For DARPP-32 mRNA D_1 and D_2 receptors the same brain areas were measured since the expression patterns were overlapping. For D_3 receptor, ventral striatal structures and the Calleja islands were measured.

Statistical analysis

Student's *t*-test comparing optical densities or fmol receptor/mg protein measured on each specific brain region considering each Roman strain as a group was used. For correlation of individual values of DARPP-32 and D_1 we used the Pearson's correlation test.

RESULTS

D_1 and D_2 receptor autoradiography

The distribution pattern obtained in the present experiment for D_1 and D_2 receptors in the Roman rats fits that previously described in rat brain (Mansour et al., 1990, 1991) except for the amygdala. We detected only slight D_1 binding in the central nucleus of the amygdala (see Fig. 1) where moderate levels of binding were described by Mansour et al. (1991). Regarding D_2, according to Mansour et al. (1990), most amygdaloid nuclei showed no binding and only in the medial portion light labeling was detected. In contrast, we found weak but detectable binding restricted to the central nucleus of the amygdala (see Fig. 2).

As shown in Table 1, compared with RLA-I rats, RHA-I rats show 171% higher binding of D_1 in the medial accumbens shell ($t(12)=3.1$ $P=0.009$), a 173% higher binding to D_1 receptors in the ventral accumbens shell ($t(12)=1.3$ $P=0.041$), and a non-significant 130% higher binding in the accumbens core. Binding to D_1 receptors is elevated by 170% in the tail of the striatum ($t(10)=2.9$ $P=0.017$) and by 204% at the level of the lateral hypothalamus ($t(10)=2.9$ $P=0.17$), the latter being the site through which the medial forebrain bundle runs from the mesencephalon to the forebrain (Paxinos and Watson, 1998). No differences between the strains were detected in D_2 binding at any measured rostrocaudal level.

Table 1. Results of *in situ* hybridization for DARPP-32 gene transcripts, and D_1 ([^{3}H] SCH 23390) and D_2 ([^{3}H] raclopride) binding in the RHA and RLA rats

	DARPP-32		D_1		D_2	
	RLA	RHA	RLA	RHA	RLA	RHA
L1: Prelimbic/Infralimbic	48.9±1.7	59.2±3.2*	42.5±6.8	49.8±8	7.9±1.2	4.2±3.2
L1: Infraorbital	36.3±1.6	42.6±3.2	24.6±4.6	16.6±1.6	1.9±1.5	0.5±0.8
L1: Agranular insular	38.3±1.9	41.3±1.7	25.3±3.5	21.6±4.1	2.1±2	4.1±1.8
L2: Caudate putamen						
Dorsolateral	137.4±3.3	141.6±2.5	831.1±97.9	994±88.5	258.1±28.5	287.4±17.1
Dorsomedial	126.8±3.2	131.4±2.1	736.9±141.1	791.9±111.2	166.1±12.3	169.9±18.6
Ventral	137.4±3.4	133±2.6	824.8±91.1	1069.9±112.6	218.7±23.5	201.3±18.8
L2: Nucleus accumbens						
Core	117.7±4.9	122.4±3.7	539.6±87.4	699.7±79.8	122.5±15.1	109.3±12.2
Shell medial	103.4±5.9	117.2±2	517.6±70.6	885.7±95.9**	123.7±13.1	105.9±12.7
Shell ventral	101.9±8.6	116.1±2.2	521.7±125.6	903.1±109.7*	84.5±7.4	71.4±7.9
L2: Cingulate cortex	25.7±0.9	30.2±0.7**	19±2.8	22.4±3.3	2.4±1.6	0.4±1.4
L2: Motor cortex	26.7±0.7	29±1.1	10.4±2.3	10.1±1.7	3.8±2	0.7±1.3
L3: Caudate putamen						
Dorsomedial	130.2±3	143.2±2.5**	240.1±71.2	415.2±145.4	84±9.5	106.2±17.3
Dorsolateral	132.8±3.5	137±4.9	537.9±85.9	488.8±61.5	184.4±26.8	183.6±15.2
Ventral	149.2±2.1	152.6±3	875.1±94.7	1016.9±63.6	421.6±30.2	406.5±23.8
L3: Cingulate cortex	27.3±1.3	34.2±1.8*	5.1±1.6	6.1±1.7	4.5±1.5	1.3±1.3
L4: Amygdala						
Central amygdala	55.7±4.1	74.3±6.4*	27.4±8.3	33±15.7	178±16.9	131.7±15.3
Intercalated nucleus			139.2±29.5	175.6±25.9		
Basolateral amygdala	21.3±2	23.6±3.9	61.6±11.3	64.1±6.7	2.2±2.9	2.7±2
Lateral amygdala	24.7±1	25.3±2.6	27.4±4.4	41.5±5.7		
L4: Caudate putamen tail	136.2±3.9	137.8±1.6	319.3±41.7	541.8±67.8*	177.1±20.2	153.7±10.9
L4: Dentate gyrus	36.5±1.7	45±2.1**	14.7±2.4	16.5±2.9		
L4: Lateral hypothalamus			133.1±40	271.6±26.9*		
L5: Dentate gyrus	33±0.8	38.8±1.7**	10.5±2.1	10.5±2.1		
L5: CA1 (molecular layer)					30.8±1.8	34.9±3.4
L6: Dentate gyrus	32.2±2	38.1±1.8*	8.9±1.9	9.8±1.7		
L6: VTA			26.8±8.7	25.5±2.8	26.3±3.9	31.7±5
L6: SNR			615.4±87.2	592±78.3	13.5±2.8	9.2±2.1
L6: SNC					65.3±7.3	44.8±7.4

Results are expressed as mean optical density in the specific areas±SEM. L1–L6 refers to the brain levels as described in the Experimental Procedures. The statistical analysis was performed using Student's *t*-test.

* $P<0.05$.

** $P<0.01$.

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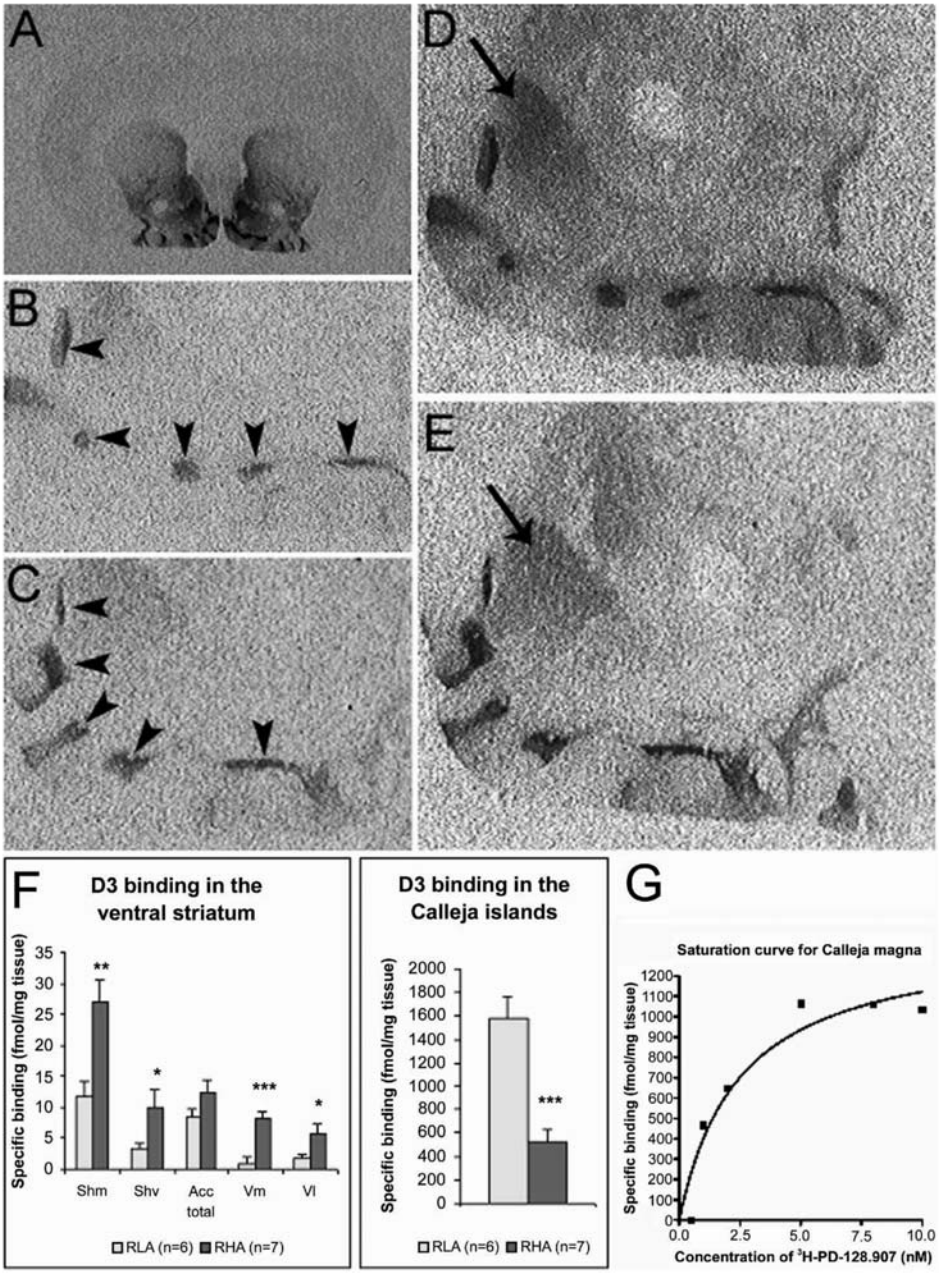

Fig. 3. D_3 binding in the Roman rats. In this figure, the labeling pattern of ^{3}[H]PD 128907 binding to D_3 receptors is depicted (A). The images obtained for the islands of Calleja after 9 weeks' exposure allow the visualization of the difference in optical density at this anatomical region between the RHA rats (B) and the RLA rats (C). The images obtained for the nucleus accumbens, after 9 months' exposure, allow the visualization of the difference in optical density at these anatomical regions between the RHA rats (D) and the RLA rats (E). With this long exposure, saturation of the optical density in the Calleja islands was observed. The specific binding (fmol protein/mg tissue) detected in the ventral striatal areas as well as in the Calleja islands of RLA and RHA rats is depicted in the bar graph (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) (F). Saturation binding experiment using graded concentrations of [^{3}H]PD 128907. The dissociation constant (K_D) estimated by non-linear regression is 2.5 nM (G).

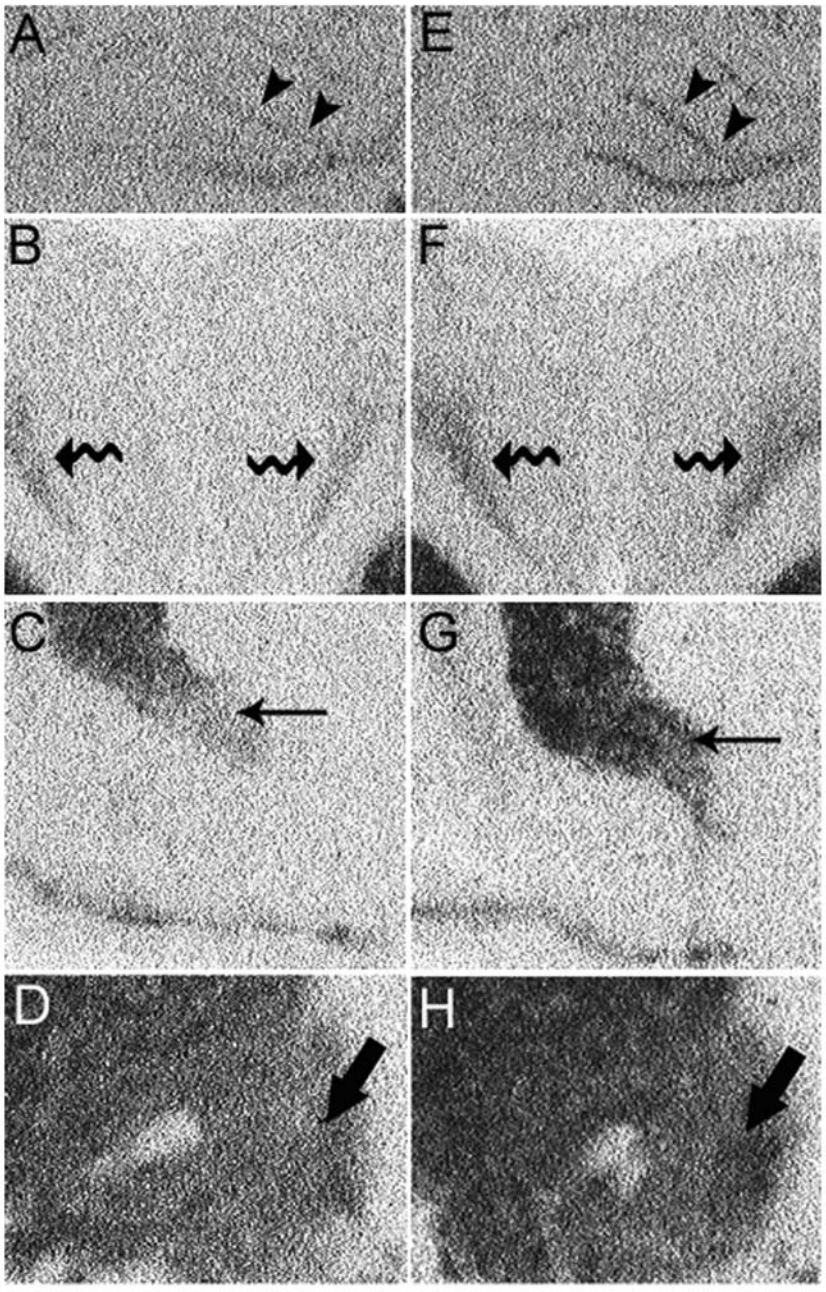

Fig. 4. DARPP-32 mRNA levels differences between the Roman rats. Several of the areas in which the two Roman strains differ in DARPP-32 mRNA expression levels are shown in this figure. Comparison of pictures representative of RHA rats (A–D) with those representative of RLA rats (E–H) shows that RHA rats express higher levels of DARPP-32 in the dentate gyrus (A and E), cingulate cortex (B and F) and the central nucleus of the amygdala (C and G). In the medial subdivision of the accumbens shell (D and H), it can be seen that in some of the animals, like the ones shown in this figure, RHA rats present higher DARPP-32 mRNA levels, although statistical analysis did not reveal a significant difference between strains.

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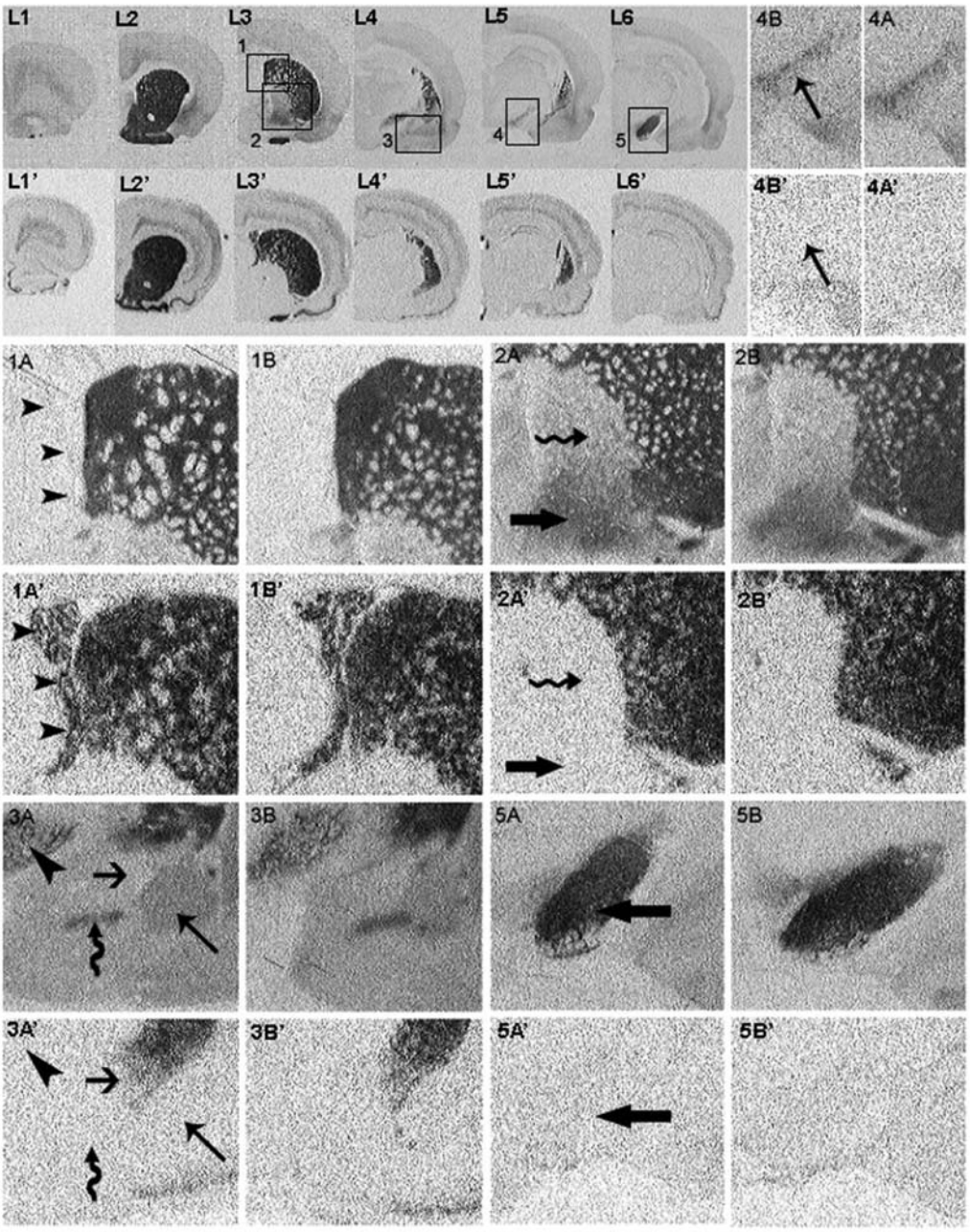

Fig. 5. Comparison of the distribution of D_1 receptor labeling and DARPP-32 mRNA expression in adjacent sections. The panels in the upper left quadrant demonstrate the levels utilized for comparison as discussed in the text, with D_1 receptor labeling with [^{3}H]SCH23390 in L1–L6 and DARPP-32 mRNA expression in L1′–L6′. The higher magnification photomicrographs of the regions demarcated with boxes (numbered 1–5) are shown in groups of four separate panels, labeled 1–5. Each pair of panels labeled A (for RLA strain) and B (for RHA strain) for D_1 receptor labeling corresponds

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D_3 receptor autoradiography

D_3 binding was detected at the ventral striatum including the nucleus accumbens and in the Calleja islands as previously reported (Stanwood et al., 1997; Bancroft et al., 1998), but in disagreement with a widespread striatal expression as described by Hillefors and collaborators (1999). The restriction to the ventral striatal areas fits with the mRNA expression pattern already described for D_3 receptors (Bouthenet et al., 1991).

As shown in Fig. 3, compared with RLA-I rats, RHA-I rats show 231% higher D_3 binding in the medial part of the nucleus accumbens shell ($t(11)=3.6$ $P=0.004$), 294% higher D_3 binding in the ventral part of the nucleus accumbens shell ($t(7.2)=2.4$ $P=0.049$), a 1046% higher D_3 binding in ventromedial subdivisions of the rostral striatum ($t(11)=4.2$ $P=0.001$), and a 315% higher D_3 binding in ventrolateral subdivisions of the rostral striatum ($t(11)=2.3$ $P=0.04$). When the total accumbens including the core was measured no difference was detected. Furthermore, RLA-I rats show much greater D_3 binding (303%) than RHA-I rats in the Calleja islands ($t(12)=4.82$ $P<0.001$). The saturation binding experiment using graded concentrations of [^{3}H]PD 128907 demonstrates that under our conditions the dissociation constant (K_D) for [^{3}H]PD 128907 is 2.5 nM, very close to the concentration used in the rest of the experiments.

DARPP-32 *in situ* hybridization

The anatomical distribution of DARPP-32 mRNA in both strains matches the pattern described in a previous publication (Schalling et al., 1990b).

As shown in Table 1, there were some areas (for some examples see Fig. 4) in which the two Roman strains differed in DARPP-32 mRNA expression. RHA-I rats showed higher expression than RLA-I rats in prelimbic cortex ($t(12)=2.86$ $P=0.015$), in the rostral cingulate cortex ($t(12)=3.77$ $P=0.003$) as well as in the caudal cingulate cortex ($t(12)=3.06$ $P=0.01$) and in all measured anatomical levels of the dentate gyrus, rostral ($t(11)=2.98$ $P=0.013$), dorsal ($t(8.7)=3.16$ $P=0.012$), and ventral areas ($t(12)=2.2$ $P=0.048$). RHA-I rats also showed higher levels of DARPP-32 than RLA-I rats in the dorsomedial portion of the caudal striatum ($t(12)=3.33$ $P=0.006$) and the central nucleus of the amygdala ($t(11)=2.37$ $P=0.037$). Different mRNA expression levels were found in the nucleus accumbens shell albeit they did not reach statistical significance ($t(7.3)=2.2$ $P=0.06$) due to the existence of great individual variability in RLA-I rats at that level.

Comparison of D_1 receptor and DARPP-32 mRNA expression

As it can be observed in Fig. 5, the comparison of the distribution obtained for D_1 receptors and for DARPP-32 mRNA respectively, shows many similarities, especially regarding striatal and prefrontal cortical areas. However, some mismatches in their expression patterns can be observed: 1) in the globus pallidus (L3, panel 2), the substantia nigra reticulate (L6, panel 5), the subthalamic nucleus (L5, panel 4) and the interstitial nucleus of the posterior limb of the anterior commissure (L3, panel 4) D_1 receptors were detected while DARPP-32 mRNA was not; 2) in the central amygdala (L4, panel 3), DARPP-32 mRNA was moderately to highly expressed while only slight D_1 receptor binding was detected; 3) in the basolateral complex of the amygdala (L4, panel 3) D_1 binding was detected while DARPP-32 mRNA was not, and that was prominent for the intercalated amygdaloid nuclei (L4, panel 3) where D_1 binding was quite intense; 4) in the CA1 field of the dorsal hippocampus D_1 binding was detected in the molecular layer while weak DARPP-32 mRNA was detected in the pyramidal layer in the CA1 and CA3 fields; 5) in the choroid plexus of the ventricles (L3, panel 1), a strong signal for DARPP-32 mRNA was detected while no D_1 binding could be seen; 6) in the area of the lateral hypothalamus (L3, panel 3) through which the medial forebrain bundle runs, D_1 binding was detected whereas no DARPP-32 mRNA signal could be seen. In RLA rats there was a correlation between the levels of DARPP-32 mRNA and D_1 receptors in the nucleus accumbens (shell medial $r=0.93$, $P=0.002$; shell ventral $r=0.88$, $P=0.01$). No correlation was found in RHA rats, which showed minimal interindividual differences in DARPP-32 mRNA.

DISCUSSION

The present experiments give a full picture of the difference between the RHA and RLA lines/strains with regard to the dopamine receptors subtypes and the intracellular signal transducer DARPP-32 in intact brain tissue. They extend previous analysis of D_1 and D_2 receptors in homogenates, and include D_3 receptors and DARPP-32 mRNA which have not been studied previously in these animals.

Quantification of dopamine receptor subtypes in the Roman strains

Inbred RHA-I rats showed higher binding of D_1 in the nucleus accumbens shell than inbred RLA-I rats, while no difference was found between the Roman strains in D_2 binding. These results are in accordance with the previous

to the panels A′ (for RLA strain) and B′ (for RHA strain) for DARPP-32 mRNA expression. (1A, 1A′) Arrowheads point to the ventricle where D_1 receptor labeling is absent but DARPP-32 mRNA labeling can be detected. In contrast the adjacent dorsal–medial caudate-putamen shows both D_1 receptor labeling and DARPP-32 mRNA labeling. (2A, 2A′) A wavy arrow points to the globus pallidus and the thick arrow to the IPAC (interstitial nucleus of the posterior limb of the anterior commissure), wherein D_1 receptor binding (2A, 2B) is present but DARPP-32 labeling absent in the adjacent section (2A′, 2B′). (3A, 3A′) A narrow filled arrow points to the central amygdala, a short arrow to the basolateral amygdala, a wavy arrow to the intercalated nucleus, and an arrowhead to the lateral hypothalamus (with medial forebrain bundle). (4A, 4A′) Arrow points to the subthalamic nucleus. (5A, 5A′) The thick arrow points to the substantia nigra reticulata.

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study of homogenates from a different set of RHA and RLA animals from an outbreeding program (Corda et al., 1997). The inbred Roman strains exhibit the same behavioral patterns as the outbred lines in behavioral tests which reflect the activity of the dopaminergic system, for example the shuttle box, the open field and other activity measures (Driscoll et al., 1998; Escorihuela et al., 1999). They also show similar behavioral responses to the direct dopamine agonist apomorphine (Giménez-Llort et al., 2005). We interpret the difference in D_1 binding in the accumbens shell to possibly underlie the behavioral differences linked to higher dopaminergic function in the RHA line/strain that is observed in a wide range of experimental situations (see introduction).

In a previous report, we suggested that the extreme difference in behavioral inhibition achieved after administration of low doses of apomorphine (RLA-I showing much greater locomotor inhibition and enhanced yawning behavior) could be due to differences in D_3 receptor function (Giménez-Llort et al., 2005). Pharmacological studies with selective D_3 agonists and antagonists have suggested that D_3 stimulation has inhibitory effects on locomotion (Richtand et al., 2001). Indeed, RLA-I rats showed much higher D_3 binding in the Calleja islands (threefold higher). However, RHA-I rats showed a much higher D_3 binding than RLA-I rats in the medial (twofold higher) and ventral (threefold higher) accumbens shell and in the ventrolateral (threefold higher) and the ventromedial (10-fold higher) parts of the striatum. The fact that RHA-I rats show higher D_3 binding in the ventral striatum than RLA-I rats does not fit with the fact that deleting D_3 receptors causes an increase in locomotor activity when D_3 knock-out mice are placed in a novel environment (Accili et al., 1996; Xu et al., 1997). In the Calleja islands as well as in the nucleus accumbens, the D_3 receptor and the D_1 receptors are coexpressed (Schwartz et al., 1998).

There is some evidence that D_3-mediated effects differ between regions, since D_3 and D_1 stimulation have similar cellular effects in the nucleus accumbens while they have opposing cellular effect in the Calleja islands (Ridray et al., 1998). Sensitization to levodopa in unilaterally dopamine-denervated striatum is mediated by D_3 overexpression in the striatum including the accumbens shell (Bordet et al., 1997), and manipulations that impair D_3 up-regulation at this level blocked the behavioral sensitization to levodopa (Guillin et al., 2001). In contrast, D_3 binding in homogenates including the accumbens, the olfactory tubercle and the Calleja islands was down-regulated in amphetamine-sensitized rats, another sensitization model (Chiang et al., 2003). The evidence for neuroanatomical differences between the Roman strains and the possible opposite role for D_3 receptors depending on the location may integrate the aforementioned published data and the present findings. In the accumbens shell, stimulation of D_3 and D_1 receptors would synergize and elicit behaviors (Karasinska et al., 2005), whereas D_3 stimulation of the Calleja islands would have an opposite, inhibitory effect. Then, dopamine modulation over spiny neurons in the accumbens shell would be stronger in RHA-I rats as compared with RLA-I, as observed in behavioral paradigms. The Calleja islands may be relevant in the neuronal mechanism underlying apomorphine-induced locomotor inhibition and yawning behavior, and dopamine would induce a stronger activation of D_3 receptors in the Calleja islands in RLA-I as compared with RHA-I rats.

Quantification of DARPP-32 mRNA expression in the Roman strains

The DARPP-32 gene expression differed between the two Roman strains, RHA-I rats showing greater gene expression than RLA-I rats in the prelimbic cortex, the cingulate cortex and the dentate gyrus. RHA-I rats also showed higher DARPP-32 mRNA expression than RLA-I rats in the dorsomedial subdivision of the caudal striatum. Our methodology, quantifying DARPP-32 mRNA using *in situ* hybridization, naturally leaves out important posttranslational regulation of DARPP-32 activity. It seems likely that this protein works in the cortex as a signal transducer as described for the striatum (summarized in the introduction), and that strain differences in expression which are always in the same direction and mainly restricted to limbic areas may have a functional significance. In order to perform a reliable interpretation of the functional implication of the present findings, it would be necessary to address the phosphorylation state of DARPP-32 in limbic areas (Svenningsson et al., 2004).

Anatomical distribution of dopamine receptors and DARPP-32 mRNA expression

The details of the amygdalar D_1 and D_2 binding patterns are of interest, since the central nucleus is one of the few locations of DARPP-32 mRNA labeling in the amygdala in which also immunostaining has been described (Ouimet et al., 1984). The anatomical distribution of D_1 and D_2 receptors in the Roman rats fits that previously described in rat brain except for the amygdala. Moderate levels of D_1 binding were reported in the central amygdala and only slight D_2 binding was reported in the medial amygdala (Mansour et al., 1990). Here, using the same radioligands as Mansour et al. (1990, 1991), D_1 binding in the central amygdala could barely be detected and D_2 binding was weak but detectable. The reason for this discrepancy in D_2 binding is unknown, since Mansour et al. did not show illustrations and their nomenclature is simplified regarding the amygdala nuclei. Comparison with Cresyl Violet and acetylcholinesterase staining in the same or adjacent sections confirmed the conclusion that D_1 binding is moderate in the basolateral amygdala but very low in the central nucleus. In the Roman rat strains, DARPP-32 in the central amygdala seems more involved in D_2 than in D_1 signaling pathways. The presence of substantial D_1 ligand binding in the basolateral amygdala but not in the central amygdala fits with the suggested role of the basolateral amygdala in the assignment of incentive value to drug-paired stimuli (Fuchs et al., 2002) and the blockade of cue-induced drug seeking by D_1 antagonists in the basolateral amygdala (see See et al., 2003). However, any D_1-mediated effects here do not seem to involve DARPP-32 (this study).

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In the basolateral complex and in the intercalated nucleus the present findings fit with those previously reported. D_1 signaling is achieved through a molecular pathway independent of DARPP-32 phosphorylation since these areas do not express DARPP-32 mRNA. A lack of DARPP-32 immunostaining at these locations has also been previously described (Ouimet et al., 1984). Dopaminergic activity in the intercalated nucleus has recently been related to the generation of anxiety behaviors in the dark–light box (Perez de la Mora et al., 2005). However, the two Roman strains do not differ in D_1 binding at this site.

The globus pallidus and the substantia nigra reticulata are other areas in which a mismatch between DARPP-32 mRNA expression and D_1 binding was detected. In these areas no D_1 mRNA is expressed (Mansour et al., 1991) and DARPP-32 immunostaining is detected in fibers but not in neuronal bodies (Ouimet et al., 1984). Therefore, it can be concluded that these markers are expressed by the terminals of striatal projecting neurons to the globus pallidus and the substantia nigra reticulata and no real mismatch between DARPP-32 and D_1 is observed. The same reasoning can be applied to the apparent mismatch observed in the molecular layer of CA1 where D_1 binding can be detected but DARPP-32 mRNA is not expressed (Mansour et al., 1991).

CONCLUSION

On balance, the present results and those of Corda et al. (1997) indicate a stronger responsiveness to dopamine in RHA than RLA lines/strains. RHA lines/strain with higher dopamine release maintain equal or show higher numbers of dopamine receptors and DARPP-32 mRNA. The elevated levels of postsynaptic markers of the dopamine system in RHA rats give some support to the proposed relevance of an active dopamine system to the behavioral phenotype of RHA line/strain (d'Angio et al., 1988; Driscoll et al., 1998; Giorgi et al., 2003). In the nucleus accumbens shell, RHA-I rats also show higher expression of the dynorphin gene transcript linked to the dopamine system (Guitart-Masip et al., 2006) as well as D_1 and D_3 binding and possibly DARPP-32 mRNA expression. Therefore, dopaminergic activity in the shell of the nucleus accumbens of RHA line/strain appears higher at the level of transmitter release, receptors and intracellular signaling pathways, supporting a role of accumbal dopamine in vulnerability to drug addiction.

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Paper III

D_3 RECEPTOR STIMULATION IN THE CALLEJA ISLANDS MAY MEDIATE LOCOMOTOR INHIBITION

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Abstract (201 words)

Dopamine D_3 receptor expression is restricted to the limbic brain areas and it is supposed to have a relevant role in the development of addiction and other psychiatric disorders such as schizophrenia. The inbred Roman high- (RHA-I) and low-avoidance (RLA-I) rats, differing in dopaminergic activity and novelty/substance-seeking profiles, also differ in the binding levels of D_3 receptors: RHA-I rats show higher D_3 binding in the accumbens shell whereas RLA-I rats show higher D_3 binding in the Calleja islands. We hypothesized that D_3 receptor activation located in the Calleja islands have an inhibitory effect on locomotor activity. To test this hypothesis we administered saline and PD-128,907 (0,01 and 0,1 mg/Kg), a putative D_3 receptor agonist, to the Roman rats and studied the locomotor activity when animals were placed in a novel environment. We found that RLA-I rats showed stronger locomotor inhibition than RHA-I rats after PD-128,907 administration. The study of the levels of NGFI-A mRNA in the striatum and the Calleja islands of these animals by means of *in situ* hybridization revealed that RLA-I rats showed stronger reduction of NGFI-A mRNA in the Calleja islands than RHA-I rats. These results suggest that D_3 receptor activation in the Calleja islands induces locomotor inhibition.

Key words: D_3 receptors, Calleja islands, striatum, locomotion, PD-128,907, NGFI-A

Introduction

The D_3 dopamine receptor was cloned by Sokoloff et al. (1990) and thereafter much effort has been made in the study of its physiological function. The expression pattern of both D_3 mRNA and D_3 receptor protein is restricted to the limbic areas with high expression in the Calleja islands, moderate to high expression in the shell of the nucleus accumbens, and lower expression in the mesencephalic dopaminergic areas as well as in the amygdala (Bouthenet et al., 1991;Diaz et al., 1995; Diaz et al., 2000; Le Foll et al., 2005). Due to its anatomical distribution, the D_3 receptor subtype has received considerable attention of researchers in the field of psychosis and drug addiction.

However, the lack of well characterized agonists with high selectivity towards D_3 has made progress understanding of the physiological role of D_3 receptors difficult. Many conflicting data have been reported that may lead to the conclusion that the locomotor effect observed after administration of putative D_3 agonists is not due to D_3 but D_2 stimulation (Heidbreder et al., 2005). Nevertheless, it has been shown that administration of putative D_3 selective agonists decrease locomotion through D_3 stimulation when animals are tested in a novel environement (Pritchard et al., 2003). Experiments performed in two separate laboratories have shown that D_3 receptor knockdown through antisense oligonucleotide administration increases spontaneous locomotor activity (Ekman et al., 1998; Menalled et al., 1999). Studies using sensitization paradigms also support an inhibitory role for D_3 receptors. Thus, D_3 binding in homogenates including the nucleus accumbens, the olfactory tubercle and the Calleja islands was down-regulated in amphetamine sensitized rats (Chiang et al., 2003), and amphetamine sensitized rats are less sensitive to the inhibitory effect of putative D_3 selective agonists (Richtand et al., 2003). In contrast, overexpression of D_3 receptors in the striatum, including the accumbens shell, is necessary for sensitization to levodopa in unilaterally dopamine-denervated striatum (Guillin et al., 2001).

The Swiss sublines of Roman high-avoidance (RHA/Verh) and low-avoidance (RLA/Verh) rats were psychogenetically selected for rapid (RHA/Verh) vs. very poor (RLA/Verh) two-way avoidance acquisition in the shuttle box (Driscoll and Bättig, 1982). We recently showed that RHA-I rats show higher D_3 binding in the shell of the nucleus accumbens than RLA-I rats, whereas

rats of the latter strain show higher D_3 binding in the Calleja Islands than RHA rats (Guitart-Masip et al., 2006b). These results suggest that D_3 receptor stimulation may have opposite consequences depending on the neuroanatomical location and that D_3 stimulation of the Calleja islands may underlie the inhibitory effects observed after administration of D_3 agonists. To address this hypothesis we tested the effect of low doses of PD-128907 on locomotor activity in a novel environment in the two Roman rat strains. We thereafter quantified, by means of *in situ* hybridization, the expression levels of NGFI-A mRNA in the striatum and Calleja islands in the brains of the same animals that were behaviorally assessed. NGFI-A transcription may be induced by CREB activation (Knapska and Kaczmarek, 2004) and therefore it is a suitable gene to study changes in cellular activity induced stimulation of dopamine receptors.

Material and Methods

Animals

Male inbred RHA-I (RHA) and RLA-I (RLA) rats, 65-75 days old, were used. The animals were bred in the animal facilities at the Medical Psychology Unit (UAB) and maintained two per cage (Macrolon, 22 x 47 x 15 cm), under standard laboratory conditions (food and water *ad libitum*, 22 ± 2ºC and 12L:12D cycles beginning at 08:00h). Since 1993, an inbreeding program (brother-sister mating) has been carried out in parallel to that of the outbred RHA/Verh and RLA/Verh rat lines, with the inbred strains (see Escorihuela et al., 1999) being presently maintained at the animal department facilities of the Medical Psychology Unit in Barcelona. Two days before the experimental procedure begun, the animals were habituated to the handling procedures that are required for drug injections. The research was conducted in accordance with guidelines and protocols approved by the European Economic Community (86/609/EEC Council) regarding the Care and Use of animals for experimental procedures and by the Ethics Commission of the Autonomous University of Barcelona.

Administration of PD-128,907 and locomotor activity test

PD-128,907 (Sigma, St Louis, USA), a 14-18x selective D_3 receptor agonist (Pugsley et al., 1995), was dissolved in 0.9% saline and injected s.c. in a volume of 1 ml/kg. Eight animals of each strain were randomly assigned to one of the treatment groups: vehicle, 0.01 mg/Kg PD-128,907 or 0.1 mg/Kg PD-128,907. Animals were weighed and carefully moved to the test room where they were immediately injected with the respective treatment dose and placed in the motor test box for 1 hour. Motor activity was determined by means of light-beam breaks (PANLAB, Barcelona) from batches of 4 animals placed individually in polyglass motor activity test cages (dimensions: 40 x 40 x 40 cm) located in a white light room with a background noise. All experiments were done between 10:00 and 14:30 h to reduce the possible influence of diurnal variation in activity. After this session, animals were sacrificed by decapitation and their brains dissected and frozen through contact with dry ice. Samples were kept at -80ºC and thereafter sent to the Swedish laboratory.

Sections for in situ *hybridization histochemistry*

The brains from the animals of the behavioral study were used. Coronal sections (14 µm-thick) were cut in a cryostat as described previously (Guitart-Masip et al., 2006a). For the identification of the different brain structures, adjacent sections to those used for *in situ* hybridization were stained with cresyl violet (Johansson et al., 1994). Equivalent sections for all brains were collected at 5 different levels, which allowed mapping of different brain areas along the rostrocaudal axis. The section levels were: level 1.- bregma: 2.2 – 1.7 (striatal anterior pole); level 2.- bregma 1.2 – 1.4 (NAc, rostral caudate putamen, rostral cingulate cortex); level 3.- bregma -0.8 – -0.92 (caudal caudate putamen, caudal cingulate cortex); level 4.- bregma -2.3 – -2.56 (rostral hippocampus and amygdala); level 6.- bregma -4.8 – -5.2 (ventral hippocampus, ventral tegmental area (VTA) and substantia nigra reticulata) according to (Paxinos and Watson, 1998) atlas.

Oligodeoxyribonucleotide probes

The NGFI-A probe (complementary to nucleotides 4-49) was synthesized and purified through high-performance liquid chromatography (Thermo Electron GmbH, Ulm, Germany). This sequence, ^{35}S-labeled, has already been used in prior *in situ* studies (Kuzmin and Johansson, 1999).The specificity was checked by the addition of a 225x excess of unlabelled probe, which blocked the signal, whereas the signal was not influenced by a 225x excess of an unrelated oligonucleotide (data not shown).

In situ *hybridization histochemistry*

The analysis of mRNA levels was carried out by *in situ* hybridization as described elsewhere (Guitart-Masip et al., 2006a). Briefly, oligonucleotide probes were 3´- end labeled with [^{33}P]-dATP, the slide-mounted sections then incubated for 16-20 hours at 42°C with the labeled oligonucleotide probe. Following hybridization, the sections were washed, air-dried and apposed to Kodak Biomax MR (Amersham) film for 5 days. Finally, autoradiograms were analyzed with a Macintosh computer using the public domain NIH Image program (US National Institutes of Health; see http://rsb.info.nih.gov/nih-image). In the striatum, we measured every striatal area which has a differentiated cortical afferent projection using the striatal sampling areas introduced by Willuhn et al., (2003).

Statistics

Behavioral study: Locomotor activity is expressed in cm, mean ± SEM, at 10-minute intervals and during the whole session. A repeated measures ANOVA with strain (RLA and RHA) and treatment dose (vehicle; 0,01 mg/Kg PD-128,907; 0,1 mg/Kg PD-128,907) as main factors and 10-minute intervals as repeated factor was used. When in repeated measures ANOVA, Mauchly sphericity was not reached, Huynh-Feldt correction was used. As a *post hoc* analysis, a transversal Duncan test comparing all 6 groups of strain and treatment was used at each interval or for the whole session.
In situ hybridization histochemistry: Two-way ANOVA with strain (RLA and RHA) and treatment doses (vehicle; 0,01 mg/Kg PD-128,907; 0,1 mg/Kg PD-128,907) as main factors was performed. When appropriate, Duncan test comparing all 6 groups of strain and treatment was used.
Correlations between locomotor activity and NGFI-A mRNA levels in the Calleja magna were analyzed with the Pearson test.

Results

Locomotor response to PD-128,907 administration

Figure 1 shows the time courses of locomotor activity after administration of different doses of the putative D_3 selective agonist PD-128,907 in the RLA and RHA rats, respectively. The two way ANOVA with repeated measures shows that both strains of rats undergo a decrease in locomotor activity during the session [time effect F(4.4, 185.5)=147.5; $P<0.001$] but with different time course [time x strain effect (F=4.4, 185.5)=5.7; $P<0.001$]. The effect of PD-128,907 administration on locomotor activity differed between the two Roman strains [time x treatment effect F=(8.8; 185.5)=7.8, $P<0.001$, as well as time x strain x treatment effect F(8.8, 185.5)=2.4; $P=0.013$]. The Duncan test comparing all 6 groups at each 10-minute interval shows that during the first interval saline treated RLA rats performed less locomotor activity (2767.2 ± 129.4 cm) than saline treated RHA rats (3563.8 ± 298.3 cm). RLA rats treated with 0.01 mg/Kg of PD-128,907 developed less locomotor activity than vehicle treated RLA rats during the first 30 minutes of the test, whereas RLA rats treated with 0.1 mg/Kg PD-128,907 developed less locomotor activity than the saline treated RLA rats during the whole session. On the other hand, RHA rats treated with the low dose of PD-128,907 did not differ in their locomotor activity from saline treated RHA rats at any interval, whereas RHA rats treated with 0.1 mg/Kg of PD-128,907 developed less locomotor activity than saline treated RHA rats during the first, second, third and fifth intervals.

When the total amount of locomotor activity performed by animals during the whole session was considered (see figure 2), the statistical analysis revealed that the two Roman strains differed in the total amount of locomotor activity performed during the test [strain effect F(1,42)=34.8; $P<0.001$] and that there is a differential effect of the different doses of PD-128,907 [treatment effect F(2,42)=34.9; $P<0.001$]. The Duncan test reveals that saline treated RHA rats developed more locomotor activity than saline treated RLA rats. Moreover, both doses of PD-128,907 were able to decrease locomotor activity in RLA rats when compared to saline treated RLA rats, whereas in RHA rats only the high dose of PD-128,907 was able to decrease locomotor activity when compared to saline treated RHA rats.

NGFI-A mRNA in situ *hybridization*

The results of the NGFI-A *in situ* hybridization are shown in table 1 and figures 3-5. The two-way ANOVA analysis revealed a treatment effect in several striatal subdivisions (the rostral pole of the nucleus accumbens [F(2,47)=4.6; $P=0.016$]; the dorsal subdivision of the rostromedial striatum [F(2,47)=6.7; $P=0.003$], the dorsomedial subdivision of the rostromedial striatum [F(2,47)=3.9; $P=0.036$]; the ventral subdivision of the rostromedial striatum [F(2,47)=3.9; $P=0.036$]; the medial subdivision of the caudal striatum [F(2,47)=4.3; $P=0.021$] and the dorsolateral subdivision of the caudal striatum [F91,47)=4; $P=0.025$]). A treatment effect was also detected in the islands of Calleja [F(2,47)=6.5; $P=0.003$], the motor cortex [F(2,44)=6.6; $P=0.003$] and the sensorimotor cortex [F(2,47)=3.3; $P=0.047$]. As revealed by the Duncan test, the administration of 0.1 mg/Kg of PD-128,907 was able to suppress the expression of NGFI-A mRNA as compared with the saline treated animals in the dorsal subdivision of the rostromedial striatum, the medial subdivision of the caudal striatum and, importantly, in the island of Calleja magna only in RLA rats, whereas in RHA rats, the same dose was able to suppress NGFI-A mRNA expression in the dorsomedial subdivision of the rostromedial striatum as compared with the respective saline-treated group. The locomotor activity performed during the test was positivelly correlated with the expression levels of NGFI-A mRNA in the Calleja magna in RLA rats ($r = 0.52$; $P= 0.009$) as well as in RHA rats ($r = 0.64$; $P= 0.001$) (see figure 6).

Administration of 0.1 mg/Kg of PD-128,907 was also able to suppress NGFI-A mRNA expression in the rostral accumbens shell in RLA rats as well as in the motor cortex both in RLA and RHA rats when compared to their respective 0.01 mg/Kg PD-128,907-treated group. In all these areas, administration of 0.01 mg/Kg of PD-128,907 increased the expression of NGFI-A mRNA compared to the saline-treated group, an effect which was only statistically significant in the motor cortex of RLA rats.

Discussion

The present results show that PD-128,907 achieves stronger locomotor inhibition effects in RLA-I rats than in RHA-I rats, the former strain showing higher D_3 receptor binding in the Calleja Islands than the latter (Guitart-Masip et al., 2006b). Moreover, after PD-128,907 administration, RLA-I rats show stronger suppression of NGFI-A in the Calleja islands than RHA-I rats. These results lend support to the hypothesis that locomotor inhibition observed after administration of a putative D_3 receptor selective agonist such as PD-128,907 is achieved through D_3 activation of the Calleja islands.

D_3 receptor activation inhibits locomotor activity in a novel environment

Experiments performed in the last decade suggest that postsynaptic D_3 receptors may play an inhibitory role when the dopaminergic system is lightly activated as it happens after placement in a novel environment. Down-regulation of D_3 receptors with antisense oligonucleotide causes increased locomotor activity when animals are placed in a novel environment (Ekman et al., 1998;Menalled et al., 1999). Although wild type and D_3 knock out mice show comparable locomotor activity when they are tested after habituation to the test cage (Xu et al., 1999; Boulay et al., 1999), when D_3 knock out mice are tested in a novel situation, they show increased locomotor activity (Accili et al., 1996; Xu et al., 1997), increased response to a low dose of cocaine (Xu et al., 1997), and PD-128,907 fails to induce locomotor inhibition in these mice (Pritchard et al., 2003). These differences between wild type and D_3 knock out mice are not secondary to changes in the presynaptic function (Koeltzow et al., 1998).

The results obtained in the present study also support the view that D_3 receptors play a role in controlling locomotor activity under circumstances that induce mild stimulation of the mesolimbic dopaminergic system. We have recently demonstrated between strains anatomical differences in D_3 receptor binding with increased levels in the Calleja islands and decreased levels in the accumbens shell in RLA-I rats when compared to RHA-I rats (Guitart-Masip et al., 2006b). Saline treated RLA-I rats show lower locomotor activity than saline treated RHA-I rats during the first 10 minutes after being placed in the test cage. Previous experiments performed with the inbred Roman strains also showed higher novelty-induced locomotor activity in the RHA-I rats when compared to RLA-I rats only during the first minutes after animals were placed in the test cage (Giménez-Llort et al., 2005). RHA rats also show higher levels of exploratory behavior in tests of novelty seeking than RLA rats (Escorihuela et al., 1999;Fernández-Teruel et al., 1997; Fernández-Teruel et al., 2002; Guitart-Masip et al., 2006a). It is known that despite a lack of difference in basal dopamine levels between the Roman lines (Lecca et al., 2004), administration of cocaine, amphetamine or morphine induce higher dopamine release in the shell of the nucleus accumbens in RHA rats than in RLA rats (Giorgi et al., 1997; Lecca et al., 2004), but the response to novelty has not been studied yet. Although RHA-I rats show higher D_3 binding in the shell of the nucleus accumbens, they resemble the D_3 knock-out mice when placed in a novel environment (Accili et al., 1996;Xu et al., 1997). Therefore, the higher behavioral activation induced by novelty in RHA-I rats may be due to the lower levels of D_3 binding in the Calleja Islands when compared to RLA-I rats.

The locomotor inhibition pattern obtained in the present experiment after PD-128,907 in the Roman strains also suggests that D_3 receptor activation induces locomotor inhibition by activation of those D_3 receptors located in the Calleja Islands. PD-128,907 shows a 14-18 fold selectivity for D_3 *versus* D_2 receptors *in vitro* (Pugsley et al., 1995), its selectivity *in vivo* being dose-dependent with higher selectivity at lower doses (as reviewed by Heidbreder et al., 2005), and its inhibitory effect in animals not habituated to the test cage is not observed in D_3 receptor stimulation knock-out mice (Pritchard et al., 2003). PD-128-907 administration in rats has a biphasic effect on locomotor activity with inhibition at low doses (< 1mg/Kg) and activation at doses higher than 10 mg/Kg (Pugsley et al., 1995). Although PD-128,907 decreases dopamine synthesis and release, the inhibitory effect on locomotor activity may already be seen at a dose of 0.03 mg/Kg without any effect on presynaptic dopamine synthesis in rats (Pugsley et al., 1995). RLA-I rats, showing higher D_3 binding in the Calleja islands, are more sensitive to PD-128,907 administration than RHA-I rats: they show inhibition of spontaneous locomotor response after administration of the lower dose (0.01 mg/Kg, a dose without effect on presynaptic function in rats, see above) which was ineffective in lowering spontaneous locomotor activity in RHA-I rats. Moreover, RLA-I rats also showed stronger inhibition of spontaneous locomotor activity than RHA-I when challenged with the higher dose. Finally, the fact that in both strains there is a positive correlation between locomotor activity and an index of neuronal activity like NGFIA-A mRNA in the Calleja magna strengthen the view that pharmacological inhibition of the neurons expressing NGFI-A in the Calleja magna results in locomotor inhibition.

D_3 receptors in the Calleja islands may underlie part of the inhibitory effect of PD-128,907

One hour after being placed in a novel environment, saline-pretreated animals show high NGFI-A in the Calleja islands. After administration of the low dose of PD-128,907, NGFI-A mRNA was only suppressed in the Calleja islands of RLA-I rats, although the reduction was not statistically significant. The suppression in NGFI-A mRNA seen in the Calleja islands after administration of the higher dose of PD-128,907 was only significant in the

RLA-I rats, although at this dose NGFI-A in the Calleja islands of RHA-I rats also show a trend towards reduction. In a previous study, stimulation of D_3 receptor also suppressed *c-fos* mRNA (Ridray et al., 1998). The gradient of suppression of NGFI-A mRNA in the Calleja islands after administration of PD-128,907 resembles the gradient of locomotor inhibition in the same groups (compare figure 2 and 3), and strongly suggests that the behavioral inhibition is achieved, at least in part, through activation of D_3 receptors in the Calleja islands. These results also suggest that D_3 receptor activation in the Calleja islands may underlie the difference in sensitivity to PD-128,907 administration between the Roman rats, and at least part of the inhibition achieved after PD-128,907 administration. More research is needed to clearly understand how activation of D_3 receptors in the Calleja islands modulates the locomotor response to novelty.

We also observed suppression of NGFI-A mRNA in some subdivisions of the dorsal striatum only in animals treated with the high dose of PD-128,907: the dorsal subdivision of the rostral striatum and the medial subdivision of the caudal striatum in RLA-I rats and the dorsomedial subdivision of the rostral striatum in RHA-I rats. This suppression in NGFI-A was not widespread in the whole dorsal striatum and it was found in both strains to a similar extent despite the differences in locomotor inhibition already described. In the dorsal striatum D_3 receptors are not expressed (Bouthenet et al., 1991; Bancroft et al., 1998). Although suppression of NGFI-A in the dorsal striatum may be related to the locomotor inhibition induced by the higher dose of PD-128,907, it is unlikely that this effect is mediated by D_3 receptor stimulation. As discussed above, PD-128,907 is a mixed D_2/D_3 agonist whose *in vivo* selectivity for D_3 decreases as the concentration increases. Administration of a dose of 0,1 mg/Kg PD-128,907 decrease dopamine presynaptic function in the ventral and dorsal striatum (Pugsley et al., 1995). However, SB-277011-A, a highly selective D_3 receptor antagonist, dose-dependently reversed the decrease in dopamine release induced by quinelorane, a mixed $D_2/D_3/D_4$ receptor agonist, in the nucleus accumbens but not in the dorsal striatum (Reavill et al., 2000). Altogether, these evidences suggest that when the doses of PD-128,907 increase and it looses selectivity towards D_3 receptors the dorsal striatum may become more involved in the inhibitory effect of PD-128,907 due to binding to presynaptic D_2 receptors.

Implications of D3 receptors in the divergent novelty-seeking profile of the Roman lines/strains

The Swiss sublines of Roman high-avoidance (RHA/Verh) and low-avoidance (RLA/Verh) rats were psychogenetically selected for rapid vs. extremely poor two-way avoidance acquisition in the shuttle box respectively (Driscoll and Bättig, 1982). An important body of concordant behavioral and neurobiological evidence indicates that these rat lines/strains are a valid laboratory model of divergent sensation/novelty and substance-seeking profiles rooted in differences on dopaminergic function (Reviewed by (Driscoll et al., 1998). These characteristics suggested that the Roman rats could be a suitable model to study the implication of the dopaminergic system in vulnerability to drug addiction. As shown in the present study, the higher novelty induced locomotor activity observed in the RHA lines/strains may be due to lower D_3 receptor activity in the Calleja islands. On the other hand, RHA rats are more prone to show behavioral sensitization than RLA rats after chronic treatments with morphine (Piras et al., 2003), cocaine (Giorgi et al., 2005b) and amphetamine (Corda et al., 2005). Moreover, only in RHA rats sensitized to amphetamine, changes in the dopaminergic outflow are observed (Giorgi et al., 2005a). The role of D_3 receptors in behavioral sensitization may be functionally dissociated between different D_3 receptor populations. Amphetamine pretreatment induced a down-regulation of D_3 binding in homogenates that included the nucleus accumbens, the olfactory tubercle and the Calleja islands (Chiang et al., 2003), and a decreased behavioral response to putative D_3 selective agonists (Richtand et al., 2003). In contrast, overexpression of D_3 receptors in the striatum, including the accumbens shell, is necessary for sensitization to levodopa in unilaterally dopamine-denervated striatum (Guillin et al., 2001). Behavioural sensitization could be related to up-regulation of D_3 receptors in the accumbens and downregulation in the Calleja islands. In this sense, naïve Roman rats would already shown a difference in this sense. The novelty-seeking RHA rats would show a sensitized-like D_3 receptor system whereas RLA rats showing lack of sensitization after chronic treatments with several drugs of abuse would show higher levels of D_3 receptors in the Calleja islands that would dampen the impact of these drugs on the dopaminergic system.

Concluding remarks

The present results show that RLA-I rats expressing higher D_3 receptors in the Calleja islands have less locomotor activity induced by novelty and stronger locomotor inhibition after PD-128,907 administration when compared to RHA-I rats. Moreover, after PD-128,907 administration, RLA-I rats show stronger suppression of NGFI-A in the Calleja islands than RHA-I rats. These results support the hypothesis that locomotor inhibition observed after administration of a putative D_3 receptor selective agonist like PD-128,907 is achieved through D_3 activation of the Calleja islands. Furthermore, the present results, together with several experimental evidences collected by other laboratories, suggest that D_3 receptors may have a differential, somehow opposing, physiological role depending on their anatomical location.

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Table 1: NGFI-A *in situ* hybridization.
Results of *in situ* hybridisation for the NGFI-A gene transcript in the RHA-I and RLA-I rats after treatment with different doses of PD-128,907 are shown in this table. Results are expressed as mean optical density in the specific areas ± SEM. The statistical analysis was performed using two-way ANOVA with strain and treatment doses as factors. Duncan test was performed when appropriate: * $P<0.05$ compared to the respective saline-treated group. † $P<0.05$ compared to the respective group treated with 0.01 mg/Kg of PD-128,907.

	RLA			RHA		
PD 128,907 dose	0 mg/Kg	0,01 mg/Kg	0,1 mg/Kg	0 mg/Kg	0,01 mg/Kg	0,1 mg/Kg
Prelimbic/infralimbic	59,5 ± 1,3	62,1 ± 1,4	58,6 ± 0,9	55,5 ± 1,1	56,1 ± 0,8	54,9 ±1,6
Cingulate cortex	64,1 ± 1,8	68,8 ± 2	64,2 ± 0,7	65 ± 2,1	66,8 ± 0,7	64,8 ± 1,9
Motor cortex	44,9 ± 2,3	53,9 ±2,6 *	43,3 ± 2,2 †	52,2 ± 3,1	56,9 ± 2,5	48,8 ± 2,2 †
Sensorial cortex	36,8 ± 2,3	41 ± 2,3	35,2 ± 1,6	37,4 ± 2,7	42,2 ± 2,3	39,2 ± 2,3
Rostral striatum						
Accumbens core	38,2 ± 1,5	36,2 ± 2,1	35 ± 1,4	36,9 ± 2,7	37,7 ± 1,5	36,1 ± 2,5
Accumbens shell	43,8 ± 4,4	50,8 ± 4,7	32,3 ± 3,2 †	37,6 ± 4	43,4 ± 2,2	38,2 ± 4,1
Rostro-medial striatum						
Dorsolateral	38,3 ± 2,3	38,2 ± 2,2	35,2 ± 1,7	40,3 ± 2	44,3 ± 3,3	37,6 ± 2,4
Dorsal	43 ± 1,8	43,4 ± 3,6	34 ± 2,4 *	43,9 ± 2	46,5 ± 1,6	38,9 ± 2,9
Dorsomedial	42,6 ± 2,7	40,9 ± 2,5	39,2 ± 1,3	46,3 ± 2	44 ± 1,6	37,2 ± 3,1 *
Medial	42,2 ± 2,7	43,2 ± 4,1	41,7 ± 2,9	42,8 ± 3	45,2 ± 3,7	38,9 ± 3,6
Ventral	31,5 ± 2,3	37 ± 3,2	29,3 ± 2,5	29 ± 2,8	36,1 ± 2,4	33,9 ± 1,5
Accumbens core	29,3 ± 1,8	30,1 ± 2,2	26,9 ± 2,9	29,1 ± 3,2	30,7 ± 3,9	25,6 ± 3,6
Accumbens shell medial	42,9 ± 1,6	45,4 ± 2,4	43,5 ± 3,5	43,8 ± 3,4	39,7 ± 2,3	37,3 ± 1,9
Acuumbens shell ventral	24,2 ± 2	27,6 ± 1,9	22,2 ± 2,7	24,8 ± 2,6	24,9 ± 2,7	25,8 ± 4,1
Calleja Magna	79,5 ±8,3	55,9 ± 9,4	38,2 ± 9,7 *	79,1 ± 6,6	73,2 ± 7,8	55,2 ± 11,6
Olfactory tubercle	38,3 ± 3,9	39,8 ± 3,9	37,7 ± 5,5	37,4 ± 3,1	40,9 ± 5,2	37,4 ± 3,5
Cingulate cortex	54,4 ± 2	57,2 ± 1,4	55 ± 0,7	52,6 ± 0,8	52 ± 0,9	49,7 ± 1,5
Motor cortex	51,1 ± 1,6	54,4 ± 2,1	51,5 ± 1,9	56,1 ± 2,9	59,9 ± 2,2	53 ± 3
Sensorimotor cortex	40,6 ± 1,9	44,1 ± 1,3	39,9 ± 1,6	44,3 ± 3	48,7 ± 2,2	42,2 ± 2,6
Piriform cortex	87 ± 2,9	86 ± 2,8	83,1 ± 3,6	85,6 ± 2,1	89,7 ± 1,3	87,8 ± 3
Caudal striatum						
Medial	45,5 ± 1,8	42,4 ± 2,5	36,2 ± 2,1 *	45,3 ± 2,1	44,8 ± 3,7	41 ± 1,9
Dorsal	38,7 ± 1,3	34,5 ± 2,9	34,6 ± 2,7	40,9 ± 2,8	41,2 ± 2,1	37,3 ± 1,8
Dorsolateral	33,3 ± 2,6	34,7 ± 3,3	27,3 ± 2,4	37,9 ± 3,2	42,3 ± 2,8	33,8 ± 2,6
Ventrolateral	32,8 ± 2,2	35,5 ± 4,6	32,1 ± 2,4	38,9 ± 2,6	37,8 ± 2,7	36,3 ± 2,8
Ventral	29,5 ± 2,7	31,5 ± 2,5	29,5 ± 2,3	29,5 ± 1,8	35,8 ± 1,8	32 ± 2,4
Central	29,9 ± 3,2	33,2 ± 2,7	26,3 ± 1,7	31,2 ± 3,2	29,2 ± 1,9	30,9 ± 2,5
Cingulate cortex	67,8 ± 1,6	67,4 ± 1,7	64,9 ± 1,7	68,9 ± 1,7	70,3 ± 2,3	67,5 ± 1,8
Amygdala						
Central	29,9 ± 1,8	29,9 ± 1,9	30,7 ± 2,3	27,9 ± 2,3	28,7 ± 1,5	32 ± 3,9
Basolateral	33,4 ± 2,7	33,3 ± 2	29,2 ± 1,9	37,3 ± 1,9	37,4 ± 1,7	35,9 ± 1,7
Lateral	43,1 ± 3,8	47,3 ±7	39,8 ± 2,1	43,4 ± 5,1	44,5 ± 2,6	38,7 ± 3,8
Medial	37,3 ± 1,9	38,8 ± 2,5	36,1 ± 2,6	36,3 ± 2,9	34,1 ± 2,7	35,7 ± 3,1
Dorsal hippocampus						
Dentate gyrus	49,3 ± 3,6	48,1 ± 4,7	49,5 ± 3,5	49,4 ± 4,7	50 ± 2,6	48,6 ± 4,4
CA1	88,1 ± 5,2	73,2 ± 4,2	76 ± 6	59,6 ± 3,5	66,6 ± 4,9	74,1 ± 5,7
CA3	48,4 ± 3,1	52,8 ± 3	46,8 ± 1,4	53,7 ± 3,5	55,3 ± 3,5	49 ± 2,5
Ventral hippocampus						
Dentate gyrus	51,3 ± 2,7	56,8 ± 2,2	50,5 ± 3,9	50,2 ± 1,8	57 ± 3,2	56,3 ± 5,2
CA1	82,1 ± 2,5	77,3 ± 2,7	83 ± 3,1	82,2 ± 3,1	78,4 ± 5,1	77,5 ± 4,4
CA3	55,8 ± 3,3	49,2 ± 1,6	52,7 ± 4,3	54,8 ± 2,5	60,3 ± 3	55 ± 3,6
VTA	18,4 ± 1,7	13 ± 0,7	18,7 ± 2,5	18 ± 3	18,9 ± 2,2	13 ± 1,6
SNR	15,6 ± 2	15,4 ± 1,9	15,8 ± 2,2	15,8 ± 1,5	16,9 ± 1,1	16,1 ± 1,7

Figure 1: Locomotor time course after PD-128,907 administration.
The time course of locomotor activity of RLA-I and RHA-I rats after administration of vehicle, 0.01 mg/Kg PD-128,907 or 0.1 mg/Kg PD-128,907 and placement in the test cage without previous habituation. Locomotor activity is expressed in cm, mean ± SEM, at 10 minutes intervals. The statistical analysis was performed by means of repeated measures ANOVA with strain and treatment doses as main factors and 10 minutes intervals as repeated factor. When appropriate, Duncan test was used at each interval. * $P<0.05$ comparing the respective saline-treated group with the group treated with 0.1 mg/Kg of PD-128,907. † $P<0.05$ comparing the respective saline-treated group with the group treated with 0.01 mg/Kg of PD-128,907.

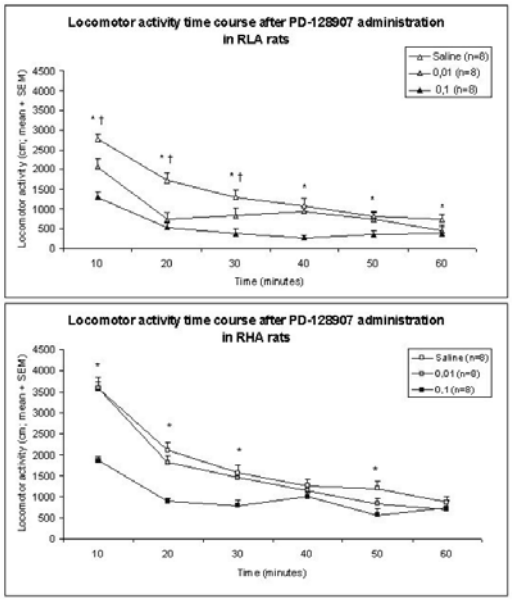


Figure 2: Accumulated locomotor activity after PD-128,907 administration.
In this figure, the total locomotor activity performed by animals during the whole 1 hour session after administration of vehicle, 0.01 mg/Kg PD-128,907 or 0.1 mg/Kg PD-128,907 to RHA-I and RLA-I rats and placement in the test cage without previous habituation is depicted. Locomotor activity is expressed in cm, mean ± SEM. The statistical analysis was performed by means of two-way ANOVA with strain and treatment doses as main factors. When appropriate, Duncan test was used. * $P<0.05$ compared to the respective saline-treated group. † $P<0.05$ compared to RLA-I saline-treated group.

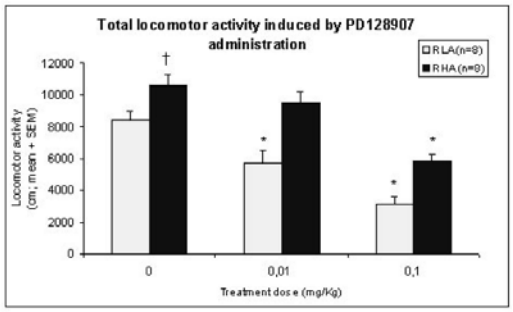


Figure 3: Expression levels of NGFI-A mRNA in the Calleja magna 1 hour after PD-128,907 administration.
In this figure, the levels of NGFI-A gene transcript in the Calleja magna in RHA-I and RLA-I rats after treatment with different doses of PD-128,907 are shown. Results are expressed as mean optical density in the specific areas ± SEM. The statistical analysis was performed using two-way ANOVA with strain and treatment doses as factors. Duncan test was: * $P<0.05$ compared to the respective saline-treated group.

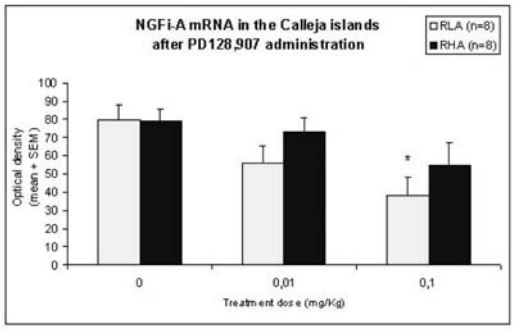


Figure 4: NGFI-A mRNA in the Calleja magna 1 hour after PD-128,907 administration in RLA rats.
In this figure, representative autoradiograms obtained after NGFI-A mRNA *in situ* hybridization in RLA-I rats treated with saline, 0.01 mg/Kg of PD-128,907 or 0.1 mg/Kg of PD-128,907 are depicted. Adjacent slides of those used for *in situ* hybridization were stained with cresyl violet and they are also shown in order to locate the Calleja magna (arrow).

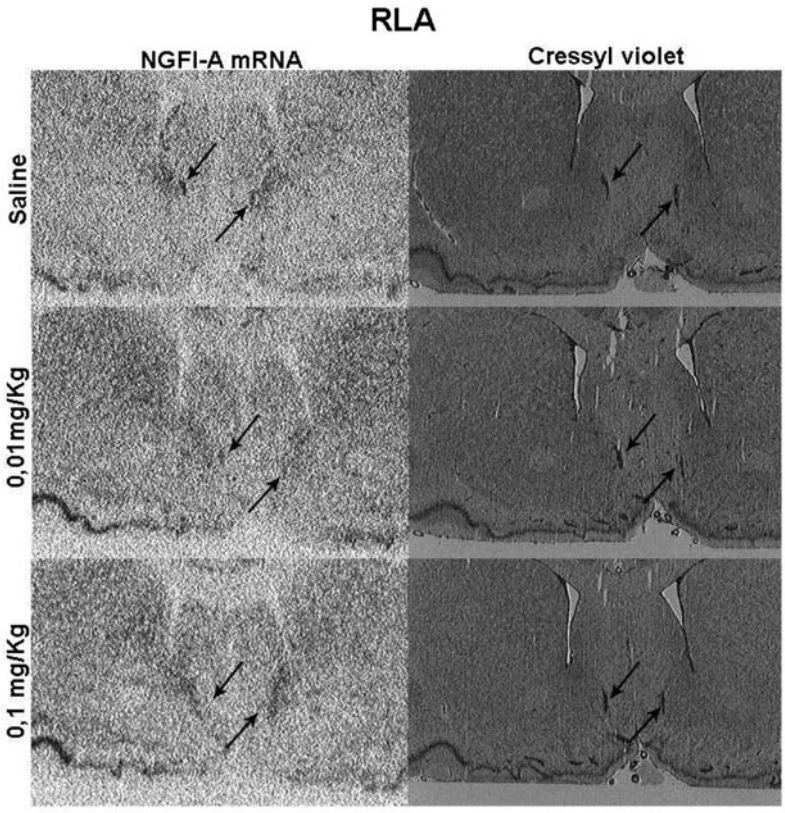


Figure 5: NGFI-A mRNA in the Calleja magna 1 hour after PD-128,907 administration in RHA rats.
Representative autoradiograms obtained after NGFI-A mRNA *in situ* hybridization in RHA-I rats treated with saline, 0.01 mg/Kg of PD-128,907 or 0.1 mg/Kg of PD-128,907 are depicted. Adjacent slides of those used for *in situ* hybridization were stained with cresyl violet and they are also shown in order to locate the Calleja magna (arrow).

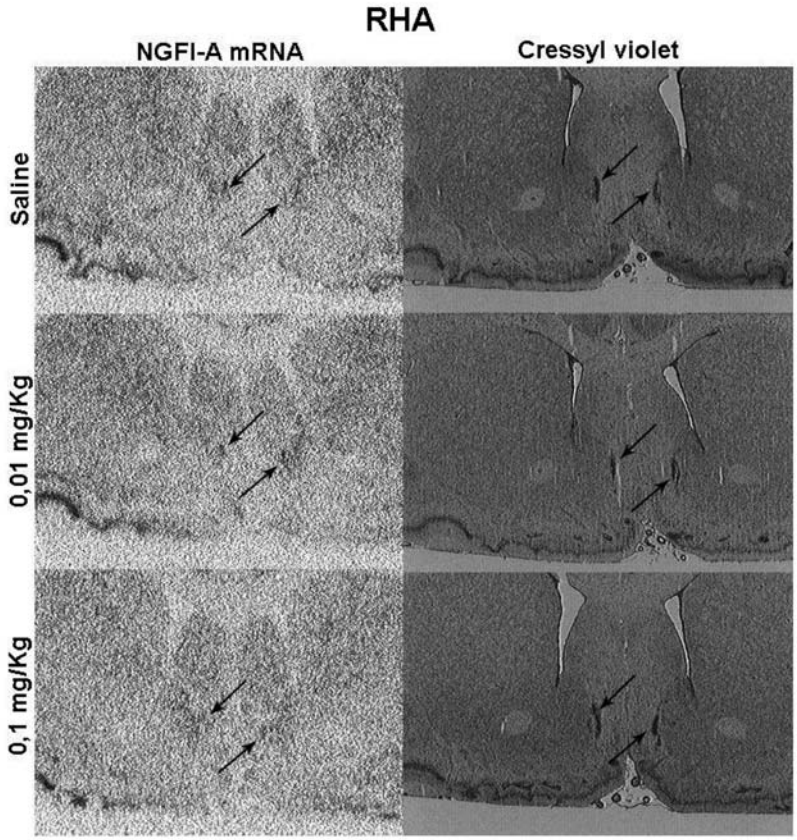
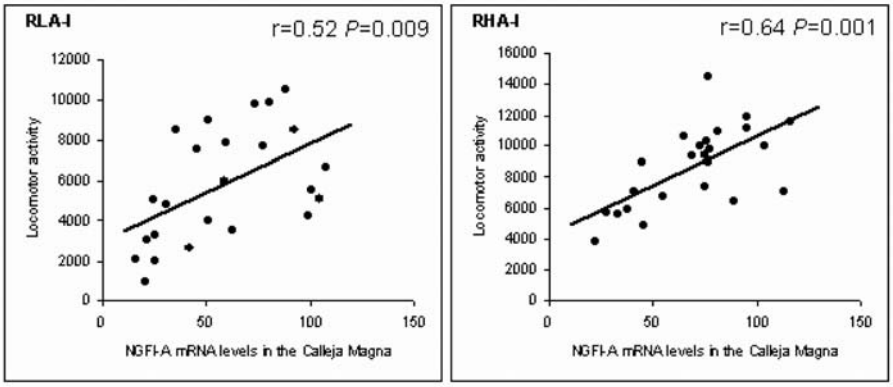


Figure 6: Relationship between NGFI-A mRNA levels in the Calleja magna and locomotor activity performed furing the test in RLA-I and RHA-I rats. The total locomotor activity performed by all animals of each strain is plotted against the levels of NGFI-A mRNA in the Calleja magna obtained by *in situ* hybridization. The correlation was analysed by means of the Pearson test.



Paper IV

INDUCTION OF BEHAVIOURAL SENSITIZATION TO AMPHETAMINE PREDICTS INCREASED VULNERABILITY OF BEHAVIOURAL SENSITIZATION IN THE ROMAN HIGH AVOIDANCE RATS

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Abstract

Behavioural sensitization after repeated exposure to amphetamine is thought to be dependent on the mesolimbic dopaminergic system. The Roman rats genetically selected for high (RHA) or low (RLA) shuttle avoidance acquisition differ in dopaminergic activity and have been used as a valid laboratory model of divergent sensation/novelty and substance-seeking profiles. We submitted the Roman rats to a sensitization regime with amphetamine and we observed the changes in behavioural response during induction and their behavioural profile was compared to that of a standard rat strain (Sprague-Dawley). We found that RHA rats already show increases in behavioural response to amphetamine after 9 days of induction treatment with amphetamine while RLA and SD rats do not show similar changes. The present results suggest that the higher vulnerability to behavioural sensitization shown by RHA upon a challenge can already be observed during induction.

Introduction

Repeated exposure to psychostimulants like amphetamine results in behavioural sensitization: a progressive and enduring increase in the behavioural effects of the drug [1];[2]. Sensitization of the dopaminergic system after repeated experiences with abused drugs has been suggested to be a relevant phenomena underlying addiction [3];[4], specially to the ability of drug associated stimuli to reinstate consumtion after long withdrawal periods [5]. A conceptualization employed in the study of mechanisms of sensitization divides it in two distinct temporal domains, termed induction and expression [2;6]. The induction of amphetamine sensitization is dependent on drug effects over the dopamine cell bodies in the ventral tegmental area (VTA) [7];[8] and on glutamatergic activity (reviewed by [9]and [10]. The expression of the enhanced response to an amphetamine challenge is better observed after relatively long withdrawal periods [2];[11], it is mediated by the drug effects on the nucleus accumbens [12];[10] and it is normally accompanied by increased responsiveness of the mesoacumbens dopaminergic pathway [11];[10].

Although much progress has been made in the description of the molecular mechanisms that lead to behavioural sensitization, they have not been completely described, and there are many conflicting results (For review see [11];[10]. Rodent strains that differ in the dopaminergic function may be a valuable tool to study anatomical and neurochemical substrates of sensitization. In this regard, the sublines of Roman high- (RHA) and low-avoidance (RLA) rats, psychogenetically selected for rapid vs. extremely poor two-way active avoidance acquisition, respectively [13], appear to be a valid laboratory model of divergent novelty- and substance-seeking profiles, as well as of differential central DAergic activity in a wide range of experimental situations (reviewed by [14]. It has been recently demonstrated that RHA rats show enhanced sensitisation to morphine [15], cocaine [16] and amphetamine [17] as well as changes in the dopaminergic outflow only in RHA rats sensitized to amphetamine [18]. In work done in our laboratory we demonstrated that RHA-I rats show higher sensitization behaviour than standard Sprague Dawley (SD-OFA) rats and replicated the lack of behavioural sensitization in RLA-I rats. Moreover, we demonstrated that the differences in the expression of behavioural sensitization between the Roman strains is dependent on a differential pattern of expression of several genes that are regulated by neuronal activity in dopamine related brain areas (manuscript IV).

The aim of the present work was to study if the higher vulnerability of RHA-I to express behavioural sensitization upon an amphetamine challenge after 14 days withdrawal could already be seen during the induction phase. Therefore, we submitted the Roman strains to a sensitization regime with amphetamine that has already been used to study differences in vulnerability to sensitization in rats [19]. We included SD-OFA rats as a standard strain to add a control for comparison of vulnerability to sensitization. We studied the changes in spontaneous and induced motor activity in an alternate days during the sensitization protocol.

Materials and methods

Animals

70-80 days old male inbred RHA (RHA-I) and RLA (RLA-I) rats were used. The animals were bred in the animal department at the Medical Psychology Unit (UAB) and maintained two per cage (Macrolon, 21.5 x 46.5 x 14.5 cm), under standard laboratory conditions (food and water *ad libitum*, 22 ± 2ºC and 12L:12D cycles beginning at 08:00h). 60-70 days old male Sprague Dawley (SD-OFA) rats were bought at the animal department of the UAB and were maintained in the same laboratory conditions for 15 days before the beginning of the experiments. SD-OFA rats were 10 days younger than Roman rats because we have previously observed important differences in weight between Roman and SD-OFA rats if the animals are the same age. 2 days before the experimental procedure begun the animals were habituated to the handling procedures that are required for drug injections. The research was conducted in accordance with guidelines and protocols approved by the European Economic Community (86/609/EEC Council) regarding the Care and Use of animals for experimental procedures and by the Ethical Commission of the Autonomous University of Barcelona for these respects.

Motor activity tests

Motor behaviour was tested on days 0, 1, 3, 5, 7, 9, 11. On the day of the test, animals were weighted and carefully moved in their homecages from the animal room to the behavioural laboratory where they were left undisturbed for half an hour before the start of the behavioural test. The motor activity was recorded in the test cages for a period of 60 minutes (spontaneous activity and habituation). Thereupon, the animals were injected i.p. with the respective drug and returned to the test cage. Motor activity was recorded for an additional period of time (treatment phase). Measurement of motor activity was run between 10:00 and 15:00 h to reduce the possible influence of diurnal variation in activity. Motor activity was determined by simultaneous video-recording of activity from batches of 4 animals placed in single polyglass motor activity test cages (dimensions: 40 x 40 x 40 cm). The motor activity cages were placed in a dimly lit room with a background noise. The videotapes were analyzed using a video-computerized system (SMART, Panlab S.L., Barcelona, Spain) which detects the position of the animal at each moment, draws its trajectory and calculates the total distance (in cm) covered by the animal during a certain period of time.

Sensitization regime

Amphetamine sulfate (Sigma, St Louis, USA) was dissolved in 0.9% saline and injected i.p. with a volume of 1ml/kg. A dose of 1mg/kg was used for induction treatment. We performed a factorial design 3 x 2 (3 strains, 2 treatments) following the protocol detailed below. In order to avoid differences in the basal activity between treatments groups of the same strain, animals were counterbalanced according to the motor activity in response to a saline challenge on day 0. On that day, after habituation to the test cage, saline was injected to all animals and motor activity was recorded during 1 hour. According to those results, animals were assigned to a treatment group (RHA: 8 rats each group; RLA: 8 rats each group; SD: 10 rats each group).

Day 1, 3, 5, 7, 9 and 11 (induction): After habituation to the test cage animals were injected with amphetamine (1mg/Kg) or saline according to the treatment group. After administration, animals were returned to the test cages for 2 hours.

Day 2, 4, 6, 8, 10 (induction): Animals were weighted and left in the home cage for 2 hours. Thereafter they were injected with amphetamine (1mg/Kg) or saline according to the treatment group. They were returned to the home cage and left undisturbed in the animal department until the next day.

Statistics

Motor activity results are expressed in mean (cm) ± SEM at 10 minutes intervals or during the whole session. When in a repeated measures ANOVA analysis Mauchly sphericity was not reached, Huynh-Feldt correction was used. Motor activity during habituation on day 0, motor activity during habituation before each treatment day, and motor activity induced by treatment each induction day were initially analyzed independently.

For the habituation activity on day 0, we used a repeated measures ANOVA analysis with strain (SD, RLA and RHA) and induction treatment (amphetamine or saline) as main factors and the 10-minute interval as repeated factor.

For habituation before each treatment day and motor activity induced by treatment each induction day, we used a repeated measures ANOVA analysis with strain (SD, RLA and RHA) and induction treatment (amphetamine or saline) as main factors and treatment day as repeated factor. To determine in which group the repeated factor had an effect we segmented the same analysis for each group. The day effect was analyzed by means of a simple contrast which compared, for each day, the total motor activity during the habituation with that on the previous day and the total motor activity induced by treatment with the one on the first day for treatment.

Results

1.1 Spontaneous activity on day 0

During habituation on day 0 (see figure 1), the three strains showed the habituation pattern already described by Giménez-Llort et al. (2005) [23]. The repeated measures ANOVA analysis detects a strain effect ($F(2,46)=10.12$; $P<0.001$) and the Duncan test ($P<0.05$) shows that SD rats developed less total motor activity than the Roman rats, while RLA and RHA rats showed no differences in the total amount of motor activity. However, the three strains presented different habituation pattern as shown by an interval x strain effect ($F(9.1, 210.1)=4.27$; $P<0.001$). As shown by Duncan test at each interval, RHA rats developed more motor activity than RLA and SD rats during the first 10 minutes interval. However, from the second interval on, RLA rats developed as much motor activity as

RHA rats. SD rats were less active than the other two strains during the whole session.

1.2 Evolution of habituation while induction (day 0-day 11)

Since the MANOVA analysis neither detects treatment effect ($F(1,46)=1.85$; ns) nor day x treatment effect ($F(8.7,200)=1.04$; ns) the data were pooled in strains as shown in figure 1A. The same analysis reveals differences in the evolution pattern of habituation before each treatment session during the induction as shown by a day x strain interaction ($F(8.7,200)=7.84$; $P<0.001$). The segmented MANOVA for each strain showed that: 1) SD rats decreased the total motor activity the second time they were placed in the activity cage for habituation while they did not decrease the total motor activity with further experiences with the cage (day effect for SD: $F(3.5,63.7)=16.5$; $P<0.001$ and contrast day 1-day 0: $D(1,18)=25.28$; $P<0.001$); 2) RHA rats did not show changes in the motor activity developed during habituation although the experience was repeated (day effect for RHA: $F(2.3, 32.5)=1.92$; ns); 3) When habituation is repeated, RLA showed an unexpected evolution pattern with an increase of activity on days 5 and 7 (day effect for RLA: $F(3.6, 50.7)=1.92$; $P=0.003$, a contrast day 5-day 3: $F(1,14)=10.98$; $P=0.005$) and a posterior decrease on day 9 (contrast day 9-day 7: $F(1,14)=4.94$; $P=0.043$).

The Duncan test ($P<0.05$) for each day shows that from day 1 on RLA rats developed higher motor activity during the habituation period than RHA rats and RHA higher activity than SD rats.

1.3 Sensitization induction treatment

The MANOVA analysis detects strain effect ($F(2,46)=97.25$; $P<0.001$), treatment effect ($F(1,46)=433.5$; $P<0.001$), and strain x treatment interaction ($F(2,46)=25.49$; $P<0.001$). As shown by the Duncan test ($P<0.05$) for each day, amphetamine (1mg/Kg) always induced more motor activity than saline treatment, and amphetamine always induced greater motor activity in RLA rats than in RHA rats, and greater in the latter strain than in SD rats.

More relevant are the effects detected when the repeated factor is included. Figure 1B-D shows the motor activity induced by the correspondent induction treatment each day the animals experienced it on the activity cage. The MANOVA analysis detects a day effect ($F(5,230)=12.82$; $P<0.001$), a day x effect interaction ($F(10,230)=4.03$; $P<0.001$), a day x treatment interaction ($F(10,230)=2.59$; $P=0.027$) and a day x strain x treatment interaction ($F(10,230)=1.96$; $P<0.039$). A segmented MANOVA for each group shows the groups in which the motor activity were modified during induction (see table 2, without covariant). SD rats showed no change in induced motor activity regardless of induction treatment. RLA and RHA rats that received saline, as well as RHA that received amphetamine showed increases in induced motor activity compared to day 1 (RLA-saline in day 5,7 and 11; RHA-saline in days 3, 5, 7 and 11; RHA-amphetamine in days 7, 9 and 11). The increase in the saline treated groups seems to be dependent on the fact that rats were repeatedly exposed to the activity cage since they parallel the habituation pattern. If this were the case, a group segmented ANOVA analysis with day as main factor and habituation motor activity each day as a covariant should eliminate the effect of repeated exposure to the activity cage. This analysis (see table 2 with habituation as a covariant) shows that RHA rats that received amphetamine were the only group that showed an increase in induced motor activity on day 9 and 11 when compared to day 1. Therefore, it seems that the changes observed during induction treatment with saline in the Roman strains were dependent on a factor already present during the habituation and independent of the treatment itself.

Discussion

Using this induction protocol, we have recently demonstrate that RHA rats show greater expression of behavioural sensitization than SD rats when they are challenged with amphetamine after a 14 days withdrawal period (manuscript IV). We also demonstrated that RLA rats do not express behavioural sensitization in a challenge (manuscript IV). In the present work, we show that this higher vulnerability to behavioural sensitization can already be seen during the induction period, since only RHA rats that receive amphetamine as induction treatment showed changes in their induced motor activity already during the induction phase.

Spontaneous activity on day 0

The pattern of spontaneous activity showed by the inbred Roman strains is comparable to that recently described in the same rats during a 90 minutes test by Giménez-Llort et al. (2005) [23]. RHA-I rats are more active than RLA-I rats during the first 10 minutes period and these two strains show the same amount of motor activity afterwards. In the present work, both RHA-I and RLA-I eventually develop the same total amount of motor activity during the 60 minutes period. This pattern of spontaneous activity shown by the inbred Roman rats is not the same shown by the outbred Roman rats, since outbred RHA rats systematically show increased spontaneous activity during a whole habituation session, normally lasting 60 minutes [20];[21];[17];[16]. A particular pattern in the habituation activity has already been described for the inbred Roman strains in different laboratories [22];[23]. However, the increased spontaneous activity shown by RHA-I rats during the first 10 minutes period is in agreement with the known higher exploratory and novelty seeking behaviour of RHA rats versus their genetic counterparts the RLA rats (reviewed by [14] which has already shown by the inbred rats [24];[25].

In the present study SD-OFA rats developed less motor activity than the Roman rats during the whole period. In a recent work using the same animal strain from the same suplier, SD-OFA and the Roman rats did not differ in the total amount of motor activity developed in a 90 minutes spontaneous activity test [23]. It may be that this discrepancy arises from the fact that the

used SD-OFA rats are a heterogeneous stock with less established traits when compared to the Roman rats.

Repetition of spontaneous activity during induction phase

SD rats show the expected pattern for a standard strain in a repeated test [26]: the second day the animals interact with the test cage they recognize it and develop less spontaneous activity during the 60 minutes before they are administered with the induction treatment. In successive experiences spontaneous activity is not modified. RHA rats do not show any change in the motor activity performed in the cage regardless of repetition. Since motor activity performed in a novel environment is dependent on the dopaminergic system [27];[28], the lack of habituation of RHA rats may be understood as a correlate of their known hyperreactive dopaminergic system when compared to RLA rats (reviewed by [14]). This characteristic would be maintained when compared to a standard strain. However, in the present experiment, RLA rats do not either show habituation to the activity cage when they experience it repeatedly; they even present an invert U shape curve with an increase in spontaneous activity the forth day they experience the test cage (induction day 5) and a decrease in spontaneous activity the sixth day that they experience it (induction day 9). This pattern was not expected with the available data about habituation sessions in the outbred Roman strains [20];[21];[17], however, this is the first time that the Roman strains are tested for motor activity in the same cage repeatedly. The possible role of the noradrenergic system in these responses is discussed below.

Amphetamine induced motor activity

RHA-I rats receiving amphetamine as induction treatment showed changes in their induced motor activity already during the induction phase whereas RHA-I rats receiving saline as well as RLA-I and SD-OFA-I regardless of induction treatment did not show changes in their saline- or amphetamine- induced motor activity. In many sensitization studies, behavioral sensitization is demonstrated upon a challenge with the drug after a withdrawal period. The study of the possible changes in amphetamine-induced motor activity during the induction period further characterize the differential vulnerability to this phenomenon recently demonstrated among RHA-I, RLA-I and SD-OFA upon an amphetamine challenge (manuscript IV). Using the present protocol, SD-OFA rats expressed behavioural sensitization upon a challenge with amphetamine, although they showed a weaker sensitization when compared to RHA-I rats (manuscript IV). Induction and expression of behavioural sensitization are two neurologically divergent processes [11];[10]. Together with the existence of experiments in which sensitization is expressed in a challenge without signs of previous sensitization while induction [29], we can conclude that SD-OFA rats show a behavioural pattern of sensitization compatible with that of a standard strain. In many experiments, an amphetamine challenge only a day after the last induction administration is not able to patent a sensitized response and this is easier to be seen after longer withdrawal periods [11];[30]. In this sense, the fact that RHA-I rats already show sensitization during induction may be interpreted as greater sensitivity of neural systems underlying induction or alternatively the appearance of the phenomenon before withdrawal. Regarding behavioral sensitization, RLA-I rats did not express it upon a challenge after 14 days withdrawal using the same induction protocol as in the present work (manuscript IV). Together with the lack of changes in motor activity during induction shown in the present experiment, we may state that RLA-I rats show protection to sensitization. This vulnerability versus protection to behavioral sensitization in the RHA-I and RLA-I rats, respectively, is in accordance with the abundant literature showing that outbred RHA rats show behavioral sensitization with several drugs whereas outbred RLA rats do not [15-18].

Although RLA-I rats do not show behavioural sensitization neither during induction (present results) nor upon and amphetamine challenge after 14 days of withdrawal (manuscript IV), they perform higher motor activity in all situations in which they receive amphetamine. Thus, RLA-I rats do not show the expected response to acute amphetamine administration. In our laboratory we have previously observed higher response to acute amphetamine in RLA-I rats than in RHA-I rats [31]. In the outbred lines, the higher response to acute psychostimulants administration in RHA-I rats has been correlated to a greater responsiveness of the mesolimbic dopaminergic system [20];[21]. This was the basis for a hypothetical vulnerability to expression of behavioural sensitization to amphetamine and other drugs that has been confirmed in the outbred lines [15];[18][17];[16] and replicated with amphetamine in the inbred rats (manuscript IV). Although this relationship between higher acute response to amphetamine and vulnerability to sensitization is maintained when RHA rats are compared to SD, it is not when compared to RLA rats. The differences in acute response to amphetamine between inbred and outbred Roman strains do not seem due to changes in the dopaminergic system. The inbred Roman strains exhibit the same behavioral patterns as the outbred lines in behavioral tests which reflect the activity of the dopaminergic system like the shuttle box, the open field and other activity measures [14];[24]. They also show similar behavioral responses to the direct dopamine agonist apomorphine [23] and, relevantly, they show very similar expression levels of dopamine D_1 and D_2 receptors in the striatum (manuscript II). The fact that RLA-I rats do not show behavioral sensitization regardless of their higher response to acute amphetamine also supports the idea that the difference between inbred and outbred Roman rats does not lay on the dopaminergic system. With this regard, it is relevant to point out that dissociation between acute response to amphetamine and its sensitization effects has been observed before [32;33]. As discussed below, this dissociation is the basis for a possible implication

of the noradrenergic system in the paradoxical hyperresponse to acute amphetamine shown by RLA-I rats.

In some circumstances, there is a group of animals that show a maximum response after acute amphetamine administration without further increases in the response in chronic treatments [34];[35]. Badiani (1995) argued that this phenomenon was observed in a subset of animals that received the drug in a familiar environment, namely the home cage. Although the animals experienced the activity cage the day before the sensitization regime begun, the protocol followed in the present study is equivalent to the one named as novel by [35]. The observed pattern of acute response to amphetamine as well as the bizarre evolution of the spontaneous activity along with repeated experiences in the test cage observed in RLA-I rats may be the result of the interaction between neurobiological characteristics of these inbred rats and some characteristics of the protocol used. In this sense, the concurrence of the noradrenergic system may help to clarify the issue. It has been described that a drug-induced decrease in the central noradrenergic tone (α1-antagonists or α2-agonists) decreases the acute response to amphetamine [36] without effect on the sensitization effect of the drug [33]. Norepinephrine mediates some aspects of amphetamine effects by means of cortical α-1 receptors activation [37] and amphetamine induces increases in dopamine, norepinephrine and in a lesser degree serotonin [38]. Moreover, norepinephrine takes part in the induction and expression of hyperreactivity to repeated saline injections [36]. The evolution of spontaneous activity shown by RLA rats in the present experiment together with the hyperresponse to acute amphetamine could be explained by increased noradrenergic activity. The noradrenergic system has only been tested in basal conditions in the outbred lines [39]. Much work should be done to clarify this issue.

Concluding remarks

In the present work we further advance in the characterization of vulnerability to behavioral sensitization that RHA lines/strains have already shown upon a challenge with amphetamine [17];[18];Guitart 2006). RHA-I rats showed induction of behavioral sensitization with amphetamine whereas RLA-I or SD-OFA did not. SD-OFA rats expressed weaker behavioral sensitization after a withdrawal period than RHA-I rats (manuscript IV). Thus, together with the present result we can state that RHA-I rats are more vulnerable to behavioral sensitization than SD-OFA rats, and RHA rats emerge as a valuable animal model to study vulnerability to behavioral sensitization. Although inbred RLA rats show an unexpected pattern of habituation and response to acute amphetamine, the lack of induction of behavioral sensitization in RLA rats shown in this experiment reinforce the already described lack of expression (manuscript IV) and establish RLA-I as a valuable tool to study intrinsic protection of behavioral sensitization.

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Table 1: The statistical analysis of the results of motor activity developed after administration of induction treatment is shown in this figure. In the left column, the day effect of the MANOVA analysis with strain and treatment as main factors and day as repeated factor is shown for each group. When repeated factor is detected a simple contrast comparing each day to day 1 is shown when it is significant. In the right column, the day effect of an ANOVA analysis with day as main factor and habituation activity as a covariant is shown for each group. When the day factor is significant, a simple contrast comparing each day to day 1 is shown when it is significant.

	Without covariant	With habituation as a covariant
SD Saline	F(5,45)=1.718; *n.s.*	F(5,53)=1.251; *n.s.*
SD Aamphetamine	F(5,45)=1. 371; *n.s.*	F(5,53)=0.903; *n.s.*
RLA Saline	F(5,35)=4.859; *P*=0.002 d5-d1: F(1,7)=10.09; *P*=0.013 d7-d1: F(1,7)=17.32; *P*=0.004 d11-d1: F(1,7)=5.649; *P*=0.049	F(5,41)=2.274; *n.s.*
RLA Aamphetamine	F(5,35)=2.424; *n.s.*	F(5,41)=2.155; *n.s.*
RHA Saline	F(3.6,25.2)=3.972; *P*=0.015 d3-d1: F(1,7)=10.805; *P*=0.013 d5-d1: F(1,7)=11.352; *P*=0.011 d7-d1: F(1,7)=11.088; *P*=0.013 d11-d1: F(1,7)=8.258; *P*=0.024	F(5,41)=1.948; *n.s.*
RHA Aamphetamine	F(5,35)=10.216; *P*<0.001 d7-d1: F(1,7)=22.901; *P*=0.002 d9-d1: F(1,7)=68.376; *P*<0.001 d11-d1: F(1,7)=42,788; *P*<0.001	F(5,41)=3.35; *P*=0.013 d9-d1: *P*=0.002 d11-d1: *P*=0.006

Figure 1: The spontaneous motor activity in cm developed by each strain during habituation period on day 0 is depicted in this figure. The results are shown in cm of motor activity each 10 minutes period as well as the total motor activity during the 60 minutes habituation. a $P<0.05$ in Duncan test comparing RHA to RLA and SD; b $P<0.05$ in a Duncan test comparing SD to RHA and RLA rats.

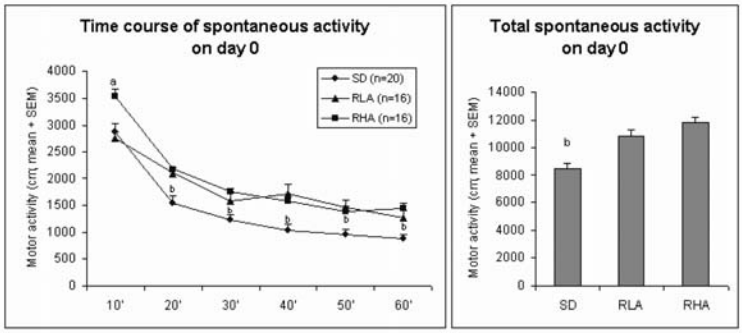


Figure 2: This figure depicts the spontaneous motor activity in cm developed by each strain previous of treatment administration every day that induction treatment was administered in the test cage. The results are shown in cm of motor activity during the whole 60 minutes period. † $P<0.05$ in an ANOVA with repeated measures using repeated contrast comparing each day to the previous one.

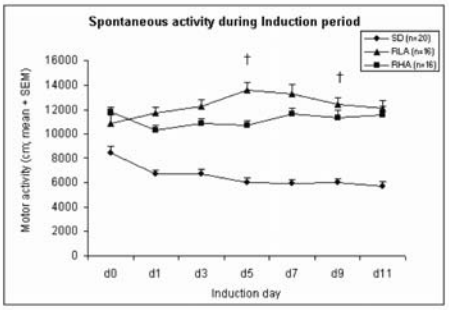
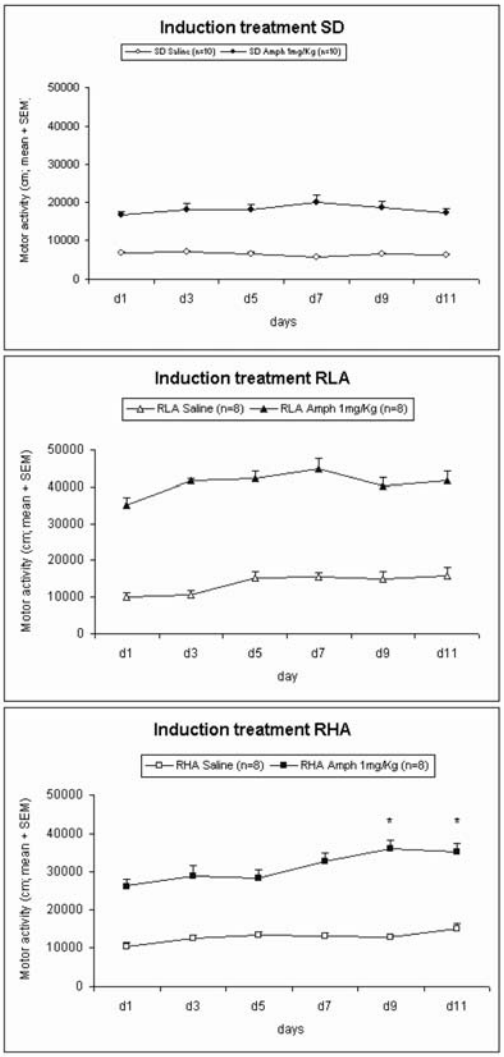


Figure 3: The behavioural results obtained during the induction treatment are depicted. Accumulated motor activity after amphetamine administration each day the behaviour was registered (every second day beginning on day 1 until day 11). Each strain is shown in one figure; the group treated with saline is depicted in open signs while the amphetamine treated group is depicted in filled signs. * $P<0.05$ in MANOVA simple contrast compared to the day 1.



Paper V

REGIONAL ADAPTATIONS IN PSD-95, NGFI-A AND SECRETOGRANIN GENE TRANSCRIPTS RELATED TO VULNERABILITY TO BEHAVIORAL SENSITIZATION TO AMPHETAMINE IN THE ROMAN RAT STRAINS

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Abstract

Genetically selected for high or low two-way active avoidance, RHA and RLA rats differ in their central dopaminergic activity, sensation/novelty- and substance-seeking profiles. These animals are, therefore, of potential value to study the anatomical and neurochemical substrates of behavioral sensitization. We submitted inbred RHA (RHA-I), inbred RLA (RLA-I) and Sprague-Dawley-OFA (SD-OFA) rats to a sensitization regime with amphetamine and studied the behavioral response to an amphetamine challenge after a two week withdrawal period. The expression patterns of NGFI-A, secretogranin, PSD-95, prodynorphin and proenkephalin mRNA were also analyzed using *in situ* hybridization, after the challenge with amphetamine. RHA-I rats showed stronger sensitization than SD-OFA rats. RLA-I rats did not show sensitization but were hyper-reactive to amphetamine. Expression of behavioral sensitization in RHA-I rats activated secretogranin and PSD-95 mRNA in the nucleus accumbens core. On the other hand, high induction of NGFI-A mRNA in the central amygdala was observed in RLA-I rats when they experienced amphetamine for the first time in the challenge. Our results reveal that 1) the acute response to amphetamine does not predict vulnerability to behavioral sensitization and 2) differences in vulnerability to sensitization may involve distinctive cellular adaptations at particular brain locations which may be related to addictive vulnerability.

Key words: amygdala; *in situ* hybridization, prodynorphin, proenkephalin

Introduction

Repeated exposure to psychostimulants like amphetamine results in behavioral sensitization, a progressive and enduring increase in the behavioral effects of the drug (Stewart and Badiani, 1993;Robinson and Becker, 1986). Although much progress has been made in the study of the molecular mechanisms that lead to this phenomenon, a satisfactory explanation is lacking. The expression pattern of gene transcripts responsive to neuronal activity permits the study of anatomical and neurochemical substrates involved in sensitization. Amphetamine administration acts on spiny neurons to profoundly change gene transcript levels of immediate early genes like NGFI-A (*zif268*) and neuropeptide precursors like proenkephalin (ENK) and prodynorphin (DYN) in the striatum (Berke et al., 1998;Nestler, 2001). Other transcripts also regulated by neuronal activity are secretogranin (Shen and Gundlach, 1996) and PSD-95 (Zundert et al., 2004;Bao et al., 2004). A constitutive low level of PSD-95 gene transcript is associated with a strong response to a cocaine challenge (Yao et al., 2004). The previously unexplored secretogranin and PSD-95 responses to a challenge in behavioral sensitization were therefore of interest in the present work.

Rodent stocks that differ in DAergic function are of potential value in the study of anatomical and neurochemical substrates of sensitization. The Swiss sublines of Roman high-avoidance and low-avoidance rats were selected for rapid (RHA/Verh) vs. very poor (RLA/Verh) two-way avoidance acquisition in the shuttle box (Driscoll and Bättig, 1982). An important body of behavioral and neurobiological evidence indicates that these selected lines are a valid laboratory model of divergent sensation/novelty and substance-seeking profiles, as RHA rats show a more reactive dopaminergic (DAergic) system in a wide range of experimental situations (Reviewed by Driscoll et al., 1998). RHA rats show enhanced sensitization to morphine (Piras et al., 2003), cocaine (Giorgi et al., 2005b) and amphetamine (Corda et al., 2005), and only RHA rats show changes in DAergic outflow when sensitized to amphetamine (Giorgi et al., 2005a). Since 1993, an inbreeding program (brother-sister mating) has been carried out in parallel to that of the outbred RHA/Verh and RLA/Verh rat lines, with the inbred strains being presently maintained at the animal department facilities of the Medical Psychology Unit in Barcelona (See Escorihuela et al., 1999). Recently, we have described constitutive differences in the expression level of DYN and

ENK mRNA between these Roman strains (Guitart-Masip et al., 2005).

The aim of the present work was to study anatomical and molecular correlates of behavioral sensitization in the forebrain in order to reveal possible key structures that may underlie differential vulnerability to this phenomenon. We studied behavioral sensitization in inbred Roman strains along with their divergent vulnerability to amphetamine sensitization, and compared them to a standard laboratory rat strain, Sprague-Dawley-OFA (SD-OFA). The latter strain was included in order to determine if the expected enhanced vulnerability to behavioral sensitization in RHA rats when compared to RLA rats is also observable when compared to a standard rat strain. Brain areas that were activated by the amphetamine challenge were identified using *in situ* hybridization of NGFI-A, DYN, ENK, secretogranin and PSD-95 gene transcripts.

Materials and methods

Animals

70-80 days-old male inbred RHA (RHA-I) and RLA (RLA-I) rats, direct descendents of outbred RHA/Verh and RLA/Verh rats (see Introduction), were used, being maintained two per cage (Macrolon, 22 x 47 x 15 cm), under standard laboratory conditions (food and water *ad libitum*, 22 ± 2ºC and 12L:12D cycles beginning at 08:00h). SD-OFA rats (provided by UAB Animal Department) were maintained in the same laboratory conditions for 15 days before the beginning of the experiments. Two days before the experimental procedure began, the animals were habituated to the handling procedures required for drug injections. The research was conducted in accordance with guidelines and protocols approved by the European Economic Community (86/609/EEC Council) regarding the care and use of animals for experimental procedures and by the Ethics Commission of the Autonomous University of Barcelona.

Sensitization treatment

In order to avoid differences in the basal activity between treatment groups of the same strain, animals were counterbalanced according to their response to saline injection prior to the beginning of the sensitization regime. On that day, after habituation to the test cage (spontaneous activity), all animals were injected and motor activity was recorded during 1 hour. Based on these results, animals were allocated to a treatment group by matching (RHA-I and RLA-I: 8 rats each group; SD-OFA: 10 rats each group) so that the two groups of each strain displayed the same amount of motor activity after this saline injection (see table 1).

Table 1 and figure 1 should be placed here

The sensitization regime to amphetamine, modified from Giorgi et al. (2005a), consisted of 11 days of induction treatment followed by 14 days of withdrawal and a challenge (see Figure 1) with a factorial design 3 x 2 (3 strains, 2 treatments). Amphetamine sulfate (Sigma, St Louis, USA) was dissolved in 0.9% saline and injected i.p. in a volume of 1 ml/kg. A dose of 1 mg/kg was used for induction treatment and 0.25 mg/kg for the challenge. In order to minimize the number of animals we only used 2 groups per strain, one receiving amphetamine and the other receiving saline during the induction phase. The comparison of these two groups gives the most relevant information in terms of neurochemical adaptations that may occur secondarily to chronic amphetamine administration. In this protocol the sensitized response to amphetamine is determined by comparison of two treatment groups (saline or amphetamine 1 mg/kg) to an amphetamine challenge (0.25 mg/kg).

Animals were treated with amphetamine or saline during 11 days. Every 2 days, animals received the treatment and were left in their home cages, whereas on the other alternate days they were habituated to the test cage for 1 hour and left in the test cage for 2 hours after administration of the respective treatment, as administration of amphetamine in an environment different from the home cage has been shown to strengthen sensitization (Browman et al., 1998; Crombag et al., 2001). After 14 days of withdrawal the amphetamine challenge was performed and all animals were treated in the same way regardless of induction treatment. On that day, animals were weighed and carefully moved to the test room where they were left undisturbed for half an hour before the start of the behavioral test. Motor activity was recorded in the test cages for a period of 60 minutes prior to injection of saline. Thereafter, they were returned to the test cage and their behavior recorded for another 60 min period. Finally, the motor activity induced by the challenge (amphetamine 0.25 mg/kg) was studied during 1 hour. Habituation to the test cage as well as the response to vehicle administration was assessed prior to the amphetamine challenge to detect other phenomena, different than sensitization, that could increase the response of the animals to the amphetamine challenge like conditioned sensitization to environment. Motor activity was determined as previously described by Giménez-Llort et al. (2005) by means of a video computerized system. After this session animals were sacrificed by decapitation, their brains dissected out and frozen on dry ice. Samples were kept at -80ºC and thereafter sent to the Swedish laboratory.

Sections for in situ *hybridization histochemistry*

The brains from the animals of the behavioral study were used. Coronal sections (14 µm-thick) were cut in a cryostat as described previously (Guitart-Masip 2006). Equivalent sections were collected at 6 different levels (see Figure 2). The section levels were: L1.- bregma: 3.7 – 3.2 (prefrontal cortex); L2.- bregma 1.6 – 1.2 (nucleus accumbens (NAc), rostral caudate putamen, rostral cingulate cortex); L3.- bregma -0.8 – -0.92 (caudal caudate putamen, caudal

cingulate cortex); L4.- bregma -2.1 – -2.3 (rostral hippocampus, tail of the striatum and amygdala) L5.- bregma -3.60 – -3.8 (dorsal hippocampus); L6.- bregma -4.8 – -5.2 (ventral hippocampus, ventral tegmental area (VTA) and substantia nigra compacta) according to Paxinos and Watson (1998). We focused our gene analysis on the striatum, the mesoencephalic DAergic areas and the prefrontal cortex, as these brain areas have been the classically studied ones for their critical role in sensitization (Pierce and Kalivas, 1997;Vanderschuren and Kalivas, 2000). The striatum was studied at many different subdivisions, including the NAc, which may be differentiated by means of their cortical inputs (Willuhn et al., 2003). We also included the amygdala and the hippocampus as these areas project to the NAc (Kelley and Domesick, 1982;Mcdonald, 1991), are related to drug dependence (Everitt et al., 1999) and may play a role in the well characterized divergence in anxiety-like behaviors between the Roman rat strains (Driscoll et al., 1998). For comparison, we included other areas such as the motor cortex as a control where no changes secondary to chronic amphetamine treatment were expected.

Figure 2 should be placed here

Oligodeoxyribonucleotide probes

NGFI-A (nucleotides 4-49) was synthesized and purified through high-performance liquid chromatography (Thermo Electron GmbH, Ulm, Germany). This sequence, ^{35}S-labeled, has been used in published *in situ* studies (e.g. Kuzmin and Johansson, 1999). ENK (nucleotides 255–299; Zurawski et al., 1986), was synthesized and purified through high-performance liquid chromatography (MedProbe, Oslo, Norway). DYN (nucleotide 871-918; Civelli et al., 1985) was synthesized in an Applied Biosystems DNA synthesizer 38lA (Foster City, CA) and purified on an OPC-column (Applied Biosystems). Secretogranin (nucleotides 661-704) was synthesized by Pharmacia Biotech (Uppsala, Sweden). The sequence of this probe gives the same labeling pattern in rat and mouse brain as other probes for this target (Kuzmin and Johansson, 1999). PSD-95 (nucleotides 801-844; Cho et al., 1992) was synthesized and purified through high-performance liquid chromatography (Thermo Electron GmbH, Ulm, Germany). The specificity was checked by the addition of a 225x excess of unlabelled probe, which blocked the signal, whereas the signal was not influenced by a 225x excess of an unrelated oligonucleotide (data not shown).

In situ *hybridization histochemistry*

The analysis of mRNA levels was carried out by *in situ* hybridization as described elsewhere (Guitart-Masip et al. 2006). Briefly, Oligonucleotide probes were 3´- end labeled with [^{33}P]-dATP, the slide-mounted sections were incubated for 16-20 hours at 42°C with the labeled oligonucleotide probe. Following hybridization, the sections were washed, air-dried and apposed to Kodak Biomax MR (Amersham) film for 2-12 days. Finally, autoradiograms were analyzed with a Macintosh computer using the public domain NIH Image program (US National Institutes of Health; see http://rsb.info.nih.gov/nih-image).

Statistics

Behavioral study: Motor activity was expressed in mean (cm) ± SEM at 10 minutes intervals or during the whole session. For the challenge activity, a repeated measures ANOVA was used. Strain (SD-OFA, RLA-I and RHA-I) and induction treatment (amphetamine or saline) were the main factors and 10-minute intervals were the repeated factor. Separate analysis was done for each period of the challenge test: habituation, saline and amphetamine treatment, respectively. When Mauchly sphericity was not reached in repeated measures ANOVA, Huynh-Feldt correction was used. As a *post hoc* analysis a transversal Duncan test at each interval, comparing all 6 groups of strain and treatment, was used.
In situ hybridization histochemistry: Data from SD-OFA rats are presented separately as supplementary information and excluded from the analysis and further discussion. Data from *in situ* hybridization histochemistry was analyzed by means of two-way ANOVA with strain (RLA-I and RHA-I) and induction treatment (saline and amphetamine) as main factors. When appropriate, a Duncan test considering the 4 groups was performed as a *post hoc* analysis.

Results

1.- Initial response to amphetamine administration

Motor activity was measured for 1 hour after administration of the respective induction treatment (amphetamine or saline) on day 1 of the induction regime (see table 2). This allowed the assessment of initial response to amphetamine administration for all strains. Although motor activity was registered for 2 hours we only include the results of the first hour to allow comparison with the results obtained the day of the challenge. Two-way ANOVA with strain and treatment as main factors revealed that amphetamine increased locomotor activity in all strains (treatment effect: $F(1,51)=324.7$; $P<0.001$) besides the differences observed between strains in motor activity (strain effect: $F(2,51)=44.5$; $P<0.001$, and strain x treatment effect: $F(2,51)=17$ $P<0.001$). Amphetamine treated animals of all strains differed from the respective saline treated group, RLA-I rats displayed higher amphetamine induced motor activity than RHA-I and SD-OFA rats, and RHA-I rats displayed higher amphetamine induced motor activity than SD-OFA rats (Duncan test, $P<0.05$).

Table 2 should be placed here

2.- Behavioral analysis of the expression of sensitization to the amphetamine challenge

2.1. Spontaneous activity: 'Strain', 'interval' and 'interval x

strain' interaction effects found during the first phase of the behavioral test on day 26 [repeated measures ANOVA, F(2,46)=45.98, F(4.7,218.2)=255.98 and F(9.5,218,2)=3.77, respectively, all $P<0.001$] revealed differences in basal spontaneous activity levels, with the Roman strains being more active than SD-OFA rats (Duncan, $P<0.05$) at all intervals of this period of the test. The strains also differed in temporal course of habituation to the test cage. There was a lack of 'treatment' effect [F(1,46)=1.15; *n.s.*] or 'interval x treatment' effect [F(4,7,218.2)=0.523; ns] during this phase. (See Figure 3).

2.2. Saline administration: 'Strain', interval' and 'interval x strain' interaction effects [repeated measures ANOVA, F(2,46)=95.28, F(5,230)=78.45, F(10, 230)=5.88, respectively, all $P<0.001$] were also found after saline administration. The evolution of motor activity was similar to that described for spontaneous activity: the Roman strains were more active than SD-OFA rats at all intervals (Duncan test, $P<0.05$) and the strains also differed in their motor activity time course. It is also important to note here the lack of 'treatment' and 'interval x treatment' interaction effects [repeated measures ANOVA, F(5, 230)=2.09 and F(1,46)=0, respectively, both n.s.). (See Figure 3).

2.3. Challenge with amphetamine: 'Strain', 'interval' [repeated measures ANOVA, F(2,46)=88.92 and F(5,230)=85.34, respectively, both $P<0.001$) as well as 'interval x strain' interactions [F(10, 230)=2.01, $P=0.034$] were found in the challenge response to amphetamine. RLA-I rats developed more motor activity than RHA-I rats, and the latter more than SD-OFA rats (Duncan test, $P<0.05$). As expected, the induction treatment modified the response to the amphetamine challenge (behavioral sensitization) as shown by a significant 'treatment' effect [repeated measures ANOVA, F(1,46)=16.2, $P<0.001$]. However, this 'treatment' effect was only manifested in RHA-I and SD-OFA rats (Duncan test, $P<0.05$) whereas RLA-I rats showed the same motor activity after the amphetamine challenge regardless of the induction treatment. The 'interval x treatment' interaction effects [F(5,230)=5.3, $P<0.001$] also indicated that the expression of behavioral sensitization to amphetamine was dependent on the interval of the test. Thus, SD-OFA rats that received amphetamine as induction treatment developed greater motor activity to the challenge than their controls (induction with saline) only at the second 10 min interval. In the RHA-I rats, the expression of behavioral sensitization was longer, as the accentuated motor activity in the group that received amphetamine during induction persisted for 50 minutes (statistically significant in the first, second, third and fifth 10 min intervals). (See Figure 3).

Figure 3 should be placed here

3.- In situ *hybridization histochemistry*

In this report we consider results regarding saline-pre-treated *versus* amphetamine-pre-treated comparisons in RHA-I and RLA-I rats, stressing the use of these two strains as representative of the two extremes in vulnerability to behavioral sensitization. Therefore, the results for RLA-I and RHA-I are presented in Tables 1-3 in the text, whereas the results for SD-OFA rats are presented as supplementary material available at the *European Journal of Neuroscience* website.

3.1 NGFI-A mRNA in situ hybridization

Two-way ANOVA for strain and treatment detected a 'treatment' effect in several areas: the rostral dorsomedial striatum [F(1,31)=6.1 $P=0.019$], the rostral ventral striatum [F(1,31)=7.3 $P=0.012$], the rostral medial striatum [F(1,31)=13.3 $P=0.001$], the caudal medial striatum [F(1,31)=4.4 $P=0.045$], the piriform cortex [F(1,31)=8.7 $P=0.006$], the CA3 field of the dorsal hippocampus [F(1,30)=5.4 $P=0.028$], and the CeA (central nucleus of the amygdala) [F(1,28)=8.5 $P=0.008$]. In this latter area a 'strain x treatment' interaction was also detected [F(1,28)=18.6 $P<0.001$]. As shown in Table 1, the Duncan test indicates that the amphetamine pre-treated RHA-I rats showed an increased induction of NGFI-A after chronic amphetamine treatment in the rostral dorsomedial striatum, the rostral ventral striatum and the piriform cortex, whereas RLA-I rats had an increased induction of NGFI-A after chronic amphetamine treatment in the rostral medial striatum and the rostral ventral striatum. In the CeA, RLA-I rats that received amphetamine for the first time in the challenge showed much higher induction of NGFI-A than RLA-I rats that were chronically pre-treated with amphetamine during the induction phase (see table 3 and figure 4).

Figure 4 and Table 3 should be placed here

3.2 DYN mRNA in situ *hybridization*

Two-way ANOVA for strain and treatment detected treatment effect in the dorsal subdivision of the rostral striatum ['treatment' effect: F(1,30)=6.5 $P=.0.17$)] and an interaction of both factors in the medial subdivision of the rostral striatum ['strain x treatment' interaction: F(1,30)=6.9 $P=.014$)]. As shown in Table 4, a Duncan test revealed an increase only in the medial subdivision of the rostral striatum of amphetamine-pre-treated RHA-I rats when compared to the saline group.

Table 4 should be placed here

3.3 ENK mRNA in situ *hybridization*

Two-way ANOVA for strain and treatment detected treatment effects in the medial subdivision of the rostral striatum ['treatment' effect: F(1,26)=10 $P=.004$)] and in the central subdivision of the caudal striatum ['treatment' effect: F(1,26)=4.4 $P=.046$)]. As shown in Table 2, the Duncan test revealed an increase in ENK expression in the medial

subdivision of the rostral striatum of RHA-I rats induced by amphetamine, and an increase in ENK expression in the central subdivision of the caudal striatum of RLA-I treated with amphetamine during induction, when compared to the respective saline group.

3.4 Secretogranin mRNA in situ hybridization

Two-way ANOVA for strain and treatment revealed treatment effects in the NAc-core ['treatment' effect: $F(1,30)=5$ $P=.034$)], where a Duncan test indicated that amphetamine-pre-treated RHA-I rats exhibited increased expression when compared to the respective saline group (see Table 5). In the infraorbital cortex ['treatment' effect: $F(1,28)=6.6$ $P=.016$)] and in CA3 field of the ventral hippocampus ['treatment' effect: $F(1,30)=7.5$ $P=.0.11$)], a Duncan test detected that amphetamine-pre-treated RLA-I rats had increased secretogranin expression when compared to the saline group (see Table 3). The two-way ANOVA for strain and treatment also detected a treatment effect in the prelimbic cortex ['treatment' effect: $F(1,28)=6.2$ $P=.02$)] but the Duncan test did not detect any statistically significant difference between treatment groups.

3.5 PSD-95 mRNA in situ hybridization

As shown in Table 5, in the NAc-core ['strain x treatment' interaction: $F(1,29)=12.4$ $P=.002$)], a Duncan test revealed an increase in PSD-95 expression in amphetamine-pre-treated RHA-I rats, but a decrease in amphetamine-pre-treated RLA-I rats, when compared to their respective saline groups.

Table 5 should be placed here

Discussion

1.- Amphetamine administration effects: initial response and response after sensitization regime

Administration of 1mg/Kg of amphetamine on the very first day of the chronic sensitization regime induced higher motor activity than administration of saline in the three strains. Inbred RLA-I rats displayed higher amphetamine induced motor activity than inbred RHA-I rats and these latter rats displayed a higher amphetamine induced response than SD-OFA rats. The locomotor response to acute amphetamine in the inbred Roman strains contrasts with that of the outbred lines (see Introduction). Outbred RHA rats showed higher locomotor activation after amphetamine administration than outbred RLA rats (Giorgi et al., 1997; Lecca et al., 2004). This was not the first time that we observed such a high response to acute amphetamine in RLA-I rats (Cañete et al., 2003). However, this hyperreaction to amphetamine does not seem to be due to a generalized difference in dopamine-related mechanisms between inbred and outbred rats since the motor activity patterns induced by apomorphine (Giménez-Llort et al., 2005) and the differences in striatal dopamine receptors subtype densities (Guitart-Masip et al., in press) are maintained despite inbreeding. Moreover, experiments performed in our laboratory have systematically demonstrated that the typical difference between outbred RHA and RLA rats in the locomotor activity induced by novelty is there despite the inbreeding: inbred RHA rats show higher locomotor activity than inbred RLA rats when they are placed in the two different hole board tests (Escorihuela et al., 1999; Guitart-Masip et al., 2006), two different open field arenas (Escorihuela et al., 1999; Fernández-Teruel et al., 2002), the plus maze (Escorihuela et al., 1999), the shuttle box (Escorihuela et al., 1999), and during the first 20 minutes after placement in the activity test box (Giménez-Llort et al., 2005). Therefore, the acute response to amphetamine in the inbred Roman strains cannot be predicted by the motor response induced by novelty as it happens with the outbred lines (Giorgi et al., 1997; Lecca et al., 2004) and with the High Reactive (HR) and Low Reactive (LR) to novelty rats (Piazza et al., 1989). However, as discussed below, high or low motor response induced by novelty predicts appearance or lack of behavioral sensitization either in the outbred and inbred RHA and RLA rats respectively.

Spontaneous and saline-induced activities before amphetamine challenge revealed strain differences, with RHA-I and RLA-I being more active and showing slower habituation of motor activity than SD-OFA. This agrees with previous observations (Giménez-Llort et al., 2005). More interesting was the lack of treatment effect during these spontaneous and saline activity phases but its presence during the amphetamine challenge, the latter of which indicated that chronic amphetamine caused selective sensitization to the response to amphetamine but not other motor responses such as a conditioned response to the test cage or to the injection procedure. When induction treatment is administered in the same cages where rats are tested for the challenge with amphetamine, an increased response to a saline injection is generally described in amphetamine pre-treated rats when compared to saline pre-treated rats (Browman et al., 1998; Crombag et al. 2001). However, in the present experiment, amphetamine injections during the induction phase were paired with the test cages only every second day which may explain the lack of an increased response to the saline injection in amphetamine pre-treated rats.

Behavioral sensitization to amphetamine was observed in RHA-I and SD-OFA, but not in RLA-I rats, as an enhanced response to the challenge with 0.25 mg/Kg amphetamine. This very low dose of amphetamine has already been used to show differential behavioral sensitization between outbred RHA and RLA rats (Corda et al., 2005), and assured that the appearance of stereotypies would not mask the expression of locomotor sensitization. The fact that, in the present experiment, this dose allowed us to demonstrate behavioral sensitization in RHA-I and SD-OFA rats discards this as a subthreshold dose. Instead, this suggests that the

neurological adaptations underlying sensitization in RHA-I and SD-OFA rats have not occurred in RLA-I rats. Thus, described differences in susceptibility to amphetamine sensitization between outbred Roman lines (Corda et al., 2005; Giorgi et al., 2005a) have been maintained with inbreeding. Moreover, the phenomenon was longer-lasting in RHA-I rats (50 minutes) as compared to SD-OFA rats (10 minutes), indicating enhanced vulnerability to behavioral sensitization in the former. In addition, repeated amphetamine administration during the induction period produced a day-by-day increased response to amphetamine only in RHA-I rats whereas SD-OFA rats did not (Guitart-Masip et al., unpublished). Therefore, the RHA-I rats exhibit higher vulnerability to both induction and expression of behavioral sensitization to amphetamine.

The RLA-I rats were hyperreactive to amphetamine regardless of the induction treatment, but did not show any increase in locomotor activity secondary to repeated treatment with amphetamine. That can be clearly seen by looking at the locomotor time course after administration of the amphetamine challenge, which was not modified by the induction treatment in RLA-I rats although they showed much higher activity than the other two groups. Previous work showed that the lack of sensitization of motor activity in RLA-I rats is not due to motor-interfering stereotypes as at doses between 0.25 and 1mg/Kg of amphetamine, sniffing was the only stereotyped behavior observed (Cañete et al., 2003). Moreover, the locomotor response shown by RLA-I rats in the challenge was far from a ceiling effect since, in the present experiment, RLA-I rats that received 1mg/Kg of amphetamine on day 1 of the induction treatment showed a much higher motor activity than when they were injected with 0.25 mg/Kg of the drug on the challenge day. There is a general assumption equating higher acute response to psychostimulants to sensitized responses to the drug (for examples see: Weinshenker et al., 2002; Yao et al., 2004). This assumption may be based on two different associations: first, the well-established relationship between high reactivity to novelty, higher acute response to amphetamine and liability to self-administer this drug in rodents (Piazza et al., 1989; reviewed by Piazza et al., 1998); and, second, the theoretical relationship between behavioral sensitization and drug addiction (Robinson and Berridge 1993; 2001), especially the fact that animals chronically treated with amphetamine which display higher motor activity upon an amphetamine administration also show more liability to self-administer the drug (Vezina, 2004). However, previous work using receptor-selective antagonists and agonists have shown that, in rats, the acute response to amphetamine can be dissociated from the effects of a chronic treatment: the blockade of the motor effect of the drug does not necessarily impair the development of behavioral sensitization and, inversely, this phenomenon may not develop in animals that perform normal motor activity after amphetamine administration (Vanderschuren et al., 2000; 2003). Our study provides genetic evidence that supports this dissociation between acute and chronic response to amphetamine. The liability to self-administer amphetamine has never been studied in the Roman rats. However, behavioral sensitization is postulated to be a model of behavioral and neurochemical plasticity induced by chronic experiences with drugs of abuse that may be behind the high risk of relapse in abstinent drug addicts (Robinson and Berridge 2001). Studies performed in our laboratory demonstrated that whereas RHA-I rats drank ethanol voluntarily, RLA-I rats did not (Fernández-Teruel et al., 2002), a fact which gives support to the hypothesis that novelty-seeking RHA-I rats may show higher liability to drug self-administration than RLA-I rats. Differences in behavioral sensitization may represent different liabilities to the aforementioned behavioral and neurochemical plasticity induced by chronic amphetamine administration. Although it may be argued that the use of a more robust or extended sensitization regime could have increased the already enhanced motor response of RLA-I rats to the initial amphetamine administration, they did not show behavioral sensitization with the protocol used in the present work. Therefore, the molecular studies may reveal neuronal events that underlie appearance and lack of behavioral sensitization.

2.- NGFI-A neural activity map

Although it is clearly established that acute administration of psychostimulants like amphetamine and cocaine induce expression of immediate early genes like c-*fos* and NGFI-A in the striatum and several cortical areas (Bhat et al., 1992; Moratalla et al., 1992; Persico et al., 1993 ; Wang et al., 1995; Badiani et al., 1998; Gonzalez-Nicolini and McGinty, 2002; Uslaner et al., 2001), this response undergoes tolerance with chronic treatments (Hope et al., 1992; Persico et al., 1993; Steiner and Gerfen, 1993). A few studies have addressed immediate early genes responses to an amphetamine challenge after a withdrawal in a sensitization paradigm: chronic amphetamine treatment does not modify *c-fos* induction in most brain structures (Ostrander et al., 2003), and negative results are also reported for NGFI-A expression (Hu et al., 2002). RHA-I rats only showed salient effects in NGFI-A mRNA expression due to chronic amphetamine sensitization in restricted striatal areas, namely the rostral dorsomedial striatum and the rostral ventral striatum. Moreover, amphetamine pre-treated RLA-I rats did not show any increase in motor activity when compared to the saline pre-treated RLA-I rats which also showed increased NGFI-A mRNA expression in restricted striatal areas, namely the medial and the ventral subdivisions of the rostral striatum. Therefore, it is likely that the increase in motor activity observed in sensitization experiments does not correlate with any widespread induction of immediate early genes as measured here but with a switch in the cellular activity from matrix to striosomes, a neurochemical compartmentalization that defines subsets of cells inside the striatum (Moratalla et al., 1996;Vanderschuren et al., 2002). The lack of sensitized NGFI-A response in the nucleus accumbens in animals that showed behavioral sensitization after the amphetmamine challenge may seem contradictory

with several recent reports showing enhanced c-*fos* immunoreactivity in the intermediate area of the accumbens shell (Todtenkopf et al., 2002) or in the nucleus accumbens in general (Crombag et al 2002; Hope et al., 2006) upon a challenge with cocaine in sensitized animals. In a study with amphetamine, increased c-*fos* immunoreactivity was found preferably in the nucleus accumbens core (Hedou et al., 2002). However, the use of a different gene to study sensitization within a distinctive challenge protocol makes comparisons difficult. In fact, in all these experiments, c-*fos* immunoreactivity was studied in animals which received the challenge after 30 minutes habituation or without habituation at all whereas in our experiment rats where habituated for an hour and then injected with saline and observed for another hour before the actual amphetamine challenge.

Upon first exposure to amphetamine, RLA-I rats showed a strong induction of NGFI-A in the CeA, an effector area of fear responses (LeDoux, 1996) and an output structure of the amygdala (Pitkanen et al., 1997). The CeA may play a role in the reinforcing effects of drugs of abuse (Koob, 1999;Everitt et al., 1999). When RLA-I rats experienced amphetamine during induction, tolerance to this response was observed. In a previous report, Roozendaal et al. (1992) showed that RLA rats display higher autonomic and behavioral response than RHA as a consequence of local infusion of vasopressin or oxitocin into the CeA, suggesting that the CeA is more active in the former rat line. NGFI-A is induced in the lateral amygdala during fear conditioning (Malkani and Rosen, 2000) and pharmacological treatments that suppress this induction abolish the expression of conditioning. However, the unconditioned fear response is preserved and NGFI-A expression in the CeA is increased (Malkani and Rosen, 2001). Therefore, activation of the CeA might be a correlate of unconditioned fear induced by the first amphetamine experience in RLA-I and may be a neuronal event that prevents sensitization to occur in that strain. Similarly, rats that experienced amphetamine in their home cage showed c-*fos* induction in the CeA (Day et al., 2001) but did not develop behavioral sensitization if they were chronically treated in their home cage (Ostrander et al., 2003). The present study, thus, is the second time that such an association between activation of an immediate early gene in the central amydala upon first exposure to amphetamine and lack of sensitization is reported. More experiments are needed to understand the exact significance of this finding.

3.- Dopamine-related transcripts

In the present work, sensitized RHA-I rats showed an increase of DYN and ENK in the medial subdivision of the rostral striatum, which has a connectivity pattern similar to that of the NAc proper (Voorn et al., 2004). Numerous studies have shown that treatments with psychostimulants like amphetamine and cocaine increase mRNA expression levels of DYN in the striatum (Jaber et al., 1995; Wang and McGinty 1996; Reviewed by Steiner and Gerfen, 1998), and the same is true for ENK (Jaber et al., 1995; Wang and McGinty 1996; Mao and Wang 2003). Although opioid peptides, specially DYN, may have a putative role in the neuronal adaptations that lead to sensitization (Steiner and Gerfen, 1998; Shippenberg and Rea, 1997; but see also Vanderschuren et al., 2000), the lack of generalized changes in opioid peptides mRNA expression in the present work is consistent with a transient increase of DYN mRNA, namely during induction treatment (Steiner and Gerfen, 1993; Wang and McGinty, 1995; Willuhn et al., 2003; Svensson and Hurd, 1998; Reviewed by Steiner and Gerfen, 1998). ENK has been less studied and results are conflicting: after chronic amphetamine treatment, ENK was not induced in the striatum after the last amphetamine injection (Jaber et al., 1995), but the contrary was obtained in another study (Wang and McGinty, 1995). Moreover, when studying expression in long term sensitization to psychostimulants no variations in the total amount of ENK mRNA were reported (Wang and McGinty, 1995). To our knowledge, no other studies addressed the effect of an amphetamine challenge after withdrawal. What is the meaning of the present result, namely, an increased response of DYN and ENK to the challenege with amphetamine in sensitized RHA-I rats restricted to the medial subdivision of the rostral striatum? The opioid peptide system has been suggested to regulate mesolimbic dopaminergic activity (Spanagel et al., 1992) and cellular responsiveness in the striatum (Reviewed by Steiner and Gerfen, 1998). Pharmacological, lesion (Li et al., 1990) and gene knock out interventions (Giros et al., 1996) modifying the dopaminergic tone corroborate this compensatory role for opioid peptides in the striatum. The coincident up-regulation of both opioid peptides in the ventral striatum may be a correlate of adaptations in cellular responsiveness underlying vulnerability to sensitization in RHA-I.

4.- Secretogranin and PSD-95: synaptic activity markers

In RHA-I rats, behavioral sensitization was associated with higher secretogranin expression in the NAc-core. In RLA-I rats, the lack of behavioral sensitization in amphetamine-pre-treated animals was associated with an increase in expression in the infraorbital cortex which projects to the central subdivision of the caudal striatum (Berendse et al., 1992), an area where amphetamine-pre-treated but not sensitized RLA-I rats showed increased ENK mRNA. Secretogranin is a glycoprotein that serves as a presynaptic marker (Iwazaki et al., 2004) and whose mRNA is increased by chronic neuronal stimulation (Shen and Gundlach, 1996). Therefore, the present results suggest increases in presynaptic activity after amphetamine induction treatment. Presynaptic differences could possibly underlie the strain differences in vulnerability to behavioral sensitization.

Expression of behavioral sensitization in RHA-I rats correlated with increased PSD-95 mRNA levels in the NAc-core. In RLA-I, the lack of sensitization coincided with decreased PSD-95 mRNA in the same structure. PSD-95 is a scaffolding protein enriched in glutamatergic postsynaptic

density and binds to proteins associated with synaptic transmission (Sheng and Kim, 2002). PSD-95 expression is increased consequent to neuronal activity both at the mRNA level (Bao et al., 2004) and at the protein level (Skibinska et al., 2001; Bao et al., 2004) and may be a marker of neuronal activity at the glutamatergic synapse (Bao et al., 2004). In genetic and pharmacological models of cocaine sensitization, constitutive levels of PSD-95 are reduced in the striatum (Yao et al., 2004), and basal extracellular glutamate levels are diminished in the NAc (Baker et al., 2003). However, a cocaine challenge after long withdrawal restores extracellular glutamate levels in sensitized animals (Baker et al., 2003). It is relevant to notice that Yao et al. (2004) found PSD-95 down-regulation in animals that had not been challenged and, therefore, the results of the present work can not be directly compared with those described by Yao et al. (2004). It may be that a decreased basal level of glutamate as observed in animals sensitized with cocaine (Baker et al., 2003) correlates with a basal down-regulation of PSD-95 as described by Yao et al. (2004). However, we measured PSD-95 mRNA levels after the amphetamine challenge, which presumably increased glutamate levels and induced PSD-95. In this sense, the coexistence of changes in secretogranin and PSD-95 mRNA in the NAc-core of sensitized RHA-I rats is suggestive of plastic changes at the glutamatergic synapses. In this regard, administration of an inhibitor of the glutamate transporter, which enhances glutamate levels in the NAc, enables a challenge of a D_1 agonist to fulfil amphetamine administration in amphetamine pre-treated rats (Kim et al., 2001).

5.-Possible framework to understand differential vulnerability to behavioral sensitization

Exposure to amphetamine in the RHA lines/strains, induces a more pronounced DA release in the NAc-shell (Lecca et al., 2004), which may lead to higher gating of cortical activity through the NAc-shell and increased cortical activity to the NAc-core (Zahm, 1999). Increased DA release in the NAc-core occurs in outbred RHA rats sensitized to amphetamine (Giorgi et al., 2005a). Plasticity at the striatal glutamatergic synapses is dependent on the local concurrence of DA and glutamate (White, 1996; Berke and Hyman, 2000). The increased secretogranin and PSD-95 mRNA expression in the NAc-core in sensitized RHA-I rats lend some support to the hypothesis that drugs of abuse take over the system that physiologically signals reward and motivational learning (Terenius, 1998;Berke and Hyman, 2000), via processes related to behavioral sensitization (Robinson and Berridge, 1993).

The lack of behavioral sensitization in RLA-I rats is associated with several neurochemical differences but their interrelation and biological relevance remains unclear. Further research will be required to better understand such phenotypical characteristics of RLA-I rats. First, the amphetamine challenge caused high induction of NGFI-A mRNA in the CeA of RLA-I rats receiving amphetamine for the first time; second, the lack of sensitized behavior in RLA-I rats may be related to a dampened response in accumbal glutamatergic synapses, as RLA-I rats chronically treated with amphetamine showed down-regulation of PSD-95 mRNA in the NAc-core; and third, amphetamine-pre-treated RLA-I rats show increased secretogranin mRNA in the infraorbital cortex and increased ENK mRNA in the central caudal striatum.

To conclude, the present results suggest that vulnerability to behavioral sensitization is not only a matter of initial sensitivity or the magnitude of neurochemical effects but may reflect the involvement of distinctive cellular adaptations at particular brain locations.

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Table 1: Motor response to saline administration previous sensitization treatment
This table shows the amount of motor activity (cm) displayed by each group of strain and treatment after a saline injection on day 0, before the sensitization regime begun. Animals were allocated to a treatment group by matching according to the motor activity that they performed after a saline injection so that the two groups of each strain displayed the same amount of locomotor activity after this saline injection. No treatment [F(1,51)=0.2; P=0.66] or strain x treatment [F(2,51)=0.1; P=0.911] effect was detected using a two-way ANOVA analysis.

Allocated induction treatment	Saline	Amphetamine
SD-OFA (n=10 per each group)	3426.8 ± 264.2	3430.5 ± 288.5
RLA-I (n=8 per each group)	4573.5 ± 743.5	4707 ± 933.9
RHA-I (n=8 per each group)	5088.8 ± 583.3	5869.7 ± 506.4

Table 2: Initial response to amphetamine administration
This table shows the amount of motor activity (cm) displayed after administration of saline or amphetamine on day 1 of the sensitization regime. Animals were habituated to the test cage for 1 hour and thereafter they were injected with the respective sensitization treatment and their motor activity registered for 1 hour. The statistical analysis was performed using a two-way ANOVA analysis with Duncan *post hoc* test: * p<.05 compared to the respective saline group and # p<.05 compared to all other amphetamine treated groups.

	Saline	Amphetamine
SD-OFA (n=10 per each group)	4241.8 ± 331.3	10806.1 ± 499.5 *#
RLA-I (n=8 per each group)	6725.8 ± 843.7	21496.5 ± 1099.2 *#
RHA-I (n=8 per each group)	5980.4 ± 504.7	16242 ± 925.1 *#

Table 3: NGFI-A *in situ* hybridization.
Results of *in situ* hybridization for NGFI-A gene transcripts in saline and amphetamine pre-treated RHA-I and RLA-I rats. The pre-treated rats were challenged with amphetamine before analysis. Results are expressed as mean optical density in the specific areas ± SEM. L1-L6 refers to the anatomical level as described in the materials and methods. The number next to each area corresponds to the identification number in figure 2. The statistical analysis was performed using a two-way ANOVA analysis with Duncan *post hoc* test: * p<.05 compared to the respective saline pre-treated group.

Strain	RLA		RHA	
Induction treatment	Saline	Amphetamine	Amphetamine	Saline
L1: Infraorbital (1)	94.6 ± 3	97 ± 2.4	88.7 ± 2.6	89 ± 4.4
L1: Prelimbic/infralimbic (2)	84.3 ± 4.5	89.8 ± 3.2	73.2 ± 3.3	81.7 ± 3.5
L2: Rostral Caudate putamen				
Dorsolateral (3)	54.7 ± 2.7	60.2 ± 2.8	54.3 ± 1.4	55.4 ± 3.2
Dorsal (4)	60.3 ± 2.3	63.8 ± 2.1	52.3 ± 2	56.2 ± 3
Dorsomedial (5)	60.6 ± 1.7	61.9 ± 1.6	49.2 ± 0.8	55.5 ± 1.9 *
Medial (6)	48 ± 2.8	55.4 ± 1.6 *	43.8 ± 1.2	52.8 ± 2.9
Ventral (7)	44.2 ± 2.8	53.7 ± 3.4 *	42.8 ± 2.7	48.3 ± 2 *
L2: Nucleus Accumbens				
Core (8)	35.6 ± 2.5	40.8 ± 4.2	28.9 ± 1.6	35 ± 3.1
Shell medial (9)	48.6 ± 4.1	49.8 ± 1.9	40.4 ± 3.2	47.5 ± 3.4
Shell ventral (10)	35 ± 2.9	39.9 ± 3.5	33.9 ± 3.2	36.8 ± 2.5
L2: Olfactory tubercle (11)	48.2 ± 3.2	54.4 ± 5.6	48.3 ± 3	48.8 ± 2.7
L2: Cingulate cortex (12)	76.3 ± 2.7	80.7 ± 2.3	63.7 ± 2.9	66.6 ± 1.8
L2: Motor cortex (13)	66.9 ± 2.4	71 ± 3.3	61.3 ± 1.4	66 ± 2.1
L2: Sensorial cortex (14)	59.5 ± 3	62.7 ± 3.9	57.7 ± 2.4	63 ± 4.7
L2: Piriform cortex (15)	115.9 ± 3.6	127.5 ± 2.8	109.9 ± 5.7	123.3 ± 4.3 *
L3: Caudal Caudate putamen				
Medial (16)	46.3 ± 2.4	50.6 ± 2.4	45.5 ± 2.7	51.2 ± 1.9
Dorsal (17)	51.6 ± 3.6	60.9 ± 3.1	47.4 ± 3.5	50.6 ± 3.1
Dorsolateral (18)	54.9 ± 3.5	51.5 ± 2.6	48.3 ± 1.8	50.1 ± 2.6
Ventrolateral (19)	59.3 ± 3.8	57.6 ± 2.3	53.6 ± 2.5	50.9 ± 2.4
Ventral (20)	47.6 ± 2.3	46.5 ± 2.9	47.4 ± 1.9	43.3 ± 2.6
Central (21)	37.1 ± 1.8	44.3 ± 1.9	35.2 ± 2	35.6 ± 1.9
L3: Cingulate cortex (22)	76.8 ± 2	77 ± 1.6	65.1 ± 2.4	65.4 ± 4.4
L4: Amygdala				
Central Amygdala (23)	80 ± 8.4	39.1 ± 2.4 *	52.6 ± 4.9	60.6 ± 2.1
Basolateral amygdala (24)	57.5 ± 4.2	59.4 ± 4.8	57 ± 4.4	50.7 ± 4.4
Lateral amygdala (25)	61.1 ± 5	68.2 ± 4.3	57.5 ± 3.9	55.3 ± 5.1
L4: Caudate putamen tail (26)	45.8 ± 2.9	47 ± 3.1	43.2 ± 2.9	48.6 ± 4
L4: Rostral hippocampus				
Dentate gyrus (27)	53.2 ± 2.7	55.7 ± 3.5	55.2 ± 3.7	56 ± 3.7
CA1 (28)	83.3 ± 7.2	95.6 ± 12.2	78.6 ± 5.7	91.3 ± 7.2
CA3 (29)	83.3 ± 5.4	89.9 ± 4.7	81.3 ± 2.6	87.2 ± 4.9
L5: Dorsal hippocampus				
Dentate gyrus (30)	56.8 ± 3.2	61.6 ± 3.6	57.6 ± 3.1	58.6 ± 2.5
CA1 (31)	145.3 ± 6.7	159 ± 3.2	132.6 ± 4.9	135.5 ± 5.9
CA3 (32)	73.1 ± 5.3	79.5 ± 2.7	70.1 ± 3.4	83 ± 4.8
L6: Ventral hippocampus				
Dentate gyrus (33)	5.5 ± 3	49.2 ± 3.2	53 ± 3.5	49.9 ± 4.1
CA1 (34)	88.7 ± 6.4	93.7 ± 6.6	106.4 ± 5.6	96.9 ± 5.7
CA3 (35)	81.4 ± 4.3	78.7 ± 3.4	83.7 ± 5.7	73.8 ± 6.9
L6: VTA (36)	24.2 ± 2.7	26.2 ± 3	25 ± 4.3	23.6 ± 1.4
L6: SNR (37)	23.6 ± 2	26.1 ± 3.2	29.5 ± 2.3	25.6 ± 2.3

Table 4: DYN and ENK *in situ* hybridization.
Results of *in situ* hybridization for DYN and ENK gene transcripts in saline and amphetamine pre-treated RHA-I and RLA-I rats. The pre-treated rats were challenged with amphetamine before analysis. Results are expressed as mean optical density in the specific areas ± SEM. L1-L6 refers to the anatomical level as described in the materials and methods. The number next to each area corresponds to the identification number in figure 2. The statistical analysis was performed using two-way ANOVA analysis with Duncan *post hoc* test: * p<.05 compared to the respective saline pre-treated group.

	DYN				ENK			
Strain	RLA		RHA		RLA		RHA	
Induction treatment	Saline	Ampheta.	Saline	Ampheta.	Saline	Ampheta.	Saline	Ampheta.
L1: Infraorbital (1)	37.2 ± 3.4	38 ± 1.5	43.2 ± 2.9	44.9 ± 2.9	48.3 ± 3.3	50.5 ± 5.5	49 ± 2.9	53.4 ± 3.6
L1: Prelimbic/infralimbic (2)	37.3 ± 2.3	31.2 ± 2.1	36.6 ± 2.5	39.7 ± 2.8	57.1 ± 5.9	49.7 ± 8	43.2 ± 2.9	45.1 ± 5.6
L2: Rostral Caudate putamen								
Dorsolateral (3)	54.5 ± 4.3	56.9 ± 2.6	45.6 ± 1.3	53.4 ± 3.7	174.8 ± 1.9	174.7 ± 2.1	170.5 ± 5.4	172.7 ± 3.6
Dorsal (4)	62.2 ± 4	71.2 ± 2.7	54.3 ± 2.4	61.8 ± 3.6	172.6 ± 1.5	174.4 ± 2.4	165.7 ± 3.1	171.4 ± 2.8
Dorsomedial (5)	57 ± 2.7	53.2 ± 1.8	54.3 ± 3	59.7 ± 2.6	169.9 ± 2.3	170.6 ± 2.9	162.9 ± 4.7	162.5 ± 3.2
Medial (6)	79 ± 2.5	74.8 ± 2.2	70.5 ± 2.1	80.3 ± 3.5 *	165 ± 1.1	171.4 ± 2.8	163.6 ± 3.1	173.1 ± 2.3 *
Ventral (7)	99.6 ± 4.3	96.7 ± 5.7	100 ± 3.4	108.7 ± 4.6	172.9 ± 2.6	174.6 ± 3.6	173.7 ± 2.5	172 ± 2.4
L2: Nucleus Accumbens								
Core (8)	71.3 ± 5	78.4 ± 3	74.3 ± 4.8	81.6 ± 3.7	169.7 ± 4.7	169.2 ± 6.5	162.1 ± 6.5	165.8 ± 6
Shell medial (9)	109 ± 1.4	104.4 ± 2.7	121.6 ± 5.4	121 ± 1.8	128.3 ± 7.6	137.7 ± 4.7	137.4 ± 5.8	149.7 ± 4.4
Shell ventral (10)	77.8 ± 4	71.3 ± 5.4	86.1 ± 4.5	86 ± 1.2	105 ± 29.1	135.4 ± 6.2	130.4 ± 4.9	141.5 ± 5.1
L2: Olfactory tubercle (11)	69.5 ± 2.8	62.9 ± 4.5	72.6 ± 2.9	66.5 ± 4.4	170.1 ± 2.7	169.5 ± 3	169.1 ± 1.6	166 ± 5.4
L2: Cingulate cortex (12)	24.6 ± 0.9	23.1 ± 1.2	23 ± 1	21.4 ± 1.2	27.6 ± 1.4	28.1 ± 1.1	29 ± 1.1	30.3 ± 1.4
L2: Motor cortex (13)	23.2 ± 1	20.4 ± 1.9	22.2 ± 1.3	23.3 ± 1.7	19.5 ± 1.1	18.6 ± 1.6	20.3 ± 1.5	20.6 ± 1.1
L2: Sensorial cortex (14)	26.6 ± 1.4	23.3 ± 2.1	25.7 ± 1.7	22.4 ± 2.4	24.1 ± 1.7	25.1 ± 0.7	21.6 ± 1.5	22.2 ± 1.4
L2: Piriform cortex (15)	19.9 ± 1.9	16.8 ± 1.8	20.7 ± 1	18.4 ± 2.3	74.9 ± 8.3	72.9 ± 3.7	76.6 ± 9.3	77.1 ± 6.2
L3: Caudal Caudate putamen								
Medial (16)	77.6 ± 3	76.2 ± 3.8	79.3 ± 4.2	75.7 ± 3.5	174.2 ± 3.3	171.3 ± 3.8	168.4 ± 3.9	172.9 ± 1.4
Dorsal (17)	53.5 ± 3.9	53.3 ± 2.2	52.5 ± 4.1	53.9 ± 2.6	162 ± 3.2	163.6 ± 3.4	152.3 ± 4	162.7 ± 2.2
Dorsolateral (18)	49.8 ± 2.8	51.4 ± 2.4	45.4 ± 3.6	49.7 ± 2.6	168.6 ± 1.5	169.5 ± 2.5	165.3 ± 1.4	164.3 ± 1.9
Ventrolateral (19)	70.1 ± 1.8	62.9 ± 2.3	60.7 ± 1.7	61.2 ± 2.4	164.5 ± 3.2	166.8 ± 3	163.3 ± 1.6	166.9 ± 2.8
Ventral (20)	118.3 ± 3.8	117.5 ± 5.5	114 ± 5.5	119.7 ± 4.6	173.5 ± 3.7	176.4 ± 3.7	176.6 ± 2.2	178.6 ± 2.4
Central (21)	43.4 ± 3.5	51.6 ± 3.1	39.5 ± 2.2	43 ± 3.4	135.8 ± 5.3	153.3 ± 5.5 *	134.5 ± 4.4	139.7 ± 5.9
L3: Cingulate cortex (22)	21.9 ± 1.3	18.1 ± 1.6	20.2 ± 1.5	19.5 ± 0.7	32.2 ± 2.1	31.9 ± 2.1	46.3 ± 3.4	49.9 ± 2.5
L4: Amygdala								
Central Amygdala (23)	70.1 ± 6.9	75.7 ± 8.4	97.8 ± 9.1	77 ± 11.4	55.5 ± 7.7	57.5 ± 6.4	148.3 ± 6.4	149.7 ± 5.3
Basolateral amygdala (24)	21.7 ± 2.7	19.9 ± 1	20.6 ± 1.9	20 ± 2.1	102.7 ± 7.1	100.4 ± 9.7	68.5 ± 7.6	62.7 ± 10
Lateral amygdala (25)	24 ± 2.6	26.4 ± 5.3	33.2 ± 4.3	27.9 ± 4.3	25.1 ± 3.6	28.4 ± 3.9	26.5 ± 2.8	31.6 ± 6.4
L4: Caudate putamen tail 26)	78.2 ± 5.8	87.4 ± 6.4	73.9 ± 2.6	67.4 ± 5.8	132.3 ± 8.3	145.2 ± 6.3	136.9 ± 6.4	140.1 ± 7.5
L4: Rostral hippocampus								
Dentate gyrus (27)	55.1 ± 4.2	70 ± 10.5	81.4 ± 8.3	89.9 ± 5.7	9.2 ± 3.9	38.8 ± 7.1	24.7 ± 1.8	29.4 ± 4.1
CA1 (28)								
CA3 (29)								
L5: Dorsal hippocampus								
Dentate gyrus (30)	167.3 ± 5.1	166.7 ± 7	167.4 ± 3.9	167.3 ± 1.7	55.4 ± 2.8	53.5 ± 3.6	46.4 ± 3.8	49.9 ± 4.9
CA1 (31)								
CA3 (32)								
L6: Ventral hippocampus								
Dentate gyrus (33)	164.9 ± 7.6	161.3 ± 4.3	158.2 ± 5.8	153.1 ± 7.8	49.6 ± 3.8	57.9 ± 4.4	46.1 ± 4.3	39.7 ± 4.4
CA1 (34)	35.3 ± 3.6	34.7 ± 3.5	38.2 ± 4	33.4 ± 5.2				
CA3 (35)								
L6: VTA (36)	16.8 ± 1.9	20 ± 3.2	17.4 ± 1.7	19.1 ± 2.3				
L6: SNR (37)	13.2 ± 1.5	16.3 ± 1.6	15.4 ± 1.5	14.3 ± 1.6				

Table 5: Secretogranin and PSD-95 *in situ* hybridization.
Results of *in situ* hybridization for secretogranin and PSD-95 gene transcripts in saline and amphetamine pre-treated RHA-I and RLA-I rats. The saline and amphetamine pre-treated rats were challenged with amphetamine before analysis. Results are expressed as mean optical density in the specific areas ± SEM. L1-L6 refers to the anatomical level as described in the materials and methods. The number next to each area corresponds to the identification number in figure 2. The statistical analysis was performed using two-way ANOVA analysis with Duncan *post hoc* test: * p<.05 compared to the respective saline pre-treated group.

	Secretogranin				PSD-95			
	RLA		RHA		RLA		RHA	
	Saline	Amph	Saline	Amph	Saline	Ampheta.	Saline	Ampheta.
L1: Infraorbital (1)	58.8 ± 2.8	69 ± 2.3 *	53.5 ± 3.2	57.3 ± 2.4	58.9 ± 1.7	61.3 ± 2.4	64.5 ± 2.1	69.6 ± 2.9
L1: Prelimbic/infralimbic (2)	56.5 ± 3.2	68.2 ± 4.4	52.9 ± 3.3	60.6 ± 4.6	61.4 ± 2.8	59.4 ± 2.1	66.3 ± 2.8	72.4 ± 2.9
L2: Rostral Caudate putamen								
Dorsolateral (3)	39.2 ± 2.4	32.8 ± 3	31.7 ± 2.8	31.6 ± 2.9	63.8 ± 2.1	62.9 ± 3.1	64.6± 2.4	66.5 ± 2.3
Dorsal (4)	44.1 ± 1.9	41.9 ± 2.3	38.9 ± 3.2	40.7 ± 1.8	59.4 ± 2.4	61.5 ± 2.2	61.1 ± 1.8	58.5 ± 3.1
Dorsomedial (5)	38.6 ± 1.6	39.7 ± 2.9	37.8 ± 1.8	42.6 ± 1.9	56.9 ± 3.2	54.1 ± 2.5	61.6 ± 2.5	56.7 ± 2.4
Medial (6)	51.1 ± 2.8	46.5 ± 2.5	43.3 ± 2.3	48.4 ± 2	62.2 ± 1.7	62.7 ± 3	63.5 ± 1.6	67.2 ± 2.2
Ventral (7)	47.9 ± 3	42.3 ± 2.8	39.5 ± 3.5	43.4 ± 2.9	68.8 ± 1.9	70.8 ± 1.3	74.2 ± 4	74.1 ± 2
L2: Nucleus Accumbens								
Core (8)	53.7 ± 4.4	57 ± 2.8	46 ± 5.1	60.1 ± 3.1 *	68.1 ± 3.5	57.5 ± 2.7 *	62.4 ± 4	75.3 ± 3.1 *
Shell medial (9)	89.1 ± 3	89.9 ± 3.8	86.5 ± 5.5	89.5 ± 2.5	59.8 ± 1.3	56.8 ± 2.3	60.2 ± 1.5	64 ± 2
Shell ventral (10)	45.1 ± 2.7	40.6 ± 3	43.5 ± 4.6	45.5 ± 2.2	61.2 ± 2.3	52.7 ± 2.8	61.5 ± 2.8	62.7 ± 2.3
L2: Olfactory tubercle (11)	57.9 ± 2.8	61.8 ± 3.5	60.7 ± 6.9	58.9 ± 5.7	91.5 ± 2.6	82.8 ± 4.6	86.7 ± 4.7	91.5 ± 2
L2: Cingulate cortex (12)	45.3 ± 2.2	48.3 ± 1.8	39.1 ± 1.6	41.7 ± 1	68.5 ± 1.2	66.5 ± 2	70.6 ± 1.2	72.6 ± 1.1
L2: Motor cortex (13)	34.3 ± 1.9	35 ± 1.2	34.3 ± 2.3	36.6 ± 1.3	60.2 ± 1.7	58.9 ± 1.7	61.3 ± 2	63.7 ± 2.1
L2: Sensorial cortex (14)	28.4 ± 1.4	29.1 ± 0.8	26.7 ± 1.3	28.8 ± 1.5	57.6 ± 1.4	55.8 ± 1.8	58.8 ± 1.4	58.2 ± 0.9
L2: Piriform cortex (15)	57 ± 3	66.9 ± 4.7	59.4 ± 4.8	65.6 ± 2.6	93.8 ± 2.3	98.7 ± 4.5	93.9 ± 3.2	95.2 ± 3.3
L3: Caudal Caudate putamen								
Medial (16)	39.1 ± 3.3	38.4 ± 2.8	32.6 ± 2.5	36.5 ± 3.2	59.1 ± 2.8	59.2 ± 2.6	65.4 ± 1.7	66.1 ± 2.2
Dorsal (17)	35.1 ± 1.4	37.6 ± 1.9	30.2 ± 2.6	32.7 ± 1.9	53 ± 2.2	58.1 ± 1.7	56.5 ± 2	58 ± 2.8
Dorsolateral (18)	33 ± 2.8	32.5 ± 1.3	27.3 ± 1.2	32.1 ± 2.3	55.8 ± 2.7	52.8 ± 2.2	59.1 ± 2.6	62.3 ± 2.1
Ventrolateral (19)	34.3 ± 1.3	37.1 ± 2.1	34.8 ± 1.2	30 ± 1.9	58.6 ± 2.3	58.5 ± 3.2	62.2 ± 1.9	62.7 ± 2.3
Ventral (20)	62.8 ± 3.9	57.1 ± 2.8	64.1 ± 3.5	62.9 ± 4.6	64.8 ± 2.6	59.2 ± 2.8	64 ± 1.7	72.2 ± 3.1
Central (21)	26.7 ± 1.6	30.4 ± 3.7	23.4 ± 1	25.2 ± 3.8	49.3 ± 2.3	49 ± 2.8	49.4 ± 2.2	51.3 ± 2.7
L3: Cingulate cortex (22)	40.3 ± 1	43.1 ± 1.3	37 ± 1.5	39.9 ± 1.7	61.9 ± 0.7	62.7 ± 1.4	65.5 ± 1	66.7 ± 1.6
L4: Amygdala								
Central Amygdala (23)	115.2 ± 5	104.8 ± 13.2	125.3 ± 7.1	118 ± 4.6	53.9 ± 2.8	58.4 ± 2.5	59.5 ± 3	59.1 ± 2
Basolateral amygdala (24)	68 ± 5.1	74.4 ± 4.8	65.7 ± 6.8	60.3 ± 7.1	56.6 ± 2.6	61.1 ± 2.5	60.3 ± 1.1	58.3 ± 3.5
Lateral amygdala (25)	41.2 ± 3.7	47.1 ± 6.8	46.2 ± 5.1	39 ± 4.9	54.7 ± 3.4	57.4 ± 3.5	59 ± 2.3	62.5 ± 4.4
L4: Caudate putamen tail 26)	40.6 ± 4	44.3 ± 5.6	37.3 ± 4.9	38.8 ± 4.6	48.3 ± 3.9	58.4 ± 1	55.4 ± 2.5	54.8 ± 2.2
L4: Rostral hippocampus								
Dentate gyrus (27)	121.1 ± 5.7	135.3 ± 7.7	137 ± 4.9	133.3 ± 5.2	92.9 ± 3.6	89.5 ± 4.5	100.5 ± 5.2	110.6 ± 7
CA1 (28)	101.6 ± 8.6	105.5 ± 16.1	88.6 ± 9.9	99.6 ± 10	90.4 ± 4.6	89.2 ± 5.7	87.2 ± 2.7	95.9 ± 6.7
CA3 (29)	96.9 ± 3.7	91.3 ± 4.4	93.3 ± 4	94.4 ± 4.5	80.7 ± 2.8	78.5 ± 2.7	80.6 ± 3	82.2 ± 1.9
L5: Dorsal hippocampus								
Dentate gyrus (30)	170.5 ± 4.8	160.3 ± 4.8	170.2 ± 3.6	169.4 ± 2.4	134.5 ± 4.2	133.9 ± 3.3	135.8 ± 4.3	129.5 ± 3.9
CA1 (31)	170.4 ± 4.1	166.8 ± 3.3	119.1 ± 4.6	129.6 ± 3.4	118.8 ± 4.5	120.2 ± 3.4	115.4 ± 4.1	113.8 ± 4.1
CA3 (32)	121.8 ± 7.8	114.9 ± 5.2	116.1 ± 4.6	122 ± 4.7	91.4 ± 4.4	93.9 ± 3.8	88.5 ± 3.5	86.5 ± 3.3
L6: Ventral hippocampus								
Dentate gyrus (33)	159.8 ± 9.6	165.8 ± 7	157.1 ± 7.9	149.6 ± 4.9	116.5 ± 5.4	120.9 ± 4.4	115.8 ± 3.5	118.7 ± 5
CA1 (34)	168.2 ± 4.9	164 ± 9.2	164.2 ± 4.4	171.8 ± 5.1	114.7 ± 2.2	112.3 ± 6.5	115.3 ± 4.1	109.2 ± 4.5
CA3 (35)	172.9 ± 2.1	185.5 ± 4.1 *	168.8 ± 4.4	176.1 ± 3.7	114 ± 2	112.5 ± 3.3	109.6 ± 3.6	101.3 ± 5.2
L6: VTA (36)	99.1 ± 3.4	93.2 ± 2.9	91.4 ± 3.8	92.5 ± 5	31 ± 1.7	33.4 ± 1.8	32.2 ± 2	32.9 ± 2.2
L6: SNR (37)	20.4 ± 1.4	26.9 ± 2.4	19.9 ± 1.5	21.4 ± 2.6	21.4 ± 1.6	20.7 ± 2.5	21.4 ± 1	21.3 ± 1.5
L6: SNC (38)	59.2 ± 3.5	62.5 ± 7.9	58.7 ± 3.7	63.8 ± 4.6	39.2 ± 1.6	36 ± 4	36.7 ± 1.8	35.3 ± 2.1

Figure 1: Description of the behavioral sensitization regime.

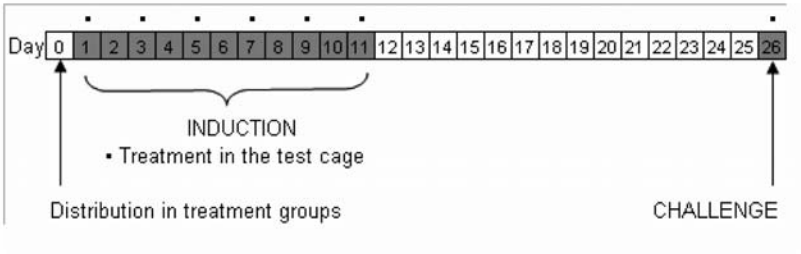


Figure 2: Anatomical location of measurements of the *in situ* hybridization experiments.
In this diagram the approximate location of measured brain areas for the *in situ* hybridization experiments are depicted in numbers. (A) **L1** (bregma 3.7 mm): 1.- infraorbital cortex; 2.- prelimbic cortex. (B) **L2** (bregma 1.6 mm): 3.- rostral dorsolateral striatum 4.- rostral dorsal striatum; 5.- rostral dorsomedial striatum; 6.- rostral medial striatum; 7.- rostral ventral striatum; 8.- NAc-core; 9.- NAc-shell medial portion; 10.- NAc-shell ventral portion; 11.- olfactory tubercle; 12.- cingulate cortex (Cg1. Cg2 rostral); 13.- motor cortex; 14.- sensory cortex; 15.- piriform cortex. (C) **L3** (bregma -0.8 mm): 16.- caudal medial striatum; 17.- caudal dorsal striatum; 18.- caudal dorsolateral striatum; 19.- caudal ventrolateral striatum; 20.- caudal ventral striatum; 21.- caudal central striatum; 22.-cingulate cortex (Cg1. Cg2 caudal). (D) **L4** (bregma -2.3 mm): 23.- CeA; 24.- basolateral amygdala; 25.- lateral amygdala; 26.- tail of the striatum; 27.- dentate gyrus of rostral hippocampus; 28.- CA1 of rostral hippocampus; 29.- CA3 of rostral hippocampus. (E) **L5** (bregma -3.6 mm): 30.- dentate gyrus of dorsal hippocampus; 31.- CA1 of dorsal hippocampus; 32 CA3 of dorsal hippocampus. (F) **L6** (bregma -4.8 mm): 33.- dentate gyrus of ventral hippocampus; 34.- CA1 of ventral hippocampus; 35.- CA3 of the ventral hippocampus; 36.- VTA; 37.- Substantia nigra pars reticulata; 38.- Substantia nigra pars compacta.

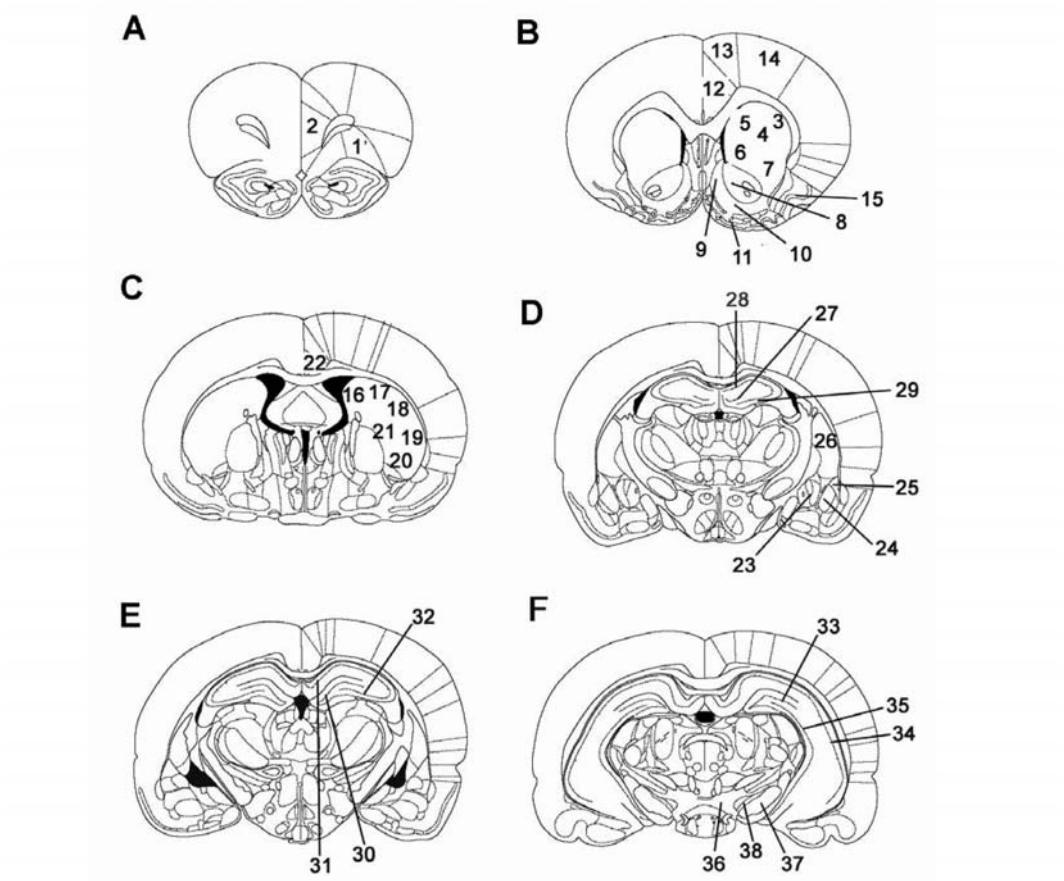


Figure 3: Expression of sensitization on the amphetamine challenge.
The behavioral results obtained on the challenge day are depicted here. The time course of the motor activity for each strain is shown. The first 60 minute period corresponds to the motor activity during habituation, the second 60 minute period corresponds to the motor activity developed after the saline challenge and the third 60 minute period corresponds to the motor activity developed after the amphetamine challenge. The arrows mark the injection times. Accumulated motor activity after the amphetamine challenge is depicted for each group of strain and treatment in a separate panel. * $P<0.05$ in the Duncan test compared to the respective saline pre-treated group.

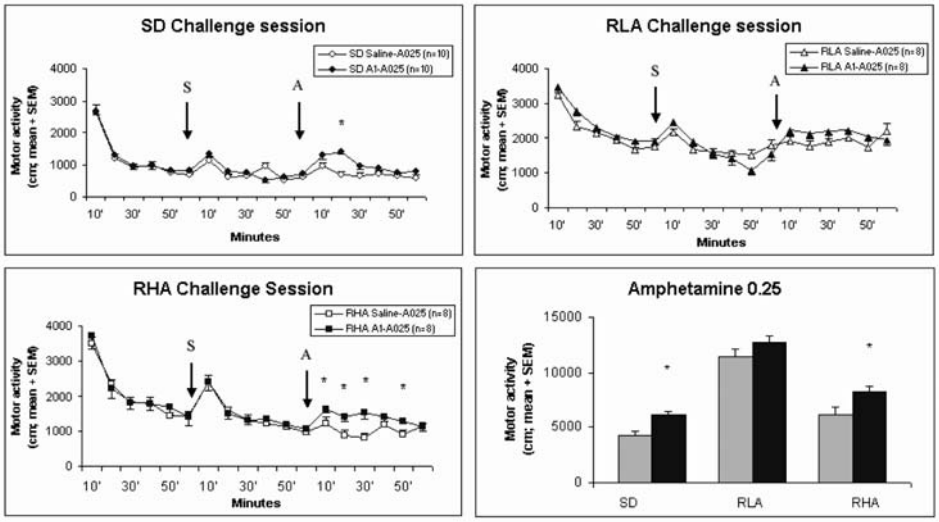
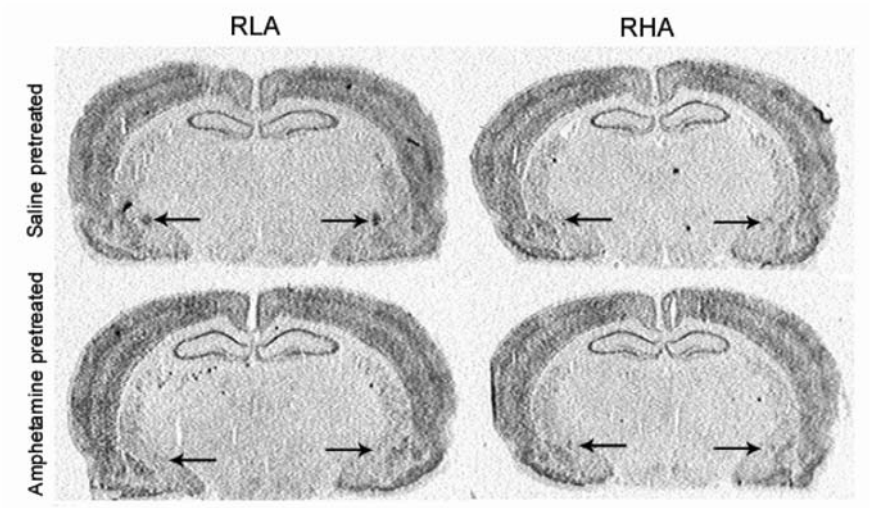


Figure 4: NGFI-A mRNA expression in the CeA
In this figure, representative autoradiograms obtained for NGFI-A mRNA *in situ* hybridization in the CeA of each group are shown: the CeA can be clearly seen as a dark spot in saline pre-treated RLA rats.



Supplementary material

	NGFI-A		DYN		ENK		Secretogranin		PSD-95	
	Saline	Ampheta.	Saline	Ampheta.	Saline	Ampheta.	Saline	Ampheta.	Saline	Ampheta.
L1: Infraorbital (1)	87.5 ± 3.6	93.9 ± 3.4	38.4 ± 2.3	35.1 ± 2.1	79.4 ± 4.3	72.2 ± 3.1	53.9 ± 3	57.6 ± 5.2	67.9 ± 1.9	65 ± 1.9
L1: Prelimbic/Infralimbic (2)	83.1 ± 3.8	88.9 ± 1.8	28.2 ± 2.9	24.4 ± 1.5	65.1 ± 7	65.1 ± 4.6	56.7 ± 5.4	54.8 ± 1.9	77.3 ± 2.2	76.2 ± 2
L2: Caudate putamen										
Dorsolateral (3)	49.5 ± 2.2	51.3 ± 2.5	39.4 ± 2.5	44.1 ± 3.6	161.8 ± 3.9	163 ± 3.5	26.1 ± 2.2	28.7 ± 1.6	62 ± 1.6	64 ± 2.3
Dorsal (4)	53.1 ± 1.1	54.9 ± 2.2	46.6 ± 1.8	50.2 ± 1.9	162.9 ± 2.5	162.8 ± 3.2	34.9 ± 1.9	34.9 ± 2.8	66.3 ± 2.3	64.1 ± 2.9
Dorsomedial (5)	52.6 ± 1.6	57.3 ± 1.2 *	46.2 ± 2.7	52.6 ± 4.3	161.4 ± 2.6	158.8 ± 3.1	34.9 ± 1.9	36.5 ± 1.7	61.3 ± 2.3	61.8 ± 1.4
Medial (6)	46.4 ± 1.8	49.1 ± 1.8	69.9 ± 3	75.5 ± 1.9	162.4 ± 2.8	165.8 ± 2.4	43.5 ± 2.3	46.3 ± 3	65.6 ± 2.7	68.1 ± 2.2
Ventral (7)	46.9 ± 1.7	50.1 ± 1.8	96.4 ± 3.6	91 ± 2.7	164.2 ± 3.8	167.4 ± 3.4	42.8 ± 2.4	44.9 ± 2.1	71.3 ± 0.9	66.7 ± 1.9
L2: Nucleus Accumbens										
Core (8)	51.1 ± 2.2	37.6 ± 4.3 *	93.5 ± 3.4	97.1 ± 4.1	165 ± 3.3	171.9 ± 3.3	61.7 ± 3.8	54.9 ± 3	66.3 ± 1.3	68.8 ± 3.8
Shell medial (9)	43.1 ± 2.5	40.5 ± 3	91.8 ± 4	94 ± 3.5	151.8 ± 3.6	152.3 ± 2.9	68.2 ± 3.9	70.1 ± 3.1	66.1 ± 0.8	61.4 ± 1 *
Shell ventral (10)	49.7 ± 2.1	44 ± 2	90.3 ± 2.9	89.2 ± 2.8	166.9 ± 2.8	158.7 ± 4.2	54.9 ± 2.8	48.3 ± 3.2	63.3 ± 2.3	63.5 ± 1.5
L2: Olfactory tubercle (11)	54 ± 2.6	61.5 ± 2.8	61.9 ± 5.6	62.3 ± 3.8	180 ± 1.6	175.5 ± 2.8	68 ± 2.8	74.3 ± 4.4	78 ± 4.6	93.2 ± 2 *
L2: Cingulate cortex (12)	69.4 ± 2.5	72.8 ± 1.6	24.1 ± 1.5	23.3 ± 1.4	33.5 ± 1.3	32.7 ± 0.8	41.5 ± 1.7	41.5 ± 1.7	71 ± 1.3	72.2 ± 0.9
L2: Motor cortex (13)	59.9 ± 1.9	61.4 ± 2.4	23.3 ± 1.5	22.2 ± 1.6	26.8 ± 1.8	25 ± 1.1	32.3 ± 1	33.9 ± 1	60.6 ± 1.6	62.8 ± 1.4
L2: Sensorial cortex (14)	54.5 ± 1.3	55.2 ± 2.6	27.1 ± 1.8	26.5 ± 1.1	30.7 ± 1.2	30.5 ± 1.4	24.7 ± 1.1	26.1 ± 0.8	58.2 ± 1.5	59.1 ± 1.4
L2: Piriform cortex (15)	115.2 ± 4	125.3 ± 3.1	21.6 ± 1.5	17.8 ± 2.6	117.7 ± 5.8	129.4 ± 7.1	70.9 ± 5	71.6 ± 2.9	90.2 ± 3.1	92.3 ± 3.4
L3: Caudate putamen										
Medial (16)	42.3 ± 2.1	43.6 ± 2.3	66.5 ± 2.9	74.8 ± 2.4	169.5 ± 1.8	169.6 ± 2	32 ± 1.7	34 ± 2.5	63.5 ± 1.4	65.3 ± 2.3
Dorsal (17)	46.2 ± 2.6	48.7 ± 1.6	46.3 ± 2.8	43.6 ± 2	150.7 ± 3.4	156.7 ± 5.8	29.6 ± 1.8	28.8 ± 2.1	54.4 ± 1.7	49.6 ± 3.8
Dorsolateral (18)	45.9 ± 1.9	45 ± 1.5	40.3 ± 2.1	38.2 ± 1.6	161.5 ± 1.1	165.7 ± 4.4	27.7 ± 1.3	25.6 ± 2.3	57.7 ± 1.3	55.7 ± 2.4
Ventrolateral (19)	46.9 ± 2.1	48.7 ± 1.2	55.2 ± 1.3	53.4 ± 1.6	163.4 ± 3.2	163 ± 4.4	23.5 ± 1.3	24.7 ± 2.4	60.7 ± 2.1	60.9 ± 2.5
Ventral (20)	39.9 ± 3.4	42.2 ± 2	96.3 ± 2.6	97.4 ± 3.2	174.8 ± 1.9	173.5 ± 2.7	48.7 ± 1.9	49.6 ± 4.4	62.4 ± 2	65.4 ± 1.9
Central (21)	35.3 ± 1.8	33.5 ± 1.2	37.3 ± 2.5	35.9 ± 2.9	136 ± 7.1	151.1 ± 6.9	20.4 ± 2	21.3 ± 2	46.5 ± 1.8	51.8 ± 3.2
L3: Cingulate cortex (22)	69.5 ± 3.4	70 ± 2	18.5 ± 0.9	19.6 ± 0.9	54.9 ± 1.8	55.4 ± 2.7	34.8 ± 1.2	35.7 ± 1.3	58.7 ± 1.3	62.8 ± 2.4
L4: Amygdala										
Central Amygdala (23)	48.3 ± 3.1	53.6 ± 5.4	56.6 ± 6.8	64.5 ± 5.1	159 ± 8.6	162.1 ± 4.5	103.6 ± 7	115 ± 10.3	57.3 ± 1.9	62.8 ± 3.5
Basolateral amygdala (24)	48.8 ± 3.2	58.5 ± 5	25.9 ± 2.4	24.2 ± 2.6	79.6 ± 3.9	65.8 ± 5.8	50.5 ± 3.3	59 ± 4.9	56 ± 3.4	59.8 ± 2.9
Lateral amygdala (25)	59.7 ± 4.5	62.9 ± 3.7	24.3 ± 2.3	26.9 ± 2.7	26.5 ± 2.5	32.9 ± 3.5	42 ± 6.6	49.9 ± 9.1	51.5 ± 2.2	57.5 ± 1.7
L4: Caudate putamen tail 26)	44.3 ± 4.1	48 ± 2.4	72.8 ± 2.9	78.6 ± 5.4	117.9 ± 7.4	137.3 ± 7.9	32.6 ± 2.7	29.2 ± 2.4	58 ± 2.3	58.6 ± 1.8
L4: Rostral hippocampus										
Dentate gyrus (27)	50.7 ± 2.3	49.6 ± 3.7	81 ± 7.3	90.9 ± 7.4	33.2 ± 4	28 ± 1.2	122 ± 5.6	123.4 ± 5.7	89.1 ± 6	88.4 ± 5.7
CA1 (28)	81.7 ± 5.1	91.9 ± 10.3					101.5 ± 7.4	98.4 ± 5.5	88.3 ± 2.5	87.1 ± 3.2
CA3 (29)	71.4 ± 4.2	80.8 ± 4.7					82.7± 4.2	83.4 ± 3.3	82.8 ± 2.8	82.1 ± 3.2
L5: Dorsal hippocampus										
Dentate gyrus (30)	53.3 ± 3.1	55 ± 2.4	171.7 ± 2.5	166.5 ± 5.1	41.9 ± 4.4	41.5 ± 2.1	155.2 ± 3.5	150.1 ± 6.9	123.7 ± 2.8	124 ± 3.2
CA1 (31)	133.3 ± 3.4	134.3 ± 6					138.2 ± 4	135.8 ± 5	122.1 ± 2.1	128.9 ± 3.7
CA3 (32)	68.8 ± 3.4	71 ± 2.6					113.3 ± 4.5	102.1 ± 3.2	88 ± 2.9	93.8 ± 3.3
L6: Ventral hippocampus										
Dentate gyrus (33)	52.6 ± 2.5	53.1 ± 3.2	187.2 ± 3.1	179.4 ± 6.3	69.3 ± 5.2	67.4 ± 2.4	184.7 ± 5.2	172.9 ± 4	121.4± 2.7	109.1 ± 3.9
CA1 (34)	90 ± 3.1	82.1 ± 6.6	43.2 ± 2	50.9 ± 6.2			153.6 ± 10.6	169.9 ± 6.7	122.3 ± 3.4	119.4 ± 4.5
CA3 (35)	76.3 ± 2.3	73.3 ± 2.4					168.4 ± 6.9	159.7 ± 5.1	115.3 ± 3.3	109.4 ± 3.9
L6: VTA (36)	22.6 ± 1.7	25.1 ± 2	21.2 ± 2.3	16.8 ± 1.9			89.9 ± 3.7	85.8 ± 3.8	33.1 ± 2.7	32.2 ± 1.4
L6: SNR (37)	24.7 ± 1.9	28.5 ± 1.6	13.2 ± 1.5	14.3 ± 1.5			21.5 ± 1.7	19.5 ± 1.7	20.6 ± 0.9	18.1 ± 2
L6: SNC (38)							67.4 ± 4.2	63.4 ± 4.7	33.1 ± 1.8	32.6 ± 1.8

Table (supplementary material): *In situ* hybridization of NGFI-A, DYN, ENK, secretogranin and PSD-95 in SD-OFA rats.
Results of *in situ* hybridization for NGFI-A, DYN, ENK, secretogranin, and PSD-95 gene transcripts in saline and amphetamine pretreated SD-OFA rats. The pretreated rats were challenged with amphetamine before analysis. Results are expressed as mean optical density in the specific areas ± SEM. The number next to each area corresponds to the identification number in figure 2. The statistical analysis was performed using a t-test analysis: * p<.05 compared to the respective saline pretreated group.

There are not widespread adaptations in gene transcripts in amphetamine-sensitized SD-OFA rats. A survey of these data show that SD-OFA rats pre-treated with amphetamine had, when compared to saline pre-treated SD-OFA rats, increased NGFI-A in the dorsomedial portion of the rostral striatum and increased PSD-95 in the olfactory tubercle but decreased NGFI-A in the core of the nucleus accumbens and decreased PSD-95 in the medial part of the nucleus accumbens shell.

Although the pattern of adaptations detected in SD-OFA is not localized in the same neuroanatomical regions as in RHA-I rats, the two groups of animals that expressed amphetamine sensitization showed to a certain degree similar changes in NGFI-A and PSD-95. Both RHA-I and SD-OFA rats pre-treated with amphetamine showed increased NGFI-A mRNA in the dorsomedial portion of the rostral striatum and increased PSD-95 in one subdivision of the ventral striatum, the nucleus accumbens core in RHA-I rats and the olfactory tubercle in SD-OFA rats. The different location of the increased challenge-induced PSD-95 may be explained by the neuroanatomical organization of the striatum. It is known that the corticostriatal loops that are the basis of the anatomical and functional organization of the basal ganglia are arranged so that ventral areas of the striatum influence neuronal activity in cortical areas that, at the same time, project to a more dorsal striatal region (Zahm, 1999; Voorn et al., 2004). It is also known that acute amphetamine administration induces dopamine release in the shell of the nucleus accumbens (Pontieri et al., 1995) whereas the increased dopamine release induced by systemic psychostimulant administration in behaviorally sensitized animals is found selectively in the core of the nucleus accumbens when shell/core subdivisions are studied (Cadoni et al., 2000; Giorgi et al., 2005). This dorsalization of the neuronal adaptations with time may be a common phenomenon in long-term psychostimulant administration as it is discussed in the main text. However, RHA-I and SD-OFA rats may differ in the sensitivity of their mesolimbic dopaminergic system as they differ in their novelty-induced motor activity (Gimenéz-Llort et al., 2005) and in their initial motor response induced by amphetamine (results shown in the main text). Therefore, it may be that given the same sensitization regime, the two strains differing in their basal dopamine responsiveness (RHA-I > SD-OFA rats) as well as in the extent of expression of behavioral sensitization (RHA-I > SD-OFA rats) showed different ventral to dorsal localization of neuronal adaptations related with expression of behavioral sensitization. RHA-I rats would show increased glutamate induced neuronal activation in the core of the nucleus accumbens whereas SD-OFA rats would show this neuronal adaptation in the olfactory tubercle.

However, we also found two neuronal adaptations in amphetamine pre-treated SD-OFA rats that do not have any equivalent in the RHA-I rats, namely decreased PSD-95 in the nucleus accumbens shell and decreased NGFI-A in the core of the nucleus accumbens. This latter finding was actually unexpected since several recent reports have shown enhanced c-*fos* immunoreactivity in the intermediate area of the accumbens shell (Todtenkopf et al., 2002) or in the nucleus accumbens in general (Crombag et al 2002; Hope et al., 2006) upon a challenge with cocaine in sensitized animals. In a study of amphetamine sensitization, increased c-*fos* immunoreactivity upon a challenge was detected preferably in the nucleus accumbens core (Hedou et al., 2002). In these reports there is no agreement in the exact location of the neuronal adaptation inside the nucleus accumbens, but in all of them an increase and not a decrease is reported. However, we studied NGFI-A mRNA expression in an amphetamine sensitization paradigm. The main difference with these cited reports is the protocol used to challenge the animals: in the experiments reported by Crombag et al. (2002) and Hope et al. (2006) animals were habituated to the test cage for 30 minutes before the challenge and in Hédou et al. (2002) animals were not habituated. In our

experiment animals were habituated for one hour and challenged with saline for 1 more hour before the actual challenge with amphetamine was administered. Considering the fast response of NGFI-A mRNA induction (Moratalla et al., 1992; Berke et al., 1998) and the low dose used for the challenge, differences in the challenge protocol may have been determinant. Moreover, the fact that we also found decreased PSD-95 in the nucleus accumbens shell in the same group of SD-OFA rats strongly suggest that in these animals, using the present sensitization protocol, behavioral sensitization is associated with increased glutamate-induced cellular responsiveness in the olfactory tubercle and decreased cellular responsiveness in striatal areas dorsal to the olfactory tubercle.

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Paper VI

EFFECTS OF NALTREXONE AND ACAMPROSATE ON ALCOHOL-INDUCED GENE EXPRESSION IN MOUSE BRAIN

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Abstract

Background: Naltrexone and acamprosate are two drugs used clinically to prevent relapse to alcohol drinking. It is widely held that naltrexone extinguishes alcohol craving by blocking its hedonic impact on the brain, whereas the pharmacological mechanism of the acamprosate effect is not well known.
Methods: In search for the substrate of naltrexone and acamprosate action on alcohol craving, we investigated the effects of ethanol alone and in combination with naltrexone or acamprosate on gene expression of nerve growth factor inducible clone A (NGFI-A; also known as *zif*268 and *egr*1). In Experiment 1 and 3, alcohol (2 g/kg) alone or in combination with naltrexone (15 mg/kg) or acamprosate (300 mg/kg) was injected intraperitoneally into mice and NGFI-A-mRNA levels in the brain were investigated by means of *in situ* hybridization. In Experiment 2, mice were treated with nor-BNI (0.5 mg/kg), a κ-opioid antagonist, injected alone to investigate whether the effect of naltrexone was related to blockade of κ opioid receptors.
Results: It was found that both ethanol and naltrexone alone induced NGFI-A in the central nucleus of the amygdala, but not in a number of other brain areas studied, and that these effects were additive. However, acamprosate alone or in combination with ethanol had no effect on NGFI-A mRNA, while nor-BNI induced NGFI-A mRNA in the basolateral amygdala.
Conclusion: The central amygdala appears to be an important target of both alcohol and naltrexone. The neuronal effect of naltrexone does not appear to be a simple inhibition of the effect of ethanol.Acamprosate may not share the site of action with naltrexone in spite of being used for the same therapeutic purpose.

Key words: amygdaloid nucleus, alcohol, zif268, NGFI-A, naltrexone, acamprosate.

Introduction

Naltrexone, an unselective opioid receptor antagonist, is often used as part of programs to prevent drinking relapse in sober alcoholics. The literature on naltrexone treatment in alcoholism is quite extensive and has been subjected to several reviews (e.g. O'Brien, 2005; O'Malley and Froehlich, 2003; Terenius, 1998), and naltrexone is often considered to act by blocking the effects of alcohol on the brain (Sinclair, 2001; Volpicelli, 1987). Clinical and pre-clinical studies have shown that naltrexone is effective when paired with drinking but ineffective when given during abstinence. This suggests that the mechanism involved is extinction, since extinction weakens responses that are made while reinforcement is not present, in this case blocked (Sinclair, 2001). The subjective rewarding responses to ethanol in alcoholics have been reported to be blocked by concurrent administration of naltrexone (Volpicelli et al., 1995) in agreement with the hypothesis that alcohol reinforcement is achieved through release of endogenous opioids (See Herz, 1997; Terenius 1998). Although tonic activity of endogenous opioid systems has been reported to be low (Gestreau et al., 2000), it has been suggested that activation of μ- and ∂-opioid receptors in the ventral striatum triggers the hedonic experience (Kelley 2004). Thus, naltrexone could cause extinction of craving for alcohol by attenuating its hedonic impact. The more recently introduced drug acamprosate has a similar clinical use as naltrexone although its mechanism is not well characterized, but supposed to involve glutamatergic rather than opioidergic mechanisms (see e.g. De Witte et al., 2005; Terenius, 1998).

The aim of the present work was to search for the anatomical substrate of the pharmacological actions of naltrexone and acamprosate in relation to alcohol-related behaviour. By measuring the expression of activity-related genes, cells that respond to drugs can be located and the primary site of drug action may be identified. Immediate-early genes (IEGs) like *c-fos* and NGFI-A, regulated by neuronal activity, can be used to identify structures responding to a drug or other

stimuli. Although an early study suggested that acute ethanol treatment has no effect on c-*fos* expression in brain (Le et al., 1990), several more recent studies have clearly shown ethanol effects on the expression of c-*fos* and other IEGs (e.g. Bachtell et al., 1999). Since NGFI-A expression is often more sensitive than the expression of other IEGs including c-*fos* (e.g. Worley et al., 1993), it was used here as a marker of neuronal activity. In Experiment 1, the effect of ethanol and naltrexone alone and their combination on NGFI-A mRNA expression levels was investigated. It was presumed that the effects of naltrexone alone on brain NGFI-A mRNA would be small, but that naltrexone would attenuate the effects of ethanol on this activity marker. In Experiment 2, the effect of the κ-antagonist nor-BNI alone on brain NGFI-A mRNA expression levels was studied to see whether the effect of naltrexone was related to blockade of κ opioid receptors. Finally, in Experiment 3, the effect of acamprosate alone and in combination with ethanol on NGFI-A mRNA expression levels was investigated. The effect of acamprosate was difficult to predict, but could be assumed to be similar to that of naltrexone, given the similar clinical use of both drugs.

Experimental procedures

Male NMRI (Naval Medical Research Institute) mice weighing 29-45g at the time of the experiments were bought from Charles River (Uppsala; Sweden) and were left for at least 5 days to habituate to the laboratory conditions. In all experiments, drugs were diluted in saline vehicle (0.9 % NaCl) at the desired concentration. The injected volume in millilitre was 1/100 of the body weight in grams. All injections were done intraperitoneally during the same time conditions (daytime, "lights on").

Treatment of animals in experiment no. 1

40 mice were divided into 4 groups of 10 animals each for different treatments. All mice were injected twice with an interval of 30 minutes between injections. Group 1 received vehicle in the first injection and 2g/kg ethanol (KemEtyl, Stockholm, Sweden) in the second injection. Group 2 received 15 mg/kg naltrexone in the first injection and ethanol (same dose as group 1) at the second. Group 3 was first injected with naltrexone (same dose as group 2) and then with vehicle. Mice in group 4 were injected at both instances with vehicle and used as a control group. One hour after first injection the mice were killed by decapitation, the brain was dissected and specimens rapidly frozen on dry ice and stored at –78°C.

Treatment of animals in experiment no. 2

23 mice were divided into 2 groups and all mice were injected once. 11 mice received vehicle and 12 mice 0,5mg/kg nor-BNI (Tocris Cookson, Avonmouth, U.K), a κ-antagonist. Mice were killed 60 min after injection, the brain was dissected and specimens kept as above.

Treatment of animals in experiment no. 3

40 mice were divided into 4 groups of 10 animals each for different treatments. All mice were injected twice with an interval of 30 minutes between injections. Group 1 received vehicle in the first injection and 2g/kg ethanol (KemEtyl, Stockholm, Sweden: 20%) in the second injection. Group 2 received 300mg/kg acamprosate (Toronto Research Chemicals, North York, Canada) in the first injection and ethanol (same dose as group 1) in the second injection. Group 3 was first injected with acamprosate (same dose as group 2) and then with vehicle. Mice in group 4 were injected at both instances with vehicle and used as a control group. One hour after first injection the mice were killed by decapitation, the brain was dissected and specimens kept as above.

Tissue preparation

Frozen brains were warmed to –20°C in a cryostat (JUNG CM 3000) and sectioned to generate 14 μm thick coronal brain sections. The levels chosen and the mapped brain areas were the following according to their distance from the bregma:

1. Approximately 1,10mm. Cingulate cortex, motor cortex, dorsomedial caudate putamen, dorsolateral caudate putamen, accumbens shell and core, piriform cortex and septum
2. Approximately -1,46mm. CA1 and CA3 fields of the rostral hippocampus, central amygdala, basolateral amygdala and basomedial amygdala
3. Approximately -2,18mm. CA1 and CA3 fields of the dorsal hippocampus
4. Approximately -3,40mm. CA1 and CA3 fields of the ventral hippocampus and entorhinal cortex
5. Approximately -5,02mm . Entorhinal cortex

The sections were fixed to the slide (VWR or Fisher Biotech) by finger heat and then stored at –20°C. To help finding the correct levels during sectioning, sections were stained with cresyl violet or for acetylcholinesterase and compared with a brain atlas (Paxinos and Franklin, 2000).

^{33}P-isotope labelling of probe

The oligodeoxyribonucleotide probe (Thermo, Ulm, Germany) for NGFI-A-mRNA was complementary to nucleotides coding for amino acids 2-16 in NGFI-A and had a length of 45 bases (5′-CCG TTG CTC AGC AGC ATC ATC TCC TCC AGT TTG GGG TAG TTG TCC-3′). This oligonucleotide, labelled with ^{35}S, has been used for *in situ*-hybridisation in published work (e.g. Kuzmin and Johansson, 1999). The oligonucleotide (approx. 4 μg/ml) was carefully mixed with ^{33}P-dATP (Perkin-Elmer; approx. 2 mCi/ml) and terminal deoxynucleotidyl transferase (approx. 500 units/ml) and incubated at 37°C for about an hour. The labelled probe was separated with the help of Qiaquick Nucleotide Removal Kit (VWR, Sweden) and the radioactive probe-fraction was measured in a scintillation counter by applying aliquots of probe to

filter paper in tubes containing 3 ml of scintillation liquid.

In situ hybridisation
The slides with cryostat sections were thawed to room temperature and dried in front of a fan for approximately half an hour. The probe was dissolved in hybridisation cocktail containing 50 % deionised formamide, 4 x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). Approximately 1 x 10^6 cpm of probe was used per ml of cocktail. This mixture was heated to 37°C and after filtering mixed so that air bubbles were avoided. Of this, an aliquot was taken to a separate tube and mixed with unlabelled oligonucleotide to obtain a 100 x excess concentration of unlabeled oligonucleotide (i.e. a negative control).
The hybridisation solution was heated to 42°C for 30 min. Then, about 125 µl of the solution was placed on each slide, in a humidified hybridisation chamber and incubated at 42°C for 16-20 hours.

Washing of slides
After hybridization, the slides were placed in a rack and washed in a beaker in 1 × SSC at 40°C, followed by 4 × 15 min in 1 × SSC at 55°C. Thereafter the slides were rinsed in distilled H_2O for 1 min and the sections were dehydrated in increasing concentrations of ethanol (60 % and 95 %, one minute in each). Then, slides were air-dried on an ordinary sheet of paper with the sections facing up in a film cassette. Finally a Hyperfilm-Betamax (Amersham, Uppsala, Sweden; experiment 1) or Kodak MR film (GE Healthcare, Uppsala, Sweden; experiments 2 and 3) was placed against the sections for exposure at room temperature for 2 to 7 days.

Detection
After the hybridization, the films were placed for 5 min in developer (KODAK D19), 10 min in fixer (KODAK 3000A) and thereafter washed under running water for 20 min and air-dried. Autoradiograms were analyzed with a Macintosh computer using the public domain NIH Image program (US National Institutes of Health; see http://rsb.info.nih.gov/nih-image). Optical densities were calculated from the uncalibrated mode by subtracting from each measurement its corresponding background and expressed in grey levels. All measurements were done on both cerebral hemispheres and data were pooled for each animal.

Staining of brain sections with cresyl violet
For a 50 ml cresyl violet solution was used: Cresyl violet 0.25 g, distilled H_2O 30 ml, 1M sodium acetate, 3 ml (1.36 g granular $Na_2C_2H_3O_2 \cdot 3H_2O$ + 9.20 ml H_2O), 1M acetic acid 1.7 ml. Distilled H_2O was then added to a total volume of 50 ml. Before staining, the solution was stirred at room temperature for one week and filtered.

Slides with sections were placed for 5 min in xylene for defatting, 5 min in 99.8% ethanol for fixation, 5 min in 95%, 5 min in 70% ethanol, a few seconds in distilled H_2O and 15 min in cresyl violet solution. After the last step, the slides were rinsed briefly in distilled water, 5 min in 70% ethanol, 5 min in 95% ethanol, 5 min in 99.8% ethanol and finally 5 min in xylene. For rapid staining the incubations were sometimes shortened to one minute.

Acetylcholinesterase histochemistry
340.0 mg sodium acetate trihydrate, 49.0 mg copper sulfate ($CuSO_4 \cdot 5H_2O$; Aldrich) and 0.60 mg glycine were diluted to 50 ml with distilled H_2O. The pH of the sodium acetate buffer was adjusted to 5.0 with 1M HCl.
A solution for incubation was prepared: 58 mg acetylthiocholine iodide was diluted in 1.2 ml prometazine (2.5 mg/ml; an enzyme inhibitor which inhibits unspecific esterases) and was diluted to 50 ml with sodium acetate buffer.

The brain sections were taken out of the freezer and after a few minutes placed in the incubation solution (buffer) over night. After incubation a white colour was seen on the sections indicating that a copper thiocholine iodide complex had been formed. For exposure or amplification of the colour slides were rinsed in distilled water and then placed in the $Na_2S \cdot 9H_2O$ solution (pH 7.5 with acetic acid) for 10 minutes. The sections received a dark brown colour in areas where acetylcholinesterase had been active. To fix the sections they were washed in distilled water and then placed in 4 % formaldehyde in phosphate buffer (Histolab, Göteborg, Sweden) over night. After fixation the slides were placed in 99.5% ethanol for 30 minutes (to get rid of excess lipids) and then in xylene for 30 minutes (for additional defatting and clearing). The sections were air dried. Then 2-3 drops of mountant was added on top of the sections and a coverslip (Knittel, Germany) was placed carefully on the glue to avoid air bubbles.

Supplier of chemicals was Sigma unless otherwise noted.

Statistics
One-way ANOVA was used for experiments 1 and 3. When appropriate, Duncan´s test considering the 4 groups was performed as a post hoc analysis. In experiment 2, the groups were compared using Student's t-test. For all experiments, $p<0.05$ was considered statistically significant.

Results
Experiment 1
Representative autoradiograms from the differently treated groups are shown in figure 1. Averages for the different groups are shown in table 1. One-way ANOVA analysis detected a treatment effect in NGFI-A mRNA expression levels measured in the central amygdala [$F(3,36)=23.02$ $P<0.001$; figure 2]. The Duncan test ($P<0.05$) revealed that mice treated with ethanol or naltrexone alone showed an increase in NGFI-A mRNA levels when compared to vehicle-treated mice. Moreover, the combination of these two

treatments had a synergic effect and induced NGFI-A mRNA expression to levels higher than those observed in mice treated with either of the drugs alone.
As shown in figure 1, this effect is easily visible upon inspection. No statistically significant effect was found in any other brain structure studied, although marginally significant effects were found in the dorsolateral striatum, the septum and the motor cortex.

Experiment 2
Results are shown in table 2. As shown in table 2, nor-BNI treated mice showed higher NGFI-A in the basolateral amygdala ($t(12)=3.16$ $P=0.008$). No other treatment effect was detected with the Student's t-test.

Experiment 3
Results are shown in table 3. One-way ANOVA analysis detected a treatment effect with ethanol on NGFI-A mRNA expression in the central amygdala ($F(3,36)=6.74$ $P<0.001$). The Duncan test ($P<0.05$) revealed that mice treated with ethanol alone showed an increase in NGFI-A mRNA levels when compared to vehicle-treated mice which was not modified by acamprosate. Acamprosate alone had no effect on NGFI-A mRNA levels and it did not modify the effect of ethanol in any measured structure.

Discussion
The present work shows that: (i) the only strong response to ethanol occurred in the amygdala, (ii) neither naltrexone nor acamprosate reduced the response of NGFI-A to ethanol and (iii) naltrexone can synergize with effects of alcohol in the central amygdala but acamprosate did not modify the alcohol effect on this structure.

This is not the first study of the effects of alcohol on IEGs, but to the best of our knowledge the first time that combinations of ethanol with naltrexone or acamprosate are tested. In some studies where c-*fos* was used as a marker, neuronal activation could not be recorded in regions where effects of alcohol had been described using other techniques. Therefore, the experiment was optimized to detect effects in brain areas associated with the action of ethanol. First, we used NGFI-A as a marker of neuronal activity instead of the more commonly used c-*fos*. NGFI-A is more sensitive to neuronal activation than c-*fos* in some brain regions but belongs to the same IEG gene family. Second, the doses of naltrexone and acamprosate were selected to obtain a strong activation to avoid that small changes in neuronal activation go undetected due to the poor dynamic quality and low sensitivity of IEG induction to rapid temporal changes, as discussed by Farivar et al. (2004). Therefore, the doses were somewhat higher as compared with those usually used therapeutically in humans, but within the range used in published animal studies (e.g. Bachtell et al., 2002). Finally, ^{33}P was used for labelling of the *in situ* probe, since it has advantages as compared to ^{35}S. By using ^{33}P, a three times stronger signal is obtained and the background (measured in the presence of excess unlabeled oligonucleotide) was almost eliminated.

Ethanol induced a strong NGFI-A response only in the CeA, and naltrexone or acamprosate did not block this effect of ethanol on NGFI-A mRNA. This lack of blocking effect was not completely unexpected in the view of previous studies. For example, in a study by Bachtell et al. (2002), the effect of ethanol on c-*fos* in the Edinger-Westphal nucleus was not blocked by naloxone. In some, but not all, areas with c-*fos* induction by ethanol in control mice, ethanol-induced c-*fos* was present also in μ opioid receptor knockout mice (especially in the supraoptic and paraventricular thalamic nuclei; Kolodziejska-Akiyama, 2005). In the present study, ethanol or naltrexone administration induced NGFI-A mRNA expression restricted to the CeA, an area not studied by Kolodziejska-Akiyama (2005). However, it has been shown that microinjection of naltrexone in the CeA suppresses alcohol self-administration in rats (Foster et al., 2004). A likely reason for a more widespread IEG induction after ethanol administration in the study of Kolodziejska-Akiyama is the fact that they used a higher dose of ethanol, and they studied c-*fos* instead of NGFI-A. Moreover, the pattern of IEG induction by ethanol differs to some extent between studies. For example, ethanol given with gastric intubation was described to reduce NGFI-A mRNA in the cerebral cortex of rats (Ueyama et al., 1999). The differences among studies is probably the administration procedure as in some studies injection is used whereas in others oral administration by intubation or voluntary drinking is used. The latter is likely to reveal brain activity related to the act of drinking as well as the direct effect of ethanol, as has already been suggested (Crankshaw et al., 2003). That c-*fos* and NGFI-A mRNA may respond differently is perhaps suggested by a study with acamprosate (200 mg/kg i.p.) in rats, where major increases in c-*fos* expression were seen in the hippocampus and cerebellum, and slight increases elsewhere (Putzke et al., 1996).

The largest effect of alcohol was found in the central nucleus of the amygdala. This was not surprising, since a number of publications indicate that ethanol can affect amygdala functions. Previous reports have indicated that increased GABAergic transmission in the CeA may mediate part of the behavioural actions of ethanol (Roberto et al., 2004). In a recent study, it was found that ethanol potentiated GABAergic transmission in CeA neurons in wild-type and CRF2 (corticotrophin-releasing factor receptor, subtype 2) knockout mice, but not in CRF1 receptor knockout mice (Nie et al. 2004). Intraperitoneal administration of ethanol at the doses used in the present experiments induced c-*fos* mRNA expression in the CeA of rats (Chang et al., 1995; Morales et al., 1998; Thiele et al., 1997), as well as mice (Hitzemann and Hitzemann, 1997). It had already been hypothesized that the reinforcing properties of ethanol, both positive and negative, are mediated by the CeA, although not

exclusively (Cowen et al., 2004). Much of previous data suggesting a role of the CeA in alcohol effects is summarized in Cowen et al. (2004). Our study, thus, adds to previous evidence that the central amygdala may be equally or more relevant than the accumbens nucleus in the effects of ethanol and naltrexone. Like the accumbens nucleus, the central amygdala receives a major dopaminergic projection from the ventral tegmental area (Asan, 1998). Recent work indicates that brain-derived neurotrophic factor (BDNF) as well as cAMP responsive element binding protein (CREB) in the CeA regulate alcohol intake (Pandey et al., 2006). Since the pathways of NGFI-A induction partly overlap with those regulating BDNF and CREB, naltrexone action in CeA might also involve the lastmentioned regulatory factors.

The paradoxical increase in activity with naltrexone contrasts with the blockade by opioid antagonists of morphine-induced IEG expression (e.g. Chang et al., 1988). Although there is strong behavioral evidence for a functional blockade of some ethanol actions by naltrexone, the present study indicates that naltrexone does not simply cause inhibition of the primary neuronal effects of ethanol. How, then, does the functional naltrexone-ethanol interaction occur? One clue may be the role of CeA in the regulation of ingestive behavior and the apparent involvement of opioids in ingestive mechanisms (Glass et al., 2002; Gosnell, 1988). The central nucleus of the amygdala and the related nucleus of the solitary tract (NTS) are involved in feeding behaviour. The amygdala receives projections from the NTS, electrical stimulation of the amygdala induces c-*fos* in NTS and naltrexone injection in the NTS increases gene expression (dynorphin) in the amygdala (Glass et al., 2002). μ-opioid agonist administration into the central amygdala caused an increase in food intake (Gosnell, 1988), whereas naltrexone into the central amygdala reduced intake of a preferred diet (Glass, 2000). Lesions of the central amygdala decrease the intake of salt after experimental sodium depletion (Johnson, 1999). In the light of the role of opioid peptides in ingestive behaviour, it may be that naltrexone potentiates the aversive effects of alcohol intake leading to reduction of its intake. We hypotesize that the central amygdala, together with other related structures like the *bed nucleus of the stria terminalis,* are the anatomical sites where hedonic value of ingested foods or liquids (orosensory reward) is neuronally represented. It seems likely that opioids regulate the threshold for orosensory reward. Gene expression and knockout studies have suggested that NGFI-A gene induction may trigger genetic changes that are necessary for maintenance of long-term potentiation and stabilization of long-lasting memories (Knapska and Kaczmarek, 2004). Such functions are best documented in hippocampus and in spatial learning (Bozon et al., 2002), but may also be found relevant in the central amygdala in relation to alcohol intake. Thus, naltrexone may "devaluate" alcohol reinforcement and through NGFI-A activation the "devaluation" may be consolidated so that relapse is prevented. Interestingly, the high alcohol consumer C57BL mice (Roger and McClearn, 1962) show lower c-*fos* mRNA induction within the CeA than the low alcohol consumer DBA mice when they are injected intraperitoneally with ethanol (Hitzemann and Hitzemann, 1997).

In contrast to naltrexone, acamprosate failed to influence the effect of alcohol effects on NGFI-A mRNA expression levels. Moreover, acamprosate alone did not show any significant effect. This result may add some support to previous studies indicating different mechanisms for naltrexone and acamprosate. A possible extension of the current study would be to measure the direct effect of the combination of ethanol and naltrexone (or acamprosate) on the electrical properties of central amygdala neurones using electrophysiology.

As naltrexone is a mixed antagonist with μ- δ- and κ-opioid receptor activity, it is interesting to define the receptor subtype(s) responsible for naltrexone´s effect on NGFI-A induction in the central amygdala. The effect of nor-BNI could not be equated with that of naltrexone; at least using this response, naltrexone does not seem to act as a κ-antagonist at the studied dose. In a previous study Fos-like immunoreactivity was not induced in the CeA of naïve rats after administration of 5 mg/kg nor-BNI (Le Guen et al. 2003), but induction in the CeA was found after naltrexone or β-funaltrexamine, the latter a selective μ-receptor antagonist (Gestreu et al., 2000). Thus, although κ-opioid antagonism does not induce NGFI-A in the central amygdala, a κ-receptor mechanism may be implicated in naltrexone induction of NGFI-A in the central amygdala through projection from the BLA to the central amygdala.

These results may be regarded as a "map" of the regions in which the opioid receptors are tonically stimulated, and indicate that the amygdala may be important in alcoholism. Although alcohol is generally believed to have very unspecific and varied effects on the brain (Oswald and Wand, 2004), the experiments with NGFI-A have indicated a fairly selective effect on the central nucleus of the amygdala. Apparently alcohol´s effects on gene expression can be highly restricted in space, e.g. CeA, and for obvious reasons difficult to detect in a homogenate of the whole amygdala and even more in a whole-brain homogenate. This might explain the conflicting literature about alcohol and gene expression (Worst and Vrana, 2005). A more detailed study of the action of naltrexone in the nuclei of the amygdala, using more sensitive methodology, is therefore warranted. Finally, it is important to stress that the effect of naltrexone does not appear to be a simple inhibition of the primary neuronal effect of ethanol.

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Table 1: *In situ* hybridization results from experiment 1. Optical density values expressed in arbitrary units are shown. The effect of ethanol, naltrexone and their combination on NGFI-A mRNA levels in different brain regions as determined by *in situ* hybridization. Duncan test was performed when appropriate. None of the observed group differences reached statistical significance (at $p<0.05$).

	Saline + Saline	Saline + Ethanol	Naltrexone + Saline	Naltrexone + Ethanol
Dorsolateral striatum	91.30 ± 8.02	82.71 ± 5.12	74.5 ± 5.15	72.67 ± 3.08
Accumbens core	27.7 ± 4.13	28.47 ± 2.64	33.43 ± 4.09	29.20 ± 1.24
Accumbens shell	41.74 ± 7.83	34.56 ± 6.63	46.05 ± 7.19	30.19 ± 6.63
Olfactory tubercle	43.38 ± 7.01	50.29 ± 3.05	47.18 ± 7.59	43.17 ± 6.89
Septum	74.12 ± 7.45	78.85 ± 6.61	90.55± 5.40	77.61 ± 6.19
Central amygdala	47.3 ± 6.6	99.3 ±6.2 *	86.2 ± 6.8 *	131.6 ± 5.8 *‡
Lateral amygdala	45.46 ± 7.02	36.84 ± 3.43	42.62 ± 4.44	39.12 ± 6.11
Basolateral amygdala	33.58 ± 4.70	34.12 ± 3.00	26.98 ± 3.22	33.28 ± 5.26
Basomedial amygdala	23.18 ± 2.92	34.88 ± 10.38	20.88 ± 1.89	29.64 ± 6.19
SNR	74.38 ± 0.79	73.45 ± 1.86	73.91 ± 0.65	77.38 ± 1.33
VTA	74.6 ± 2.64	75.58 ± 2.46	78.82 ± 7.98	78.32 ± 1.48
Periaqueductal grey	82.55 ± 1.14	83.16 ± 2.26	81.39 ± 3.33	83.32 ± 1.95
Motor cortex	76.98 ± 8.65	78.17 ± 4.30	81.12 ± 7.06	75.94 ± 8.02
Cingulate cortex	117.16 ± 8.29	127.79 ± 4.38	131.36 ±5.89	109.88 ± 8.17
Rostral hippocampus				
CA1	120.92 ± 9.61	140.12 ± 10.09	135.70 ± 5.96	132.98 ± 10.73
CA3	116.620 ± 9.20	115.76 ± 5.95	92.18 ± 5.23	90.92 ± 10.48
Dorsal hippocampus				
CA1	120.92 ± 9.61	140.12 ± 10.09	135.70 ± 5.96	132.98 ± 10.73
CA3	58.24 ± 3.98	65.03 ± 2.98	69.39 ± 4.22	61.83 ± 3.46
Ventral hippocampus				
CA1	135.55 ± 8.26	137.52 ± 8.73	145.53 ± 12.07	145.99 ± 8.83
CA3	82.31 ± 8.27	84.31 ± 4.72	83.69 ± 8.21	84.266 ± 4.98

Table 2: *In situ* hybridization. Experiment 2. The results of the *in situ* measurements are shown in table below. N is typically 7-10 per group. Student's t test was performed: * $P<0.05$ compared to the respective saline-treated group.

	Saline	Nor-BNI
Dorsomedial striatum	33.38 ± 3.66	34.49 ± 2.23
Dorsolateral striatum	30.62 ± 2.85	36.30 ± 1.48
Septum	24.96 ± 2.79	27.06 ± 2.40
CeA	21.58 ± 4.36	19.07 ± 4.31
BLA	16.60 ± 3.34	31.24 ± 2.87 *
Piriform cortex	58.83 ± 2.53	63.87 ± 2.30
Motor cortex	29.40 ± 2.30	34.76 ± 2.23
Cingulate cortex	54.56 ± 2.60	55.74 ± 1.61
Rostral hippocampus		
CA1	54.83 ± 9.53	45.08 ± 8.35
CA3	30.20 ± 4.89	37.77 ± 4.69
Dorsal hippocampus		
CA1	84.72 ± 6.03	95.00 ± 5.67
CA3	34.60 ± 2.60	38.46 ± 2.77
Ventral hippocampus		
CA1	79.62 ± 7.37	75.71 ± 5.13
CA3	29.44 ± 1.80	32.98 ± 2.24
Enthorrinal cortex 1	30.75 ± 1.66	36.11 ± 2.86
Enthorrinal cortex 2	35.25 ± 5.85	33.38 ± 3.26

Table 3: *In situ* hybridization results from experiment 3. The effect of ethanol, acamprosate and their combination on NGFI-A mRNA levels in different brain regions as determined by *in situ* hybridization (Mean±SEM). N is typically 7 - 10 per group. dm=dorsomedial, dl=dorsolateral, CA=cornu Ammonis, a subfield of hippocampus, numbers The numbers 1-3 after a colon (:) indicate different rortrocaudal levels of the same structure. Duncan test was performed when appropriate. None of the observed group differences reached statistical significance (at $p<0.05$).

	Saline-Saline	Saline-Ethanol	Acamprosate-Saline	Acamprosate-Ethanol
Dorsomedial striatum	30.35 ± 1.52	34.37 ± 2.46	28.40 ± 2.46	32.95 ± 2.40
Dorsolateral striatum	29.98 ± 1.29	30.64 ± 2.11	24.29 ± 1.82	28.70 ± 2.12
Septum	31.32 ± 2.17	30.25 ± 2.86	30.09 ± 2.43	30.95 ± 3.07
CeA	92 ± 2.9	110 ± 4.4 *	93.9 ± 3.4	108.2 ± 3.2 *
BLA	24.84 ± 1.07	22.48 ± 2.09	27.78 ± 4.42	24.74 ± 1.99
Piriform cortex	55.72 ± 4.12	63.94 ± 1.75	59.52 ± 3.02	60.92 ± 3.20
Motor cortex	31.28 ± 1.43	31.68 ± 1.30	28.73 ± 1.51	30.50 ± 2.17
Cingulate cortex	53.83 ± 2.07	56.83 ± 2.25	50.25 ± 2.12	53.28 ± 2.85
Rostral hippocampus				
CA1	54.44 ± 9.64	48.98 ± 6.39	43.42 ± 8.00	42.08 ± 6.18
CA3	39.01 ± 3.78	35.90 ± 3.20	37.28 ± 2.47	38.78 ± 2.80
Dorsal hippocampus				
CA1	80.01 ± 4.02	81.81 ± 2.60	69.36 ± 7.02	69.53 ± 5.01
CA3	34.55 ± 2.07	34.70 ± 1.90	30.26 ± 4.32	27.45 ± 3.00
Ventral hippocampus				
CA1	46.72 ± 5.18	52.88 ± 5.32	68.39 ± 6.75	58.67 ± 5.46
CA3	23.19 ± 1.93	28.34 ± 2.20	28.18 ± 3.11	27.81 ± 3.07
Enthorrinal cortex 1	26.61 ± 1.62	28.72 ± 0.76	28.11 ± 2.27	29.84 ± 2.42
Enthorrinal cortex 2	31.06 ± 6.28	33.58 ± 3.70	33.75 ± 2.15	37.36 ± 2.90

Figure 1: Representative autoradiograms of NGFI-A-mRNA at the rostrocaudal level of the amygdala are shown. All treatment groups of experiment 1 are included: (A) saline-saline, (B) saline-ethanol, (C) naltrexone-saline or (D) naltrexone-ethanol. The amygdala is visible upon inspection and it can be appreciated that ethanol and naltrexone induced NGFI-A in the central nucleus of the amygdala (arrow) when administered alone and that the two drugs have a synergic effect on NGFI-A mRNA.

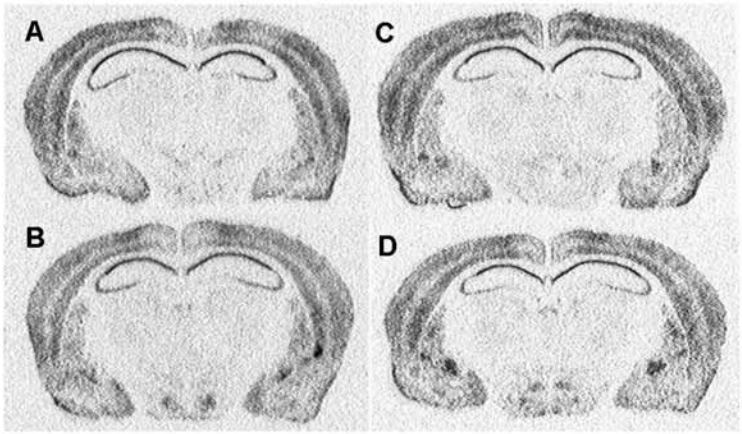

Figure 2: NGFI-A mRNA expression levels in the central nucleus of the amygdala after different treatments. In A, the normalized results of Experiment 1 are depicted: the effects of different treatments are outlighted as(SAL) saline-saline, (EtOH) saline-ethanol, (NTX) naltrexone-saline or (NTX-EtOH) naltrexone-ethanol. In B, the normalized results of Experiment 2 are depicted: the effects of different treatments are outlighted as (SAL) saline-saline, (EtOH) saline-ethanol, (ACM) acamprosate-saline or (ACM-EtOH) acamprosate-ethanol. Duncan test: * $P<0.05$ compared to the respective saline-treated group; ‡ <0.05 compared to all other groups.