UNIVERSITA' DEGLI STUDI DI CATANIA

FACOLTA' DI FARMACIA

Dottorato di ricerca in Biotecnologie

XXIV ciclo

Potential involvement of gap junctions in pathology of addiction

Dott. MASSIMO LAZZARO

Coordinatore e tutor *prof. Federico Cicirata*

Supervisors *prof. PierVincenzo Piazza, prof. Guillaume Drutel*

Ai miei genitori

Abstract

Cocaine abuse represents a huge social problem for the widespread in the world and for the many health risks associated. Moreover, a significative percentage of cocaine users develop an addiction with loss of control on drug intake. Several molecular modifications occurs with cocaine use, some of which seem occur only in addicted individuals.

Gap junctions (GJs) allow cellular communication, indeed they are fundamental to regulate cellular synchronization, cellular growth and metabolic coordination in tissues.

We analyzed the role of GJs proteins in the cocaine's mechanism of action and furthermore we verified the potential role of GJs in addiction.

Our experiments show several modifications occurring at early and after prolonged cocaine exposure. In addition are shown differential alterations depending by addiction-like behaviour in animals, suggesting a potential involvement of GJs in transition to addiction.

Contents

1 Introduction

Drug abuse represents a serious health issue worldwide. Many are the drugs of abuse and between these, cocaine has taken over the years a prominent place. Only in Europe, it represents the second drug of abuse used after cannabis. It is therefore a question of great importance and extensive studies have been carried out in order to understand the physiological mechanisms of action and try to cure addiction. In fact, it is possible to distinguish two types of individuals, there are the majority of users who occasionally use cocaine for recreational purposes, but they are able to control the frequency and doses and a second group of individuals who show a real addiction, with loss of control on drug intake. Cocaine use is a huge social problem due to the numerous health risks it causes; indeed, it is in relation to cardiovascular and neurological damage, and furthermore to risk of overdose. It entails also an increase of social and health costs sustained for actions of detoxification and rehabilitation.

Recent studies, as regards the dependency, highlight the presence of a real pathology. In many animal models are present alterations in the limbic system at receptorial level and in the mechanisms of signal transduction together with alteration in genes expression. Mechanisms of long-term synaptic modifications seem also involved, including those associated with disturbance of learning and memory.

From the foregoing, it is evident that studies are needed to understand the molecular mechanisms and then potentially identify drug targets to treat addiction.

1.1 Cocaine

1.1.1 History and administration methods

Cocaine (benzoylmethylecgonine $C_{17}H_{21}NO_4$) is extracted by Erythroxylon Coca, a plant native to the Andes and other parts of South America. For hundreds years has been used by Andean Indians that chew the leaves to control symptoms associated with living at high altitude. This form of administration is not very dangerous because the content of cocaine in leaf is about 0.5%, oral absorption is low (about 1 hour) and the hepatic metabolism degrades up to 80% of the ingested dose; so it doesn't reach important blood concentration, the toxicity is rare and phenomena of habituation are mild [1, 2].
The most common form of cocaine as drug of abuse is cocaine hydrochloride, produced by dissolving the alkaloid (as free base) in hydrochloric acid. The salt that is obtained after dehydration represents the white powder that can be taken by nasal sniffing, orally or intravenously.
The free base is water insoluble, can be obtained by dissolution of hydrochloride in alkaline water with use of baking soda; the crystals are vaporizable at temperature lower than chloride and can be smoked together tobacco. In this form has termed "crack", for the popping sound during smoking. Crack produces, respect to hydrochloride, an instantaneous action, high plasma levels but a short-lived euphoria (about 15 minutes). The absorption at the pulmonary level induces, thanks to the considerable absorption area, very high blood levels (similar to intravenous injection), and the passage into the venous circulation produces direct availability in brain, rendering it more addictive.
The intranasal administration of hydrochloride is characterized by low absorption from mucosa, due also to vasoconstrictive properties of cocaine.
The behavioural effect starts after 3-5 minutes and the blood peak occurs between 10 and 20 minutes but after 1 hour, cocaine is no longer detectable. Intravenous administration cause effects after about 1 minute and the action persists up to 20 minutes [3, 4].
After whatever form of administration, cocaine is rapidly distributed from plasma to different district, among which the brain and the fat. Indeed, it crosses the blood brain barrier rapidly due to its lipophilic properties, and more slowly is distributed in other

compartments. 5% of cocaine is excreted unchanged in the urine (detectable after 3-6 hours of use), while 85% is metabolized by the liver and plasma esterases to give ecgonine methyl ester and benzoylecgonine, detectable in urine for more than 14 days after the assumption [5].

1.1.2 Side effects

Cocaine can cause many damages in brain, heart, blood vessels and lungs; moreover for the strong involvement of vital organs, it can cause sudden death [6].

Cardiovascular system is the most common site where appear complications among cocaine users and these can occur after acute or chronic use. Cocaine increases heart rate, blood pressure and myocardial oxygen demand and in addition vasoconstriction and tachycardia. The result can be infarction and ischemia, even in people without heart disease [7, 8].

Cocaine use damages lungs and respiratory system; nose and sinuses are the first to be affected for the direct contact with the substance, indeed a chronic use can degrade the cartilage in the septum leading to nasal perforation. Smoking “crack” irritates the lungs and can cause asthma, bronchospasm, dyspnoea, haemoptysis, diffuse alveolar infiltrates pulmonary and systemic eosinophilia, chest pain, lung trauma pneumonitis, vascular lesions and pulmonary edema [9-11].

Pathological effects of cocaine in central nervous system (CNS) may vary from cerebrovascular effects, as intracranial haemorrhage and infarction due to rapid increase in blood pressure and vasoconstriction, to arrive at neurological effects as seizures, which can compare also at the first intoxication and hyperthermia. There are also psychiatric consequences largely associated to abuse, as psychosis, schizophrenia, depression, suicidal ideation, obsessive-compulsive disorders. To add sexual dysfunction, even though low doses may increase sexual excitement, high doses have the opposite effect with disinterest and impotence [12-19].

On gastrointestinal system cocaine cause constriction of blood vessels and hence a reduction in mesenteric blood flow leading to gastritis or even perforation of the stomach or intestines. It has been reported also hepatocellular necrosis and ischemic hepatitis [20, 21].

As regard the renal system, cocaine can cause sudden and overwhelming kidney failure through a process called rhabdomyolysis. In people with high blood pressure a regular cocaine use can accelerate the long-term kidney damage causing renal infarction, glomerulosclerosis [22, 23].

1.1.3 Psychological effects of cocaine

The use of drugs of abuse may be considered as behaviour that is maintained by its own consequences. A drug of abuse may reinforce the behaviour by inducing pleasurable effects of intake (positive reinforcement) or putting an end to an unpleasant (negative reinforcement). Cocaine is an addictive drug both rewarding, seen as intrinsically positive, and has positive reinforcement effect, because pushing the individual to repeat the intake.

The effects of cocaine can be distinct in short-term and long-term effects.

The principal effect is a powerful stimulation of nervous system. Use in acute induces a rapid sense of euphoria, depending on the dose, by the route of administration and by the state of individual tolerance; this euphoric state can last from about 30 minutes to two hours. This phase, termed *high*, is the most widely effect recognized among the users and hence the reason for which people taking cocaine, at least the occasional users. It increases the alertness, the feeling of well-being and supremacy, an high sense of energy and motor activity, feelings of competence and sexuality. Athletic performance may be enhanced in sports. Indeed the hallmarks of cocaine users are principally high levels of energy and activity, excitement, exuberance, dilated pupils.

The cocaine *high* involves psychological and physical changes. The effects of cocaine on the brain and nervous system cause some of these, and others are due to personal feeling of cocaine users.

This first state, characterized by a complete sense of security is followed by onset of bad mood and anxiety, that taking the users to a *binge,* that is a repeated use of drug at short intervals. With higher dose for prolonged time the effects can be the opposite of the high, with a blunting of the emotions, sadness, anxiety, irritability, paranoia; in addition

there are different physical effects as tremors, convulsions, tachycardia, hyperthermia, as a result of an increased catecolaminergic tone [24].

1.1.4 Exposure time-dependent effects

Acute cocaine use acts in deep areas of the brain, among which areas that reward us for "good behaviour", as those activities that lead to food, sex and healthy pleasure. The stimulation of these areas provides a sense of well-being, but it can create a powerful *craving*. The *craving* is the moment in which there is a strong and compelling need to assume the substance, but this need becomes uncontrollable. It may occur even if there is no physical dependence and may be triggered by seeing objects or experiencing moments that are associated with the drug or usage of it. In some case, this feeling may persist for the rest of the life.

Chronic cocaine use induces changes in brain reward centres and consequent chronic dysphoria. This last together with the sensation of depression, felt after the initial *high*, are caused by the lack of normal amounts of serotonin and dopamine in the brain. The dysphoria magnifies *craving* for cocaine, because cocaine reward occurs rapidly and improves mood, this leads the individual to continue a compulsive drug intake even if there is a worsening of conditions. At the end, the long-term effects are opposite those occurring after acute use.

The molecular changes occurring in the brain, after chronic use, can give the phenomena of tolerance, dependence and withdrawal.

Tolerance is a state characterized by the need to increase the dose of drug to maintain constant the intensity of the effect produced by it. This phenomenon is the manifestation of the processes that occur in brain cells, in order to adapt functionally themselves to strong imbalances of transmitter levels caused by chronic use of cocaine. The mechanisms by which cells induce a tissue response are different and maybe are the summa of different factors. A main factor is the modification at receptorial level that can be obtained in different ways, with the modulation of receptor affinity for the ligands, with mechanisms of down or up-regulation but also with modifications in intracellular pathways or modulation of second messengers. In addition, there are losses of vesicular

monoamine transporters, of neurofilament proteins and other morphological changes that in the long-term cause damage of dopamine neurons. All these effects contribute a rise in tolerance thus requiring a larger dosage to achieve the initial effect.

When dependence is present, stopping cocaine suddenly leads to withdrawal.

Cocaine determines the appearance of a withdrawal syndrome, characterized by an immoderate search of the substance in order to alleviate the physical dependence that produced the drug. The withdrawal is caused by a physical alteration in the normal physiological state of the individual, to adapt itself to the substance. It is characterized by deleterious symptoms that can only be offset with the additional intake of the drug.

The symptoms of cocaine withdrawal (also known as “comedown” or “crash”) range from moderate to severe and are more psychological than physiological, so usually are not visible physical symptoms such as those due to others drugs (like vomiting, chills, tremors) but are present dysphoria, fatigue, difficulty concentrating depression, anxiety, psychological and physical weakness, pain and compulsive craving. Sometimes may cause suicidal thoughts. Some users also report formication: a feeling of a crawling sensation on the skin also known as "coke bugs". These symptoms can last for weeks or in some cases for months.

[25, 26]

1.1.5 Addiction

Cocaine addiction is a persistent state in which drug use escapes control, even when serious negative consequences ensue. These behavioural abnormalities develop gradually and progressively during the course of repeated exposure to a drug of abuse and can persist for months or years after discontinuation of drug use. Indeed, usually the withdrawal symptoms resolve in 1-2 weeks, but craving may returns even many years after the last use. The major stimuli that precipitate this state are three, a stressful life event, an environmental stimulus associated with a drug taking event or a re-exposure to the drug. As a result, drug addiction can be considered a form of drug-induced neural plasticity [27-29].

Definition of addiction is in accord with the criteria for substance dependence contained in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV): substance dependence is a chronic disorder characterized by compulsion to seek and take the drug, loss of control in limiting intake and the emergency of a negative emotional state. The substance intake is continued despite knowledge of having a persistent or recurrent physical or psychological problem, that is likely to have been caused or exacerbated by the substance [30].

There is no "safe" frequency of use for cocaine. It is impossible to predict whether a person will become physically or psychologically dependent on cocaine but only a small proportion (15-20%) of individual become addicted. In fact, the majority of cocaine users maintain a controlled use of the substance and although they manifest pharmacological tolerance never develop a real addiction, contrary to what happens with opioids.

The principal problem is to establish why some individuals move from occasional use to a compulsive use, independently by the time of use.

Several theories attempt to explain transition to addiction; these theories are the result of numerous and different perspectives. Despite the heterogeneity, they can be grouped into two broad theoretical paradigms; the first is linked to the drug while the other is linked to an individual predisposition. Theories centred on drugs are probably the most explored. According to these theories, the transition to dependence arises from neurobiological and psychological effects caused by prolonged use of a drug, which,

through profound changes in the brain and behaviour would render the individual addicted.

In fact neurobiological researches have shown that chronic use of a drug is associated with profound changes at molecular, cellular or synaptic level in the brain; hence for these theories addiction is an iatrogenic disease, a side effect of repeated application of cocaine.

The theories centred on the individual are more recent and are based on the observation that only a small number of people that use the cocaine for long time become dependent. Therefore, the transition to addiction would be a pathological response to the drug and it would depend by specific characteristics of the individual. Addiction would be indeed, an abnormal response to the drug in some individuals and not an inevitable consequence of prolonged use. The drug dependence, according to these theories, is a real pathology involving presumed biological bases associated with a greater or lesser susceptibility of individual to the drug. The biological changes that cause the vulnerability concern many of motivational systems that control the physiological dependence by natural rewards the vulnerable systems, but these are activated aberrantly in response to the drug [31-34].

.

1.1.6 Molecular mechanisms of cocaine

The main goal in the study of addiction is to understand how the effects of a drug of abuse with a prolonged use, progressively lead to permanent molecular and cellular changes.

Cocaine, at synaptic level, inhibits reuptake of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) such as inhibits the action of monoamine oxidases (MAO), enzymes required for catabolism of these neurotransmitters (NTs). The consequence is a high concentration of these molecules after the normal process of depolarization that causes a prolonged activation of the sympathetic nervous system and consequently with effects on hearth, blood pressure and other systems.

Although cocaine acts on different neurotransmitters, growing researches indicate dopamine, as the principal amine involved in cocaine effects. Cocaine binds to dopamine re-uptake transporters (DAT) on the pre-synaptic membranes of dopaminergic neurones; this binding inhibits the removal of dopamine from the synaptic cleft and its subsequent degradation by monoamine oxidase in the nerve terminal. The excess of DA in the synaptic space makes it free to bind to its receptors on the post-synaptic membrane, producing further nerve impulses. This increased activation of the dopaminergic transmission pathway leads to the feelings of euphoria and the *high* associated with cocaine use [35-38] (**Figure 1.1**).

The principal mechanism of action of cocaine is the activation of reward circuitry in the brain. This circuit is principally constituted by the dopaminergic neurons of ventral tegmental area (VTA) in the midbrain, that project to the other regions of mesocorticolimbic system as nucleus accumbens (NAc), dorsal striatum, amygdala, hippocampus and some regions of prefrontal cortex (PFC).

The NAc seems to be the area mostly involved in the *high* phase; indeed this area is implicated in the biological base of survival and reproduction. The DAergic stimulation in NAc induced by cocaine, causes release of DA and the consequences are the sensation of well-being and the desire to repeat the experience pleasant. In limbic system furthermore there are amygdala and hippocampus, memory centres that allow to associate and remember the pleasure had with increase of DA in NAc but also everything associated with the drug; maybe for this, is enough only a place, an image or an emotion to desire repeat experience. The other region involved is the prefrontal cortex; this

mediates several cognitive functions including working memory, behavioural flexibility and attention. Here are processed and analyzed the information received by the others areas, and in normal condition it acts as brake for the other limbic regions when the pleasure can be followed by negative consequences [39-42].

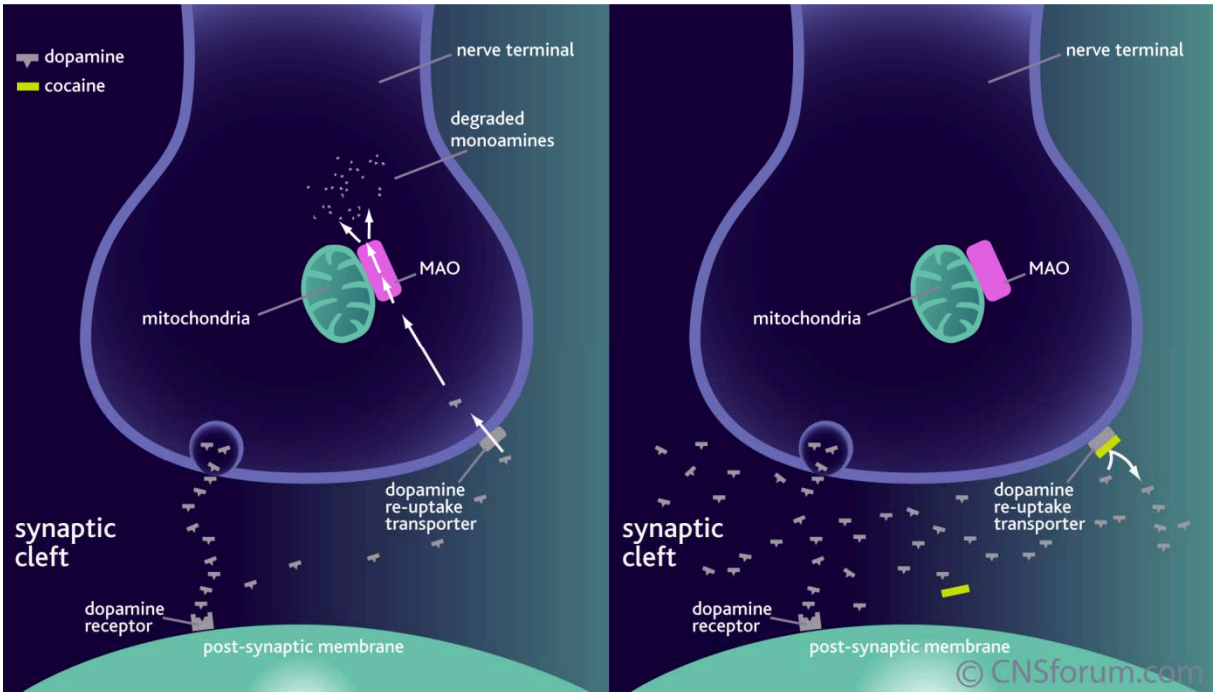

Figure 1.1 Cocaine binds to dopamine transporter blocking the reuptake of DA molecules and then the subsequent degradation by monoamine oxidase (MAO). The result is an excess of DA molecules in synaptic cleft that cause an over activation of the dopaminergic system. (Image adapted from web site http://www.cnsforum.com/)

1.1.6.1 Modifications in genes expression

The behavioural alterations consequent to addiction can persist even for years; this suggests that probably long-term changes in the expression of certain genes may play a decisive role in the transition to addiction. It has been proposed that mechanisms of epigenetic regulation may be modified by chronic exposure to drugs of abuse and this determine adaptations and changes that lead to dependence. Between these mechanisms there are activation and inhibition of transcriptional factors, modification in chromatin and DNA structure (throughout histone tail modifications, DNA methylation), induction of non-coding RNAs. These alterations may last for many years and so it may explain the relapse after long periods of abstinence [43, 44] (**Figure 1.2**).

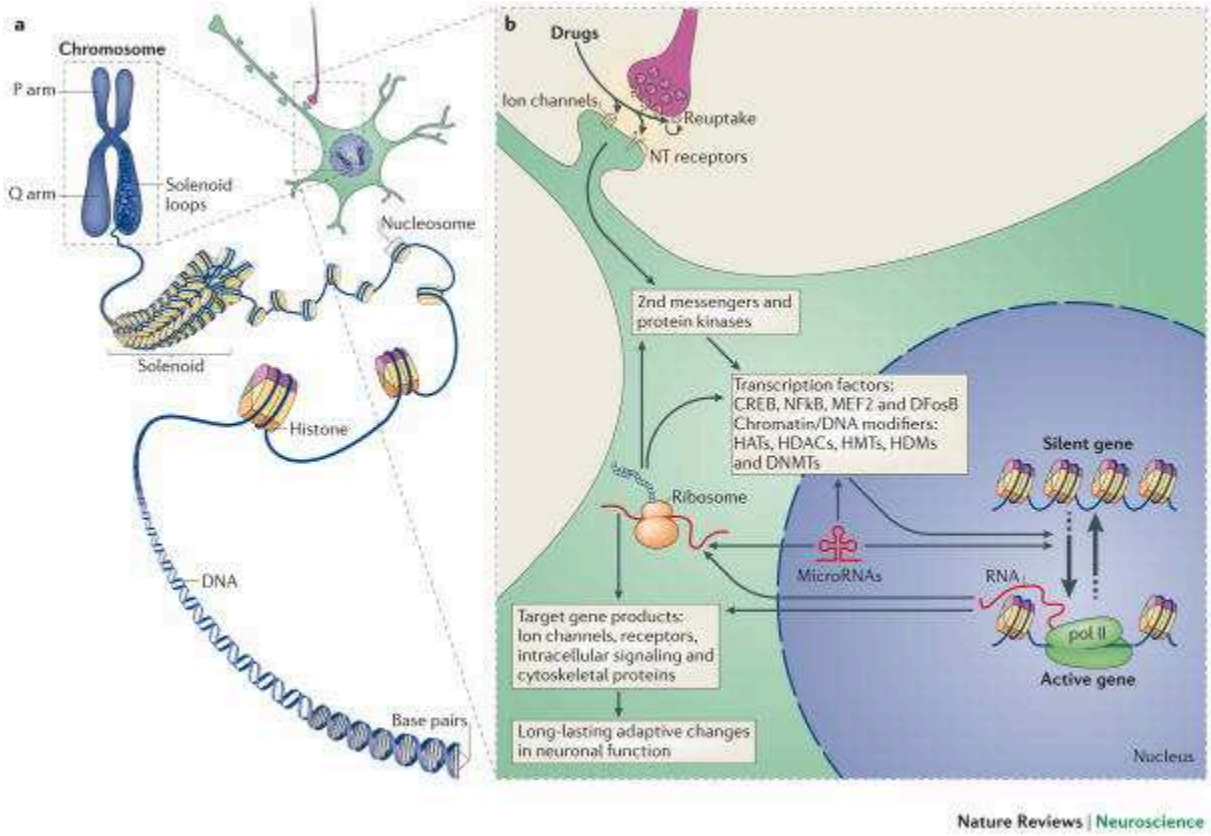

Figure 1.2 Chronic exposure to drugs of abuse causes alteration of intracellular signaling cascades. These lead to the activation or inhibition of transcription factors and of many other nuclear targets including chromatin regulatory proteins. These mechanisms of epigenetic regulations may lead to the induction or repression of particular genes, including those for non-coding RNAs; the altered expression of some of these genes can in turn further regulate gene transcription, determining adaptations and changes that lead to dependence. (Image adapted from Robinson and Nestler, *Nature Rev. Neurosci.*, 2011 [44])

Following treatment with cocaine, amphetamine and other drugs there is an activation of cyclic adenosine monophosphate (cAMP) pathway that can lead to different events depending by the exposure time.

After acute administration of cocaine has been observed an increase of dopamine receptor signaling (in particular of D1 subtype). This D1 activation increases cAMP levels, leading to activation of protein kinase A (PKA) that finally causes an increase in phosphorylation of DARPP-32 at Thr-34 and a decrease in phosphorylation at Thr-75 via a signalling mechanism depending on protein phosphatase-2A (PP2A).

DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, Mr 32kDa) is a phosphoprotein having different phosphorylation site. The phosphorylation of Thr-34 by protein kinase A (PKA), transforms DARPP32 as a strong inhibitor of protein phosphatase-1 (PP-1), while the phosphorylation at Thr-75 level by cyclin-dependent kinase 5 (Cdk5), convert it as an inhibitor of PKA.

PP-1 and PKA are central proteins for the regulation of intracellular events triggered by the activation of D1 receptors and involved in neuronal excitability. The effectors include voltage-gated Na^+ and Ca^{2+} channels, ligand-gated GABA-A channels and glutamate channels such as AMPA/kainate and NMDA.

Instead, after chronic administration of cocaine there is an up-regulation of D1/PKA signaling system and the activation of ΔFosB that cause the increase of Cdk5 expression in caudatoputamen and NAc. This protein kinase may be involved in the locomotors effects of cocaine through the regulation of dopamine signalling. Indeed Cdk5 phosphorylates DARPP-32, co-localized in medium spiny neurons of nucleus accumbens, at Thr-75, becoming inhibitor of PKA.

The effects of chronic cocaine use cause activation of ΔFosB and Cdk5 and maybe these may cause modifications in other pathways, giving stable compensatory adaptations that lead to mechanism of drug addiction [45, 46] (**Figure 1.3**).

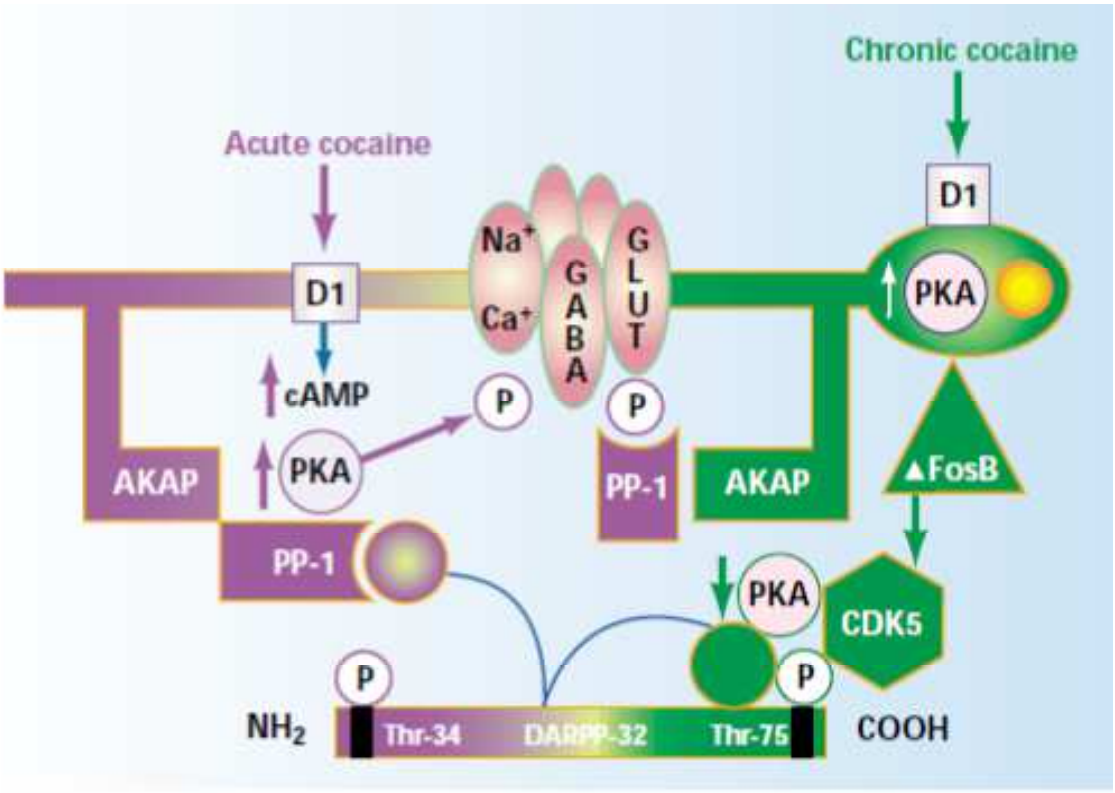

Figure 1.3 Acute and chronic effects of cocaine on the dopamine receptor (D1) signaling pathway.
Acute cocaine administration increases D1 receptor signaling by blocking the presynaptic re-uptake of dopamine. D1 receptor activation increases cAMP levels, leading to activation of protein kinase A (PKA). Anchoring proteins (AKAP) serve to position PKA close to its substrates, facilitating PKA-mediated phosphorylation (P) of these targets. PKA substrates include voltage-gated Na^+ and Ca^{2+} channels, ligand-gated GABA-A channels (GABA), and glutamate channels (GLUT) such as AMPA/kainate and NMDA channels. Phosphorylation of DARPP-32 at Thr-34 causes inhibition of phosphatase-1 (PP-1). Chronic cocaine administration up-regulates D1/PKA signaling, and also leads to the activation of the transcription factor ΔFosB. ΔFosB induces the cyclin-dependent protein kinase Cdk5 that phosphorylates DARPP-32 at Thr-75, which then inhibits PKA. (Image adapted from Bibb JA et al. *Nature* 2001 [45])

Drugs of abuse cause changes in many proteins in the various brain regions. There are a few numbers of well-characterized changes, which seem contribute to certain features of addiction.

Among these, the transcriptional factors ΔFosB and CREB (cAMP response element binding protein), but also the plasticity-related protein Arc (activity-regulated cytoskeletal associated protein).

Following treatment with cocaine there is an increase of CREB activity in some brain regions among which NAc. Once time activated by phosphorylation (for activation of different pathways and involving different kinases), CREB can bind to cAMP-responsive elements (CRE), hence it allows recruitment of CREB-binding proteins mediating genes

transcription. It has been shown that over expression of CREB reduces the rewarding effects of cocaine. So it has been led the hypotheses that CREB mediates tolerance to cocaine's positive hedonic effects, but also increases responsiveness to stress and may mediate the negative and dysphoric aspects associated with cocaine withdrawal [47-51]. FosB belong to Fos family of transcriptional factors; after binding to Jun family proteins, it forms a complex activator protein-1 (AP-1). This complex, by binding to AP-1 sites on DNA, regulates transcription of some genes. Several studies show the induction of c-Fos, FosB and others Fos family components in NAc and dorsal striatum after acute administration of cocaine, but their levels return normal after few hours by drug intake. Instead, after chronic cocaine administration, a many stable protein ΔFosB, derived by alternative splicing of FosB, is induced at high levels in NAc. This protein has been linked to typical behaviour of cocaine abuse observed in laboratory animals, as increased locomotor sensitivity, increased conditioned place preference and increased self-administration. In fact, all these behaviours are reduced in mice blocking this protein. Thus ΔFosB may represent the switch from occasional use to addiction, because alone is sufficient to sensitize the animals to reward of drugs and its long stability can persist for months, even if this would be the starting conditions for other stable modifications [52-56].

The increase of ΔFosB after cocaine administration causes transcriptions of some genes among which, cyclin-dependant kinase-5, Arc and others that would mediate increasing of dendrites spine in medium spiny neurons of NAc. In this way, NAc may receive more information from other regions and so lead to long-lasting neurobiological effects of addiction. ΔFosB regulates also proteins important for glutamatergic transmission and plasticity as AMPA receptors and Ca^{2+}/calmodulin-dependent protein kinases II (CAMKII) [45, 57-60].

Activity-regulated cytoskeletal associated protein Arc, belongs to the class of Immediate Early Genes. Studies show that Arc is index of neuronal activity because it plays an important role in activity-dependent synaptic plasticity. It has also been shown that Arc influence directly homeostasis and cellular function.

Arc encodes for a protein principally localized in the neurons at the level of dendritic spines. It is important in the regulation of long-term depression (LTD) activated by NMDA and metabotropic glutamate receptors. The translation of Arc is induced within five minutes after the activation of these receptors, and leads to endocytosis of AMPAR

and then to depression of excitatory synaptic transmission. Arc plays also a crucial role in the consolidation of long-term potentiation (LTP) and then in synaptic plasticity. One of the regulators of this process seems to be BDNF (brain-derived neurotrophic factor) and the LTP BDNF-dependent would be associated with the transport to dendrites of Arc mRNA. Here Arc protein participates in the organization of cytoskeleton proteins promoting the growth of dendrites. This process is also observed in amygdala following exposition to several drugs. So this suggest an involvement of Arc in the modulation of others genes and proteins with use of drugs of abuse [61-63].

Many others transcriptional factors have been implicated in transition to addiction, among these NF-κB (nuclear factor-κB), equally involved in dendrites sprout and sensitization to drug's reward or MEF2 (multiple myocite-specific enhancer factor 2), involved in the structural and behavioural changes. Others evidences regard glucocorticoid receptors, early growth response factors (EGR) and signal transducers and activators of transcription STATs [44].

1.1.7 Neurobiological substrates of cocaine: brain reward system

Mesocorticolimbic system regulates the reward mechanisms to natural stimuli as food, water and sex. The drugs of abuse, such as cocaine, produce their reinforcing effects acting on the same areas of limbic system responsible for influence of motivational, emotional and affective information.

This system includes several structures interconnected. It is constituted by dopaminergic neurons that from VTA innervate NAc, amygdala, PFC and hippocampus. Amygdala, hippocampus and prefrontal cortex send glutamatergic projections to NAc. NAc send GABAergic projections to the ventral pallidum and to VTA/substantia nigra. VTA transmits GABAergic inputs to the medial dorsal thalamus. This last sends glutamatergic efferents to PFC [64](**Figure 1.4**).

The major responsible of rewarding and reinforcing effects of cocaine is the dopaminergic system that projects from VTA to NAc and to others forebrain areas including dorsal striatum. Beside to dopaminergic transmission cocaine affects also the glutamatergic system, inducing long-term adaptations that lead to typical behaviour of addiction as cocaine craving and seeking [64].

Nucleus Accumbens (NAc) is divided in two sub regions, the core and the shell, having each one distinct function. The shell is classified as a part of limbic system and is implicated in reward effect of cocaine, hence it regulates the response in presence of motivational stimuli; while the core, considered part of basal ganglia, mediates seeking and locomotor activity under stimuli linked to drug consumption. The NAc functions translating the rewarding/reinforcing effects of drugs of abuse into drug-seeking behaviour. It processes, consolidates and integrates information from limbic nuclei to basal ganglia structures, including the ventral pallidum, thalamus and motor cortex. After acute cocaine administration, there is a selective activation of DAergic transmission in NAc shell, due to activation of Daergic projections from VTA; the huge increase of DA returns to basal levels stopping the administration. However, a prolonged cocaine use causes depletion of DA in NAc, PFC and cerebral cortex and in addition in NAc there is also a decrease in glutamate levels [65].

The ventral tegmental area (VTA) is the origin of the dopaminergic cell bodies of the mesocorticolimbic dopamine system; indeed, it is constituted by about 50-60% of DA neurons. These neurons are stimulated by excitatory glutamatergic afferents and they

are negatively regulated by GABAergic neurons. It is widely implicated in the drug and natural reward circuitry of the brain but also in cognition and motivation [38, 64-66].

Medial prefrontal cortex (mPFC) is the region involved in higher-order cognition (decision-making) and emotion. In drugs addiction, it has implicated with salience attribution and inhibitory control and in compulsive behaviours, as craving and compulsive drug intake. It receives DAergic stimulation from VTA and sends glutamatergic projections to NAc.

The dorsal region of mPFC send efferents to NAc core, while the ventral region to the NAc shell. After cocaine intake there is an increase in metabolic activity and blood flow in PFC. During the phase of seeking there are changes in glutamatergic communication, followed by an increase of Glut in NAc that may cause the motor behaviour. Maybe this is due to the increase of DA stimulation of PFC from VTA that increase the excitability of glutamatergic neurons [67-69].

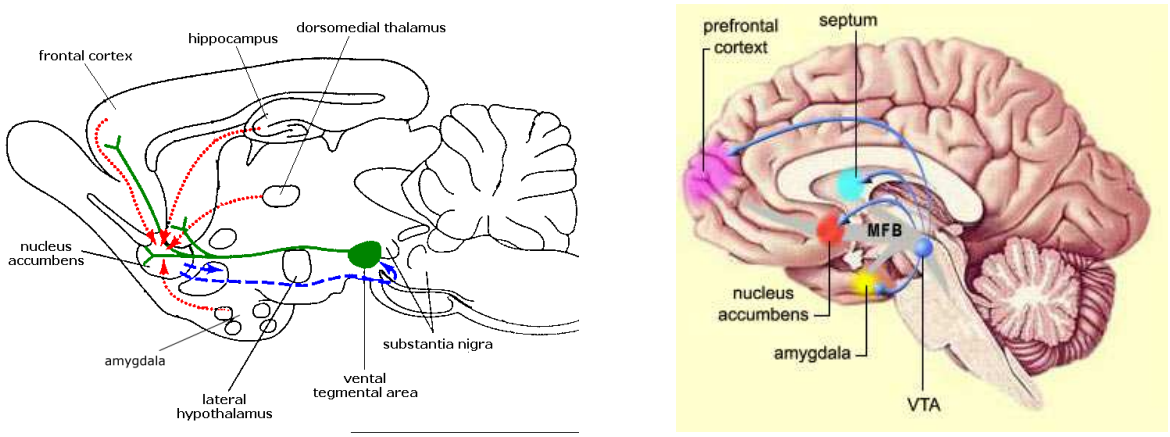

Figure 1.4 Organization of mesocorticolimbic system on rat brain (on the left) and human brain (on the right). The medial prefrontal cortex (mPFC) sends glutamatergic afferents to the nucleus accumbens. The core and shell sub regions of the NAc also receive excitatory glutamatergic projections from hippocampus and basolateral amygdala nuclei. Dopaminergic projections from the VTA and substantia nigra (SN) modulate the flow of emotional, declarative, and procedural memories. The activity of VTA and SN dopamine cells is regulated by excitatory glutamatergic projections from the mPFC, hippocampus, and amygdala, and by inhibitory GABAergic projections from the NAc and ventral pallidum (VP). In rat brain are indicated in green the DAergic, in red the Glutamatergic and in blue the GABAergic projections; in human brain, in blue are indicated Daergic projections. (Adapted from Schmidt et al., *Ann. N Y Acad. Sci.;* 2010 [64]).

1.1.7.1 Dopaminergic and glutamatergic transmission

Dopaminergic system is the main actor of rewarding mechanisms in the brain. The dopamine (DA) is the neurotransmitter most involved in the regulation of pleasure and reinforcement. It mediates its physiological effects by binding with a series of five G protein-coupled receptors. The dopamine receptors consist of the D1, D2, D3, D4, and D5 receptors and are categorized into two groups based on their properties and effects. The D1 and D5 receptors belong to the D1-like subfamily, they are coupled to Gs protein and increase the cellular concentrations of cAMP by the activation of the enzyme adenylate cyclase. The D2, D3, and D4 receptors belong to the D2-like subfamily; they are coupled to Gi/Go proteins and decrease the cellular concentrations of cAMP by inhibition of adenylate cyclase.

Even though is clear the involvement of DAergic transmission in the behaviour of cocaine seeking, it is still controversial which class of receptors are involved.

Some studies report that the administration of D2-like receptors agonists in blood flow or in NAc shell, give cocaine seeking, while antagonists attenuate this behaviour. Other works report that D1-like agonists prevent cocaine-seeking behaviour induced by cocaine, even if there are contrasting results, indeed others report that administration of D1 agonists into NAc shell reinstates drug seeking. These controversial results demonstrate however a role for both D1- and D2-like receptors in cocaine seeking, even if the dissociation of effects observed between the two types, may depend by the site of expression [70-74].

In the last years, it has become evident a fundamental role of glutamatergic transmission in the cocaine effects. In fact, persistent changes in this system are implicated with cocaine craving and seeking behaviours, but also with learning and memory processes linked to development of addiction [75, 76].

Glutamatergic system plays an important role in almost all physiological functions. Glutamate (Glut) is the most abundant excitatory neurotransmitter in the vertebrate nervous system, accounting over 60% of neurons and two types of receptors regulate its action: the ionotropic and the metabotropic. The ionotropic receptors (iGluR) are ion channels activated by glutamate. Their activation increases the influx of cations, as sodium and potassium, causing depolarization of the membrane. They are divided into three subtypes receptors: N-methyl-D-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-

methyl-isoxazol-4-yl)propanoic acid (AMPA) and kainate receptors.

The metabotropic receptors (mGluR) are eight G protein-coupled receptors and are divided into three groups, I (mGluR1-5), II (mGluR2-3) and III (mGluR4-6-7-8), based on sequence homology, mechanisms of signal transduction and their pharmacological selectivity. Ionotropic receptors tend to be quicker in relaying information, but metabotropic ones are associated with a more prolonged stimulus.

Cocaine does not act directly on glutamatergic neurons; acute administration has no effect on glutamate levels on NAc, but during withdrawal, the glutamate levels are reduced, perhaps due to a reduced activity of cystine-glutamate antiporter in glial cells (plasma membrane transporter for the cellular uptake of cystine in exchange for intracellular glutamate). Moreover it has been found that cocaine-induced reinstatement of drug-seeking behaviour is accompanied instead by an increase in glutamate levels in NAc, due to the glutamatergic stimulation from dorsal prefrontal cortex [77-79].

The metabotropic glutamate receptors (mGluR) are potentially involved and may be a pharmacological target for cocaine addiction. Indeed, they are subjected to variations in transcription and membrane trafficking during withdrawal. For example, expression of mGluR5 and mGluR2/3 mRNAs, are increased and decreased respectively in the NAc after 3 weeks of withdrawal. Agonists for mGluR2/3 attenuate cocaine self-administration and the reinstatement of cocaine seeking, while mGluR5-KO mice are insensitive to the reinforcing effects of cocaine and mGluR5 antagonists attenuate the reinstatement [64].

As regard the ionotropic glutamate receptors , NMDAR in NAc seem to have a role in the neuronal plasticity during addiction. The use of NMDAR agonists promote reinstatement of cocaine seeking while antagonists decrease it.

The AMPA receptors antagonists decrease cocaine self-administration when are infused in NAc core but not in shell. After abstinence, there is also an increase in the number of these receptors. It seems that an increased glutamatergic transmission performed by AMPA receptors may mediate the reinstatement of cocaine-seeking behaviour. AMPA receptors have been also implicated in neuronal plasticity observed in several regions of mesocorticolimbic system; in particular changes in the ratio between GluR1 and GluR2/3 subunits (this last increases as trafficking at cell surface), would be responsible of alterations in forms of long-term potentiation (LTP) and long-term depression (LTD), observed after cocaine administration [64, 80-82].

1.1.8 Synaptic plasticity in the addiction

Growing researches support the hypothesis that forms of long-lasting synaptic plasticity may be implicated in mechanisms of addiction.

Synaptic plasticity is the ability of the synapse to change its strength in response to use or disuse of transmission, over synaptic pathways. Plasticity can be divided as short-term, lasting a few seconds or less, or long-term, which lasts from minutes to hours. Short-term synaptic plasticity results from an increase or decrease of probability that synaptic terminals will release transmitters in response to action potential.

Long-term potentiation (LTP) and long-term depression (LTD) are two forms of long-term plasticity, lasting minutes or more, that occur at excitatory and inhibitory synapses. These two forms of synaptic plasticity are principally involved in the mechanisms of learning and memory, but seem to have also an important role in the consequences of drug use.

LTP is a long-lasting enhancement in signal transmission between two neurons resulting from their synchronous stimulation, while LTD is an activity-dependent reduction in the efficacy of neuronal synapses following a long patterned stimulus.

LTP is induced by *high frequency stimulation* (HFS) and requires activation of NMDA receptors through the binding of glutamate, glycine or D-serine. The opening of NMDAR causes a strong increase of Ca^{2+} in the post-synaptic neuron; moreover, the strong depolarization displaces the block of NMDAR by Mg^{2+}, allowing more Ca^{2+} to enter. The strong increase of Ca^{2+} causes activation of several proteins such as Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), protein kinase-A (PKA), mitogen-activated protein kinase (MAPK). These kinases are strongly involved in the increased membrane trafficking and in the activation of excitatory post-synaptic receptors, the AMPAR. These, enhance the cations afflux in the cells, and as a results there is an enhancement of synapse's strength.

Other mechanisms can activate an LTP. In mossy fiber-CA3 hippocampal synapses or in cerebellar parallel fiber synapses, LTP is induced by a rise of calcium in presynaptic terminal. Here, Ca^{2+} causes the activation of calcium-stimulated adenylate cyclase, then the rise of cAMP and the consequent activation of PKA, activates Rab3a and RIM1α, proteins involved in the long-lasting increase of glutamate release.

LTD can be induced by *low frequency stimulation* (LFS) with three different mechanisms. In hippocampus LFS causes a weak depolarization that open NMDAR but determine a low Ca^{2+} concentration in post-synaptic neuron; this activates different phosphatases, such as protein phosphatases calcineurin and protein phosphatase-1 (PP1), that revert the LTP process, inhibiting the opening and internalizing the AMPAR. In cerebellum it has been found an LTD mGluR-dependent, in which the activation of group I of mGluR activates the protein kinase C, taking to endocytosis of AMPAR. Another mechanism is LTD eCB-mediated, found in striatum and neocortex, where the activation of group I of mGluR (which leads to activation of phospholipase C) or an increase of intracellular Ca^{2+} in the postsynaptic neuron, initiate the synthesis of endocannabinoids (eCB). The eCB are subsequently released from the postsynaptic neuron, travel retrogradely to bind to presynaptic cannabinoid-1 receptors (CB1R) and this prolonged activation of CB1Rs depresses neurotransmitter release [83] (**Figure 1.5**).

Physiologically in mesocorticolimbic system are expressed both LTP and LTD.

In VTA, the excitatory DAergic synapses exhibit LTP and LTD. For LTP are necessary NMDAR and the consequent increase in post-synaptic calcium, instead LTD can be inducted with two mechanisms, activation of NMDAR and voltage-dependant calcium channels or activation of mGluR [84-87].

In NAc forms of LTP and LTD occurs in medium spiny neurons. LTD requires activation of mGluR to increase Ca^{2+} and production of endocannabinoids (although exist also a mechanism eCB-independent). As regard LTP, its activation requires NMDAR and calcium increase [88-91].

The PFC is undergone to dynamic neuronal adaptation processes through the induction of synaptic plasticity LTP and LTD. It has been shown that several neurochemical substances, such as dopamine, noradrenalin, serotonin and acetylcholine, modulate synaptic plasticity in this region. In particular serotonin, that in PFC regulates cognition and emotion, seems to have a principal role in the LTD induction; indeed it in collaboration with group I of mGluR, facilitates LTD induction through increase of AMPAR internalization. Another characteristic of synaptic plasticity induction in the PFC is its dependence from dopamine [92, 93].

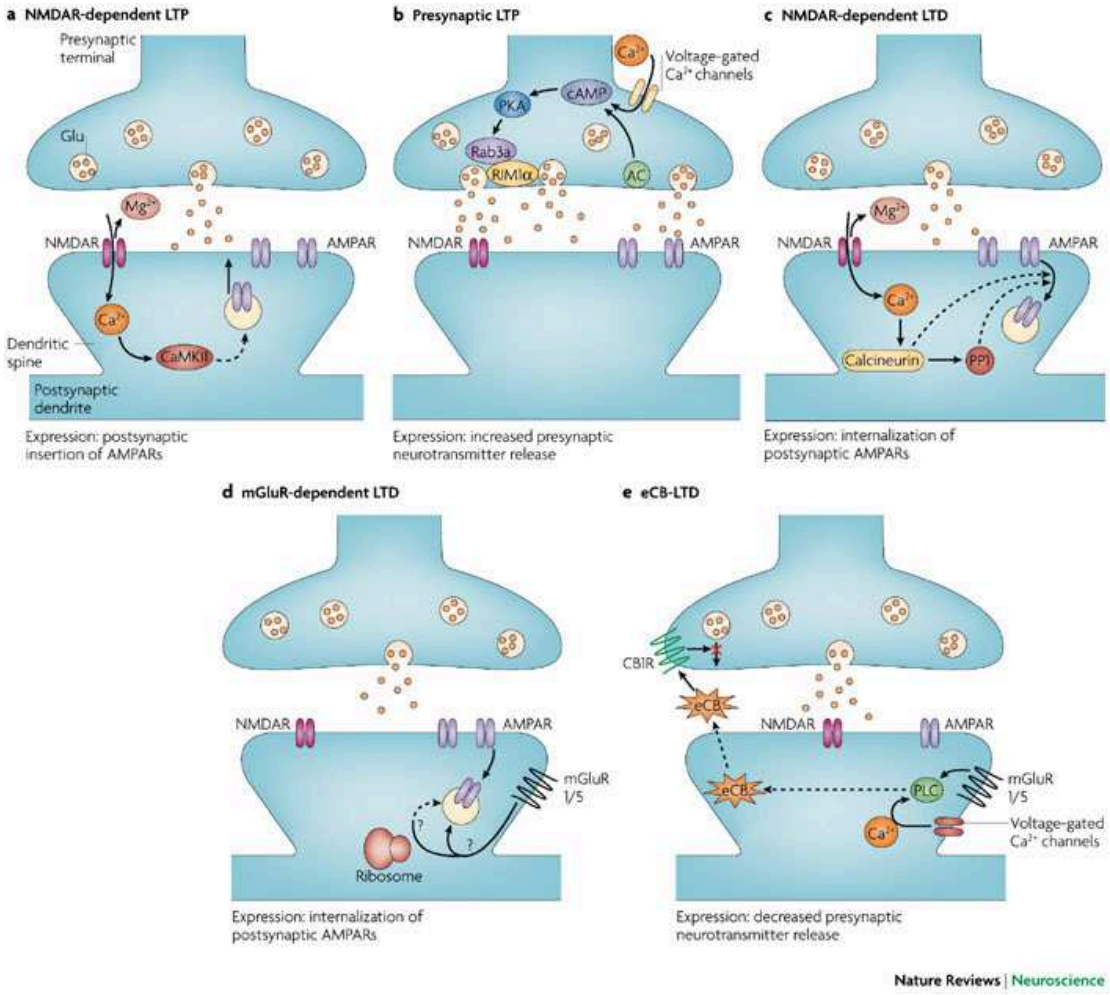

Figure 1.5 Simplified Diagrams of synaptic plasticity observed in the rodent brain. **a**: NMDAR-dependent LTP is dependent on postsynaptic NMDAR activation and CAMKII for its initiation. This causes the insertion of AMPAR into the postsynaptic membrane. **b**: Presynaptic LTP at mossy fiber–CA3 hippocampal synapses and in parallel fibre–Purkinje cell cerebellar synapses. The entry of presynaptic Ca2+ activates adenylate cyclase (AC) leading to a rise in cAMP and the activation of PKA. This modifies the functions of Rab3a and RIM1α leading to increase in glutamate release. **c**: NMDAR-dependent LTD is triggered by Ca2+ entry through postsynaptic NMDAR channels, leading to increases in the activity of the protein phosphatases calcineurin and protein phosphatase 1 (PP1). This causes internalization of postsynaptic AMPARs and a downregulation of NMDARs. **d**: mGluR-dependent LTD. Activation of postsynaptic mGluR1/5 triggers the internalization of postsynaptic AMPARs. **e**: Endocannabinoid-LTD. mGluR1/5 activation or an increase of intracellular Ca^{2+}, lead to activation of PLC in the postsynaptic neuron, causing the synthesis of endocannabinoid (eCB). The eCB is subsequently released and it binds to presynaptic cannabinoid 1 receptors (CB1R) and this prolonged activation of CB1Rs depresses neurotransmitter release via unknown mechanisms. (Image adapted from Kauer & Malenka, *Nature Rev. Neurosci*, 2007 [65])

Under cocaine exposition occur numerous rearrangements. In VTA, only 1 or 7 days of passive cocaine administration is enough to potentiate the glutamatergic transmission in DAergic neurons. This potentiation is mediated by an increase in AMPAR and particularly with the disappearance of GluR2 subtype, normally present in AMPAR, and an increase of GluR1subunit. However, this LTP-like is transient, lasts 5 days and disappears after 10 days, and it precludes an ulterior LTP stimulation [94-96]. Surprisingly the induced LTP AMPA-mediated last for about 3 months if the cocaine is self-administrated, while a natural reward cause an LTP of only 7 days. From this, appears important the involvement of mechanisms of learning and memory associated with the drug. In addition, should be considered also the GABAergic transmission, even influenced by drug of abuse [97, 98].

In the NAc, one single cocaine injection does not alter AMPAR activity, but it abolishes the normal LTD eCB-mediated, probably by decreasing of mGluR5 levels. After repeated exposure and during early withdrawal the AMPAR functions are reduced but this occurs only in the NAc shell [99, 100].

After chronic cocaine self-administration, there is a disruption of LTP in all NAc and a loss of LTD only in the core. The loss of LTP may be due to a reduced trafficking of AMPAR, indeed the amount of AMPA receptors relative to NMDA receptors decrease in medium spiny neurons. This decrease in AMPA receptors may occur through the mechanism of LTD NMDAR-dependent, because this form of plasticity is reduced after cocaine use [101].

In the AMPA receptors is also observed an increase of subunit GluR1 and interestingly, the administration of interfering molecules for GluR1 and GluR2 in the NAc reduces the cocaine reinstatement, indicating a possible involvement of the two subunits in drug-motivation [102, 103].

Several studies show that medial PFC, after few days of cocaine administration, become highly sensitive to the induction of LTP, due to a reduction of inhibition of pyramidal neurons for decrease of GABA(A) receptors. Repeated cocaine administration impairs also the LTD mGluR-mediated. In both cases is evident an involvement of DA receptors, because their block prevent the effects, cocaine-induced on LTP and LTD [104-107].

The equip of PV Piazza in Bordeaux has developed an animal model, through which, sessions of prolonged cocaine self-administration (SA) allow to discriminate animals with an addiction-like behaviour from non-addict animals. Experiments carry out on

slices obtained after 24 hours by the last cocaine infusion, show no modifications in LTP or in LTD after one week of SA in NAc, while after 17 days of SA, LTD NMDAR-dependent is completely abolished. Instead, with a prolonged cocaine exposure (50 to 72 days), LTD returns normal in non-addict animals while in addict remains suppressed. For contrast, the LTD mediated by mGlu2/3 receptors is not affected in all groups. For this, it has been postulated that maybe the persistent impairment in LTD is associated with transition to addiction even if all individuals start by the same impairment at early drug use [108, 109] (**Figure 1.6**).

The same experiments have been conducted on PFC slices. In this region both addict and non-addict animals show no variations as regard LTD eCB-mediated after 17 days of SA, but this, is suppressed in all individuals after prolonged drug exposure.

On the contrary the LTD mGlu2/3-mediated is normal in the animal controls and in both non-addict and addict animals after 17 of SA, while after 72 days of SA, this form of synaptic plasticity is suppressed only in addict rats. There is moreover a reduced expression level of mGlu2/3 proteins. Changes are observed also in basal synaptic strength (measured as AMPA/NMDA ratio), that is normal in controls and non-addict animals but is increased of 50% in addicted rats after prolonged cocaine exposure [110] (**Figure 1.7**).

These discoveries suggest reorganization into the brain following drug use, and this may explain the loss of control occurring in some individuals. Indeed the addicted individuals exhibited a form of "anaplasticity", i.e. the brain is unable to adapt and change in response to the loss of LTD, which becomes permanent. This may explain why gradually the control of drug intake is lost, the craving become more intense, and appears the addiction in which the need for the drug becomes very strong, despite the negative consequences of its use.

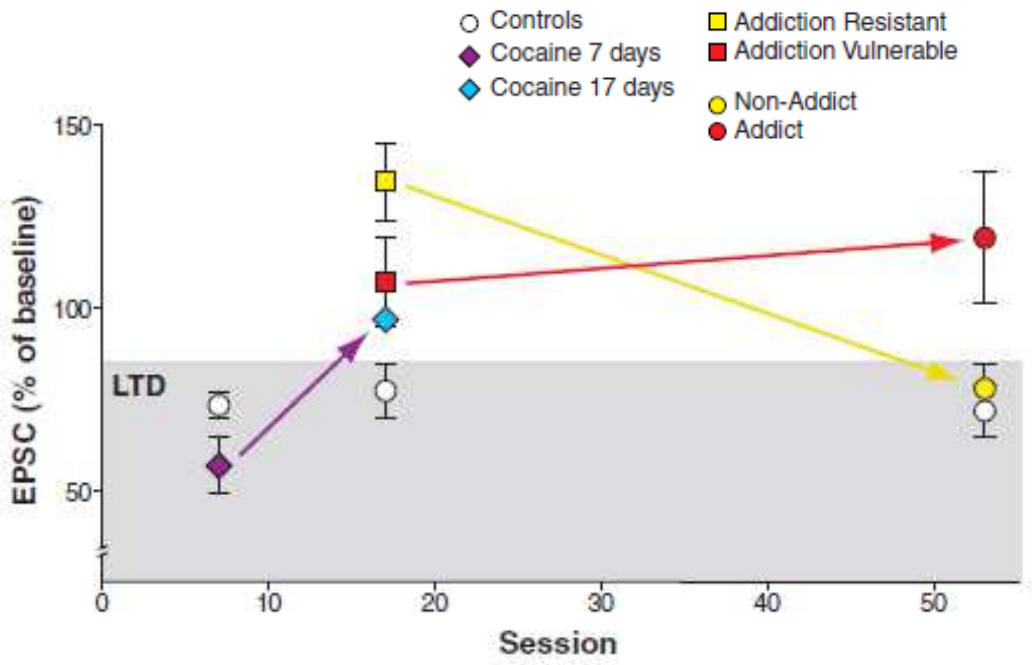

Figure 1.6 NMDAR-dependent LTD is disrupted in Addict animals. In the graph are shown the averaged data of representative excitatory postsynaptic current (EPSC) traces. LTD is induced in controls and in all animals treated for 7 days. After 17 days of cocaine SA the LTD NMDAR-dependent is suppressed in both resistant and vulnerable animals, but after prolonged exposure to cocaine, non-addict animals restore their LTD while in addict animals remains suppressed. (Image adapted from Kasanetz F et al. *Science* 2010 [109])

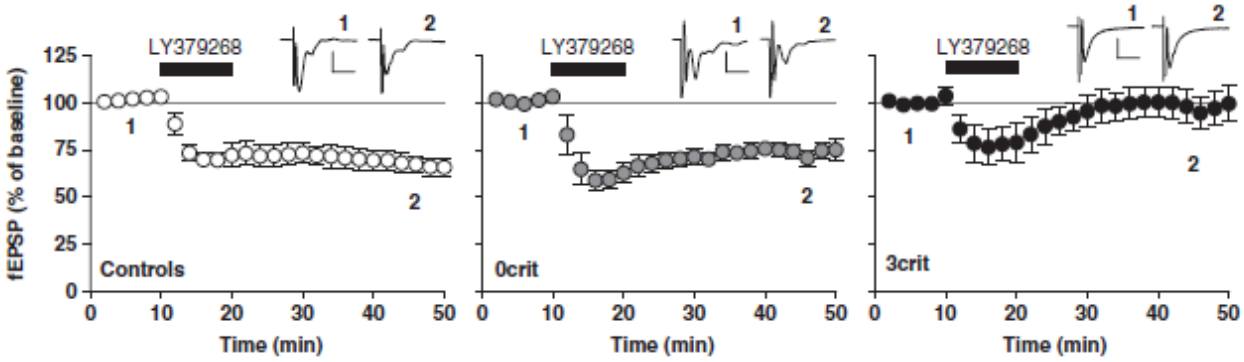

Figure 1.7 mGluR2/3-dependent LTD is selectively impaired in addict-like rats. After 17 days of cocaine SA, LTD was normal in all groups, but 72 days of SA completely abolish the LTD only in addict rats (3crit), while in controls and non-addict animals (0crit) is normal. Representative fEPSP traces recorded during baseline (1) and 35 min after LTD induction (2) are depicted. (Image adpated from Kasanetz et al. *Mol. Psychiatry* 2012 [110])

Moreover, these mechanisms of synaptic plasticity, similar to what occurs in the mechanism of memory, are associated with formation of new connections that cause changes in the synaptic transmission. Dopamine denervation reduces dendrites density but repeated treatments with cocaine increase dendritic spine density and the number of branched spines in the shell of NAc and on pyramidal cells in PFC; these alterations last for 4 weeks after drug exposure [39, 111] (**Figure 1.8**).

In this way, it is possible that cocaine self-administration experience alters patterns of synaptic connectivity within limbo cortical circuitry. These alterations may contribute to cocaine's incentive motivational effects and have neuropathological effects in frontal areas involved in decision-making and judgment.

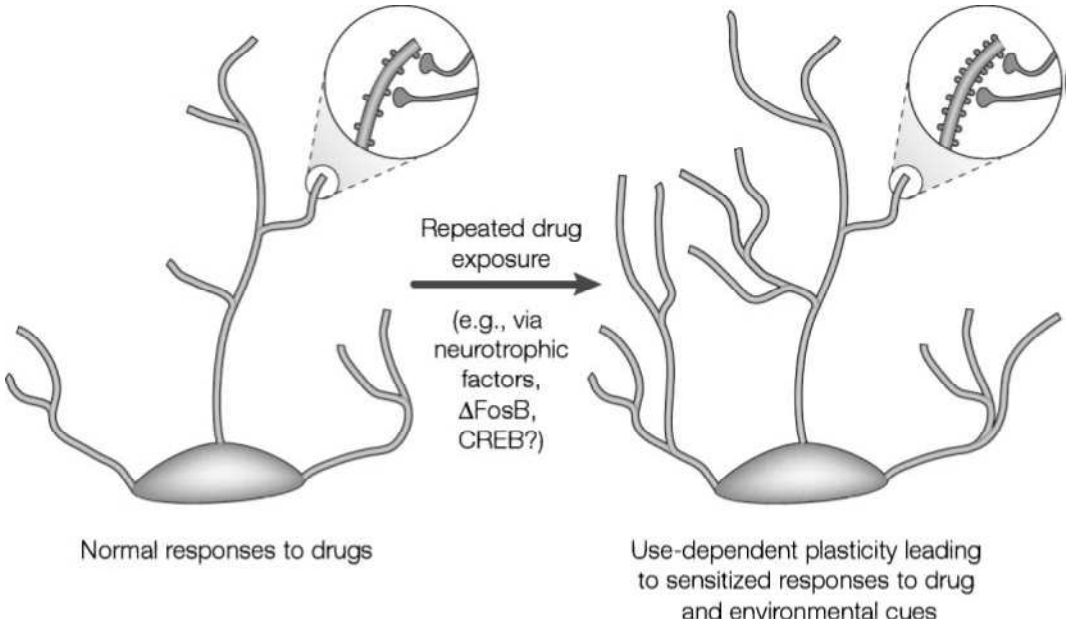

Figure 1.8 Regulation of dendritic structure by drugs of abuse. The figure shows the expansion of a neuron's dendritic tree after chronic exposure to a drug of abuse, as it has been observed in the NAc and PFC for cocaine and related psychostimulants. (Adapted from Nestler AJ, *Nature Rev Neuroscience;* 2001 [112])

1.1.9 Animal models of addiction

The need to understand the mechanisms underlying the phenomenon of addiction has led to the development of animal models that mimic the typical behaviours occurring in humans after taking drugs. In the past years have been used principally techniques of passive administration of the substance, procedures that showed direct results about the pharmacology of the substance and neurobiological aspects. Other models have been used to scan the molecular mechanisms induced by drugs and to understand why some individuals pass from normal use to a compulsive use. However, with these models the behavioural effects of drugs depend by several factors as the species, routes of administration, time of administration, dose concentration. Nevertheless, differences arise depending on type of administration, if non-contingent (passive) or contingent (voluntary).

However, the best animal model to mimic the human behaviour seems to be the self-administration model, in which the animals learn to achieve behaviour to obtain the drug and after they can decide whether or not assume the drug.

Deroche-Gamonet *et al*. developed an experimental animal model to identify the subjects with a predisposition for addiction. In this model, as in humans, only a small proportion of individuals show the hallmarks characteristics of addiction. In according to substance dependence criteria of DSM-IV, several parameters are considered, as the difficulty in stopping drug intake, the high motivation to take the drug and the continued use of substance despite harmful consequences.

One of the most important findings in these studies has been the demonstration that the total amount of drug consumed by addicted rats is the same than that consumed by the animals maintaining a perfect control of drugs use. This would demonstrate the importance of individual vulnerability to the drugs respect to the iatrogenic theories that predict how the amount of drug taken is directly proportional to the probability of fall into addiction. Then these experimental animals show a real addiction, and the data indicate that the transition would be the result from the combination of two main factors: prolonged exposure to a substance with abuse potential and a vulnerable individual phenotype [108] (**Figure 1.9**).

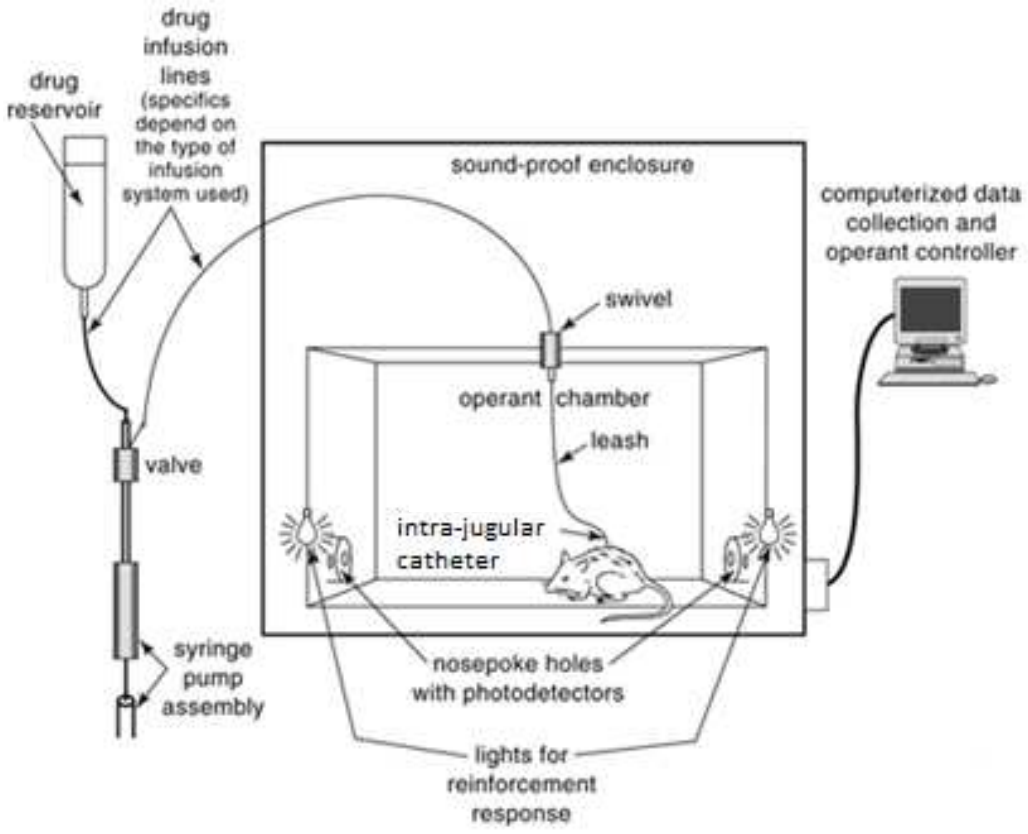

Figure 1.9 Intravenous self-administration apparatus used to deliver response-contingent drug infusions and collect data during self-administration sessions. (Image adapted from Grahame NJ, *Curr Protoc Neurosci*. 2002 [113]

1.2 Gap Junctions

Gap Junctions (GJs) allow cellular communication, a key condition for the existence of pluricellular organisms. Without this type of direct transmission, the exchange of information would not be possible. Indeed GJs regulate cellular synchronization, cells growth and metabolic coordination in tissues.

1.2.1 Formation and structure

Gap Junctions are intercellular channels composed by transmembrane proteins belonging to three families: connexins (Cxs), pannexins (Panxs) and innexins (Inxs). They are present in all Metazoan kingdom. The first proteins identified have been the connexins, found only in Chordates. In invertebrates are present instead, the innexins, similar in the structure and membrane topology to connexins but not in the amino acidic sequence. In recent years, by sequencing of mammalian genomes, the pannexins have been identified as genes homologues to innexins and present in many groups including Chordates [114-116].

These proteins are characterized by a similar characteristic structure, comprising four alpha helix transmembrane domains (TM1-TM4), N- and C-terminal intracellular regions, two extracellular loops (E1-E2) and one cytoplasmatic loop (I1) [117, 118]. This structure is essential for the formation of a hemichannel; indeed six Cxs, Panxs or Inxs oligomerize to form a hexameric pore complex, respectively called connexon, pannexon or innexon. Two opposing hemichannels, each arising out from a cell, give rise to a gap junction, commonly assembled as GJs plaque, characterized by a reduced space between the cells (about 2-4 nm) and composed by clusters of few or hundreds of gap junction channels. The association of the two hemichannels is mediated by H-bonds occurring between the extracellular loops of the proteins (**Figure 1.10**).

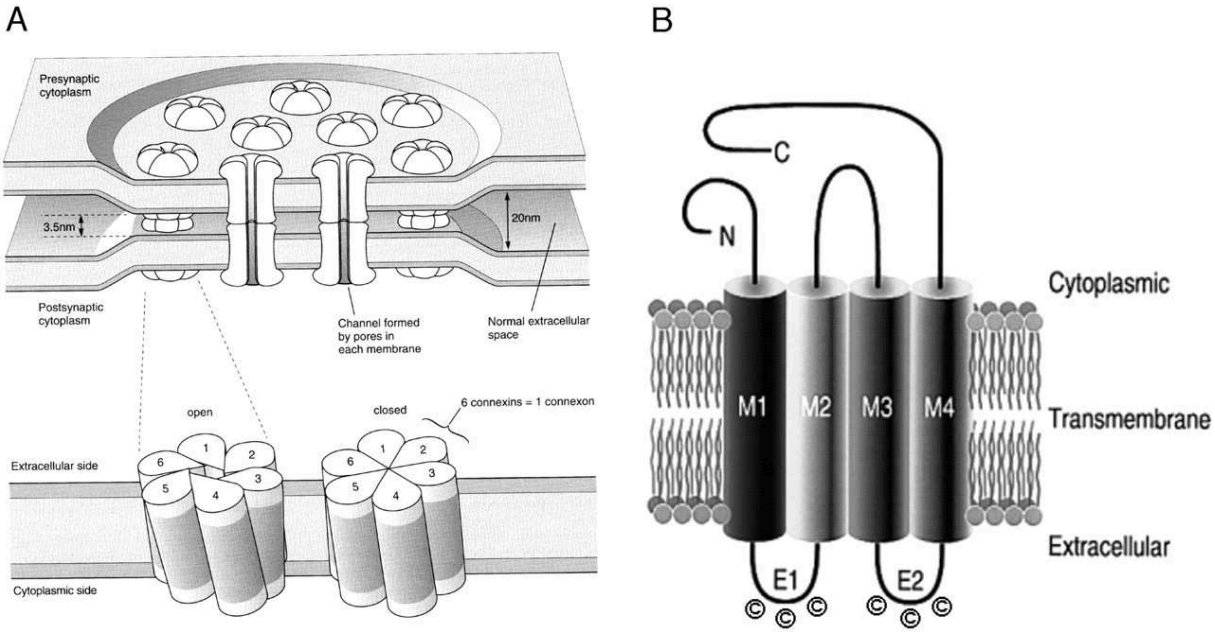

Figure 1.10 Schematic representation of gap junctions and their components. **A**: Two cells are interconnected by a plaque of gap junction channels, every formed by the opposition of two hemichannels. **B**: Structure of a protein forming GJs characterized by: four transmembrane domains (M1-M4); N- and C-terminal intracellular regions; two extracellular loops (E1-E2) and one intracellular loop. With © are indicated the cysteine residues. (Image adapted from Sohl et al., *Cardiovascular Res.*, 2004 [119])

The residues of cysteins (Cys) localized in the extracellular loops are necessary for intercellular docking between GJs hemichannels. Indeed, there are three Cys residues in each loop of connexins and two in pannexins and innexins. Not surprisingly, the extracellular loops, together with the transmembrane domains, are the most conserved regions of these proteins, which differ however for the sequence and the length of the intracellular loop and C-terminal tail.

Moreover the pannexins proteins, present a unique feature, constituted by a consensus sequences for glycosylation localized in the extracellular loop [120, 121] (**Figure 1.11**).

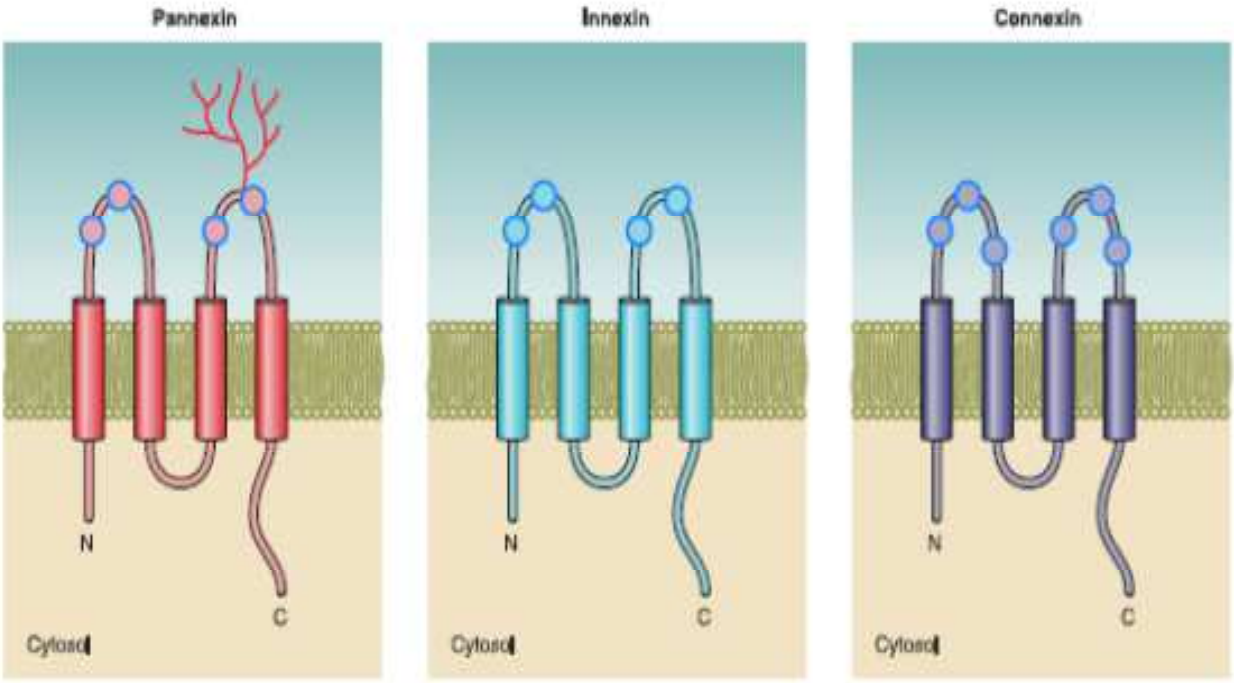

Figure 1.11 The three families of gap junction proteins share a similar membrane topology. All are formed by four transmembrane domain, the N-term and C-term are intracellular together the loop that link TM2 to TM3. In the extracellular loop are present two cysteine residues (blue circles) in Panxs and Inxs, while three residues in Cxs. In Panxs is present also a consensus sequence for glycosylation. (Image adapted from Bosco et al., *Physiol. Rev.,* 2011 [122]).

1.2.2 Connexins

The connexins proteins are encoded by a family of genes divided in five subfamilies α, β, γ, δ and ε. The five groups show differences in the structure and sequence due to a different phylogenetic origin (**Figure 1.12**).

Up to now, the family of connexins genes comprises 21 members in human and 20 in mouse genome, of these 19 are orthologous.

The nomenclature of connexins includes the indication of the species of origin, "h" for human and "m" for mouse (the species in which they have been first studied), the family name "Cx" and the number corresponding to the predicted molecular mass deduced on the basis of cDNA sequence (range of 23-62 kDa) [115, 119]. There is also another nomenclature, based on the use of the abbreviation "Gj", for gap junction, the group they belong (A, B, C, D, E for α, β, γ, δ and ε respectively) and by a number in according to the order of discovery. For example, mCx43, the first discovered connexin, belonging to the α-group, is also called Gja1 (**Table 1.1**).

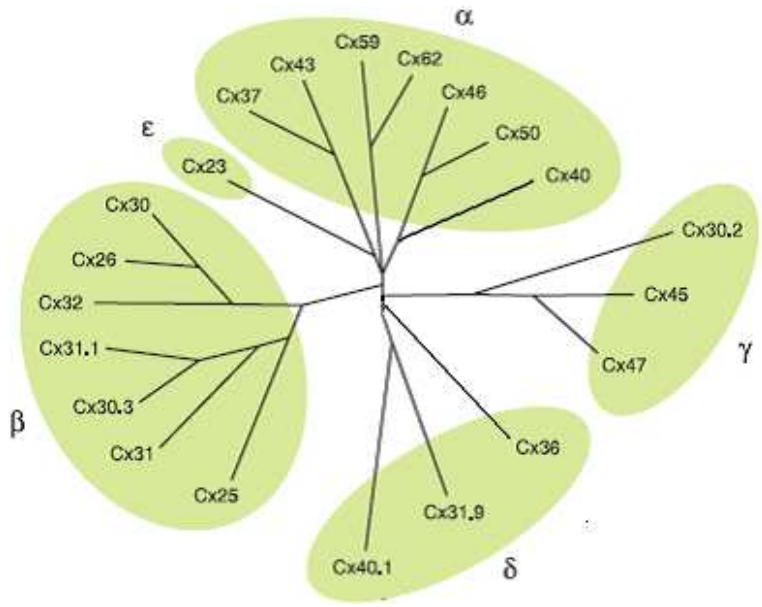

Figure 1.12 Phylogenetic tree of human connexins family. The five subfamilies α, β, γ, δ and ε are divided based on genes sequence and structure. (Image adapted from Bosco et al., *Physiol. Rev.,* 2011 [122])

Human				Mouse			
Cx	Gene	ID no.	Chromosome	Chromosome	ID no.	Gene	Cx
hCx23			**6**	**10**			mCx23
hCx25	GJB7	(375519)	**6**q15				
hCx26	GJB2	(2706)	**13**q11-q12	**14**d1-e1	(14619)	Gjb2	mCx26
hCx29	GJE1	(349149)	**7**q22.1	**5**g2	(118446)	Gje1	mCx29
hCx30	GJB6	(10804)	**1 13**q11-12.1	**14**c2	(14623)	Gjb6	mCx30
hCx31.9	GJA11	(125111)	**17**q21.2	**11**d	(353155)	Gja11	mCx30.2
hCx30.3	GJB4	(127534)	**1**p34.3	**4**d1-d3	(14621)	Gjb4	mCx30.3
hCx31	GJB3	(2707)	**1**p34	**4**d2.2	(14620)	Gjb3	mCx31
hCx31.1	GJB5	(2709)	**1**p35.1	**4**d2.2	(14622)	Gjb5	mCx31.1
hCx32	GJB1	(2705)	**X**q13.1	**X**d-f4	(14618)	Gjb1	mCx32
				Xf4	(414089)	Gja6	mCx33
hCx36	GJA9	(57369)	**15**q14	**2f3**	**(14617)**	**Gja9**	**mCx36**
hCx37	GJA4	(2701)	**1**p35.1	**4**d2.2	(14612)	Gja4	mCx37
hCx40.1		(219770)	**10**p11.21	**18**a1	(225152)		mCx39
hCx40	GJA5	(2702)	**1**q21.1	**3f2.1**	**(14613)**	**Gja5**	**mCx40**
hCx43	GJA1	(2697)	**6**q21-q23.2	**10**b4	(14609)	Gja1	mCx43
hCx45	GJA7	(10052)	**17**q21.31	**11**e1	(14615)	Gja7	mCx45
hCx46	GJA3	(2700)	**13**q11-q12	**14**c2	(14611)	Gja3	mCx46
hCx47	GJA12	(57165)	**1**q41-q42	**11**b1.3	(118454)	Gja12	mCx47
hCx50	GJA8	(2703)	**1**q21.1	**3**f2.1	(14616)	Gja8	mCx50
hCx59	GJA10	(81025)	**1**p34	**4**a5	(14610)	Gja10	mCx57
hCx62		(84694)	**6**q15-q16				

Table 1.1 Family of human and murine connexins. For each protein is reported also the gene name and the position on chromosomes. (Table adapted from Bosco et al., *Physiol. Rev.*, 2011 [122])

Various tissues and cell types can express more than one type of connexins. Connexins can form a large variety of channels; consequently, the connexons can be homomeric, if composed by the same type of connexins, or heteromeric in the case of two or more type of Cxs. Furthermore, a gap junction can be homotypic, if constituted of two identical hemichannels made from one type of connexin, or heterotypic, if formed by two different hemichannels, each of which is made of a different type of connexin. Not all the connexins are able to form a heterotypic GJ. The different composition dictates different physiological properties as gating, single-channel conductance and the permeability to biological molecules [123-126].

1.2.2.1 Assembly and degradation

The oligomerization of the connexins into a hemichannel (HC) occurs in the endoplasmic reticulum (ER), where the neoformed connexons pass in the Golgi apparatus and then in *trans*-Golgi network. They interact with chaperone proteins and inside to vesicles are transported along microtubules and actin filaments to the membrane cell, through which they freely diffuse and are integrated in the outer edges of existing plaque. The connexon arrived at the membrane cell, is aided by E- and N-cadherins, to bind with another connexon arising from adjacent cells and to form the gap junction channel [124, 127-129].

Gap junction plaques are highly dynamic regions, in which new connexons are added at the periphery and old connexons are removed from the centre of the plaques. The removal occurs with invagination of a vesicle containing a portion of membrane with all or a part of GJ. This structure, named "annular junction", is released in cytosol and its components are degraded through the lysosomal and proteosomal pathway [130-132].

Gap junctions biosynthesis and assembly are tightly regulated, indeed these structure have a half-life of only few hours. This rapid process is probably fundamental for a quick adaptation of the cells to mutated physiological or environmental conditions (**Figure 1.13**).

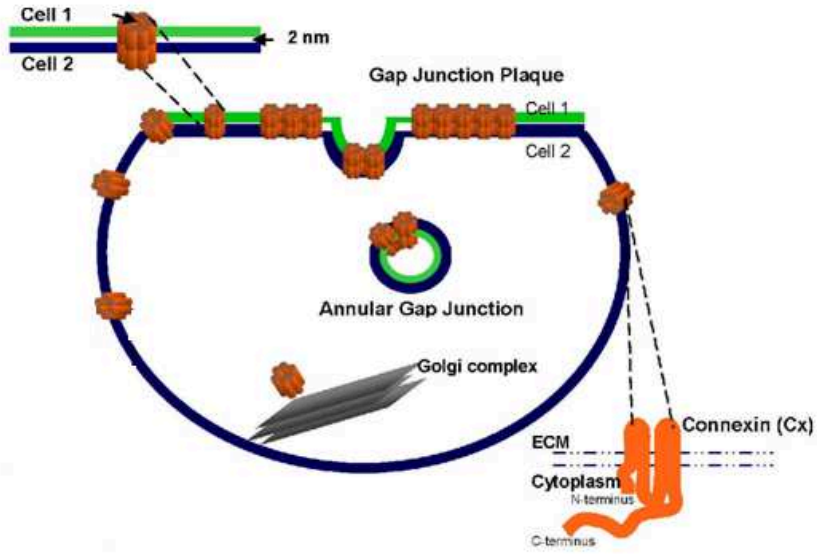

Figure 1.13 Formation of an annular gap junction. The removal of a gap junction occurs with invagination of a vesicle containing a portion of membrane with a connexon or the whole GJ. (image adapted from DeFranco BH, *Doctoral Thesis*, 2009)

1.2.2.2 Gap junctions functions

Gap junctions are implicated in a large variety of functions as embryonic development, morphogenesis, cell differentiation, cell proliferation and migration, electrical and mechanical synchronization (cardiac, muscular and cerebral cells), transmission of trophic or death molecules. All these functions have been discovered using targeted mutated connexins or through the over expression of some connexins isoforms.
Connexins proteins are expressed in all tissue except in differentiated skeletal muscle, erythrocytes and sperm cells. This almost ubiquitary presence is a further confirmation of their importance for the correct functioning of the organisms (**Table 1.2**).
The importance of these proteins and hence, of gap junctional communication is evident by the large number of human genetic diseases associated with connexins mutations or with pathogenic single nucleotide polymorphisms. Among this for example the X-linked Charcot-Marie-Tooth syndrome, a peripheral neuropathy with atrophy of distal muscles and low number of myelinating fibers, has been linked to mutations in Cx32 gene, suggesting its participation in myelination of peripheral nerves. Mutations in Cx43 can cause oculodentodigital dysplasia characterized by craniofacial, neurologic, limb and ocular abnormalities. Still mutations of Cx46 and Cx50 result in cataracts [133-137].

Recent studies show that connexons are also active in single plasma membranes as hemichannels (HCs). HCs might be essential in intercellular signalling in different physiological and pathological process, indeed they act in the cells of various organs in response to extracellular signaling, injury, ischemic preconditioning and mechanical stimulation. In contrast to GJs, they show low open probability and low permeability to small molecules under resting conditions. HCs have been implicated in autocrine/paracrine signalling to provide a pathway for release of ATP, glutamate, NAD^+ and prostaglandins [138-140].

Human connexins	Mouse connexins	$g_{i\ (pS)}$	Expression pattern of connexins in different tissue
* Cx23	* Cx23	ND	By human and mouse genomes. Transcription and translation have not been demonstrated in human
* Cx25		ND	Human genome
Cx26	Cx26	115-150	Breast, skin cochlea, liver, endometrium, glial cells, airway epithelium, pancreas
Cx30	Cx30	160	Skin, brain, cochlea, airway epithelium, exocrine gland
Cx30.3	Cx30.3	ND	Skin
Cx31	Cx31	85/15	Skin, cochlea, airway epithelium, placenta
Cx31.1	Cx31.1	ND	Skin
Cx31.3	Cx29	ND	Oligodendrocytes, skeletal muscle, liver, pancreas, kidney
Cx31.9	Cx30.2	15	Mouse heart, mouse brain
Cx32	Cx32	58-70	Liver, skin, Schwann cells, Oligodendrocytes, endometrium, gland cells
	Cx33		Testis
Cx36	Cx36	5-15	Retina, pancreatic beta cells, neurons
Cx37	Cx37	219-300	Vascular smooth muscle, endothelium, ovaries, skin
Cx40	Cx40	158-198	Skin, nervous system, endothelium, hearth
* Cx40.1	Cx39	ND	Human genome. Developing muscle mouse
Cx43	Cx43	90-110	Most widely expressed connexin. Found in about 34 tissues and 46 cell types
Cx45	Cx45	30	Pancreatic epithelial cells, SA and AV nodes, neurons, oligodendrocytes, astrocytes, vascular system, skin, osteoblasts, retina, uterus
Cx46	Cx46	140-152	Lens, alveolar epithelium
Cx47	Cx47	55	Brain, spinal cord, Oligodendrocytes
Cx50	Cx50	212	Lens
* Cx58		ND	Human genome
Cx62	Cx57	57	Mouse oocytes, horizontal retina cells

Table 1.2 Expression patterns and single channel conductance (gi) of human and mouse connexins. Some connexins proteins (signed with *) have been identified by analysis of humane and murine genomes , but they are not still found in tissue. Single channel conductance is expressed in pico Siemens (pS). (ND not determined). (Table adapted from Rackauskas et al., *Biophysical Journal*, 2007 [126])

1.2.2.3 Permeability and regulation

A gap junction forms a hydrophilic channel pore of about 100-150 Å in length and 12.5 Å in width. It allows the passage of small molecules under 1kDa like ions, water, nucleotides, small peptides, metabolites. In this way, GJs provide to ionic and metabolic coupling among the cells. For some substances, this coupling is bidirectional and is driven by an electrochemical gradient, for others GJs there is a high degree of selectivity. Indeed the channels composed by different connexins show different permeability; for example some channels are specific for the cations or the anions, while others are able to discriminate between similar molecules as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).

The junctional conductance (g_j), i.e. the passage of molecules or ions through GJ channel, is subjected to regulation by a number of physiological factors, such as voltage, intracellular pH and calcium, second messengers, or phosphorylation. Conductance of a single homo-connexins channel ranges from ~10 picoSiemens (pS) to ~300 pS [125, 141-143].

The most important factor for the permeability is the structure of channel pore. Indeed the pore width, the electrical field and the electrical charge on the pore surface affect the permeability of ions and molecules passing through the channel.

The permeation pathway of a gap junction channel consists of an intracellular channel entrance, a pore funnel and an extracellular cavity. The pore funnel surface is formed by the six N-terminal regions of connexins. Because the funnel forms a constriction site at the cytoplasmic entrance of the pore, the size and electrical character of the side-chains in this region should have a strong effect on both the molecular cut-off size and the charge selectivity of the channel. Indeed the substitutions or deletions of residues in N-terminal regions can affect single channel conductance, molecular permeability and charge selectivity [144] (**Figure 1.14**).

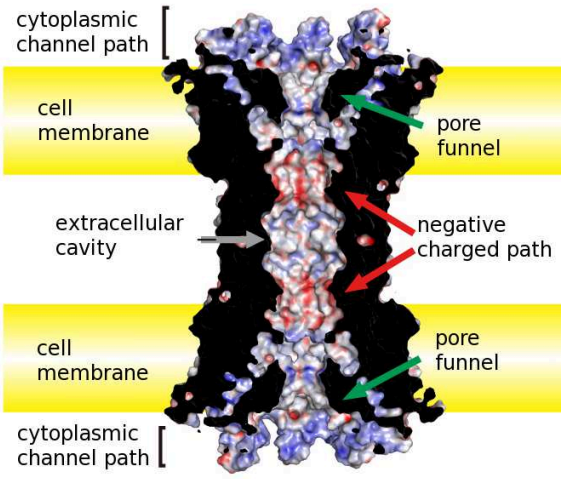

Figure 1.14 Pore structure of the Cx26 gap junction channel. The permeation pathway of Cx26 GJ consists of an intracellular entrance (the pore funnel formed by N-terminal regions), a negative charged path and an extracellular cavity. (Image adapted from Maeda et al., *Cell. Mol. Life Sci*, 2011 [144])

The gap junctional communication is regulated at multiple levels. The regulation can be at short or long-term; the more rapid mechanism involves changes in the conductance or in the probability of opening of a single channel while in the slow mechanism occur an alteration of number of channels for changes in synthesis and degradation.

Gap junction channels have different gating mechanisms and as some ions channels are voltage sensitive. Indeed, they are under the influence of two types of electrical field, the membrane voltage (V_m), i.e. the voltage difference between intra and extracellular space, and the transjunctional voltage (V_j), that is established when the membrane voltages are not equal in the two coupled cells. Some connexins are sensitive to both V_j and V_m, others only to V_j. As regard the dependence by V_m, different connexins show different sensitivity; for example Cx43, Cx30 and Cx26 channels close with depolarization, whereas Cx45 upon hyperpolarization. The dependence of junctional conductance by Vj regards all GJs analyzed; so this mechanism is specific for GJs channels and is characterized by two forms of gating, fast or low, depending by the time reaction of opening and closing.

Different studies revealed that N-terminal domain, forming the pore funnel, determine the magnitude and polarity of Vj. A cytoplasmic movement of the N-terminal portion, where the voltage sensor is believed to reside, has been suggested to initiate voltage-dependent gating [144] (**Figure 1.15**).

Therefore, the substitution of the residues on N-terminal, changes the conductance or sensitivity. Indeed there are hemichannels that are close at positive voltage, as Cx26, Cx30, while others at negative voltage, Cx32, Cx43, Cx45 [145-149].

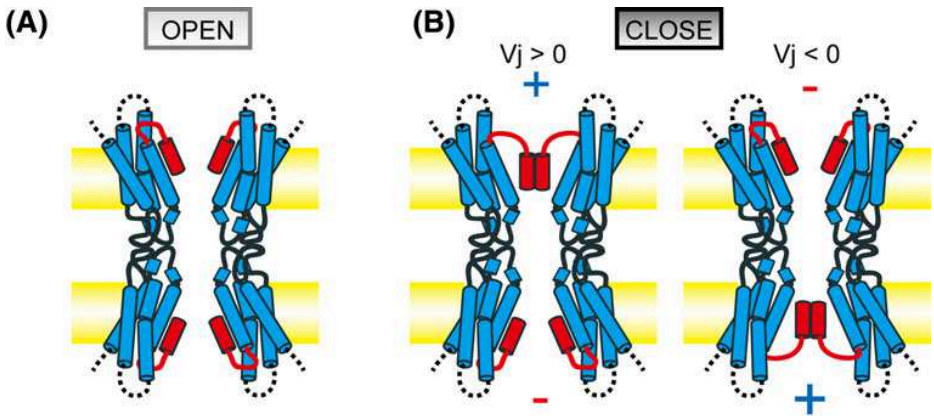

Figure 1.15 Plug gating model for transjunctional voltage-dependent gating of the Cx26 gap junction channel. **(A):** When there is no difference in membrane voltages between two neighboring cells N-term region (in red) form the pore funnel and attach to TM1 by hydrophobic interactions. **(B):** When there is a difference in membrane voltages between two cells, the positive electric field causes the releasing of N-term from TM1. Once released, N-term region will assemble on the top of the pore with the others N-term region. (Image adapted from Maeda et al., *Cell. Mol. Life Sci*, 2011 [144]).

Others factors modulating gap junction conductivity are the Ca^{2+} and pH. An increase in intracellular calcium concentration reduces GJ communication. Indeed it is a signal to protect the other cells by the damaged cells, even if the sensitivity depend on connexin and cell types, in a range from 500-600 nanomolar to micromolar concentration of Ca^{2+}. There are also experimental evidences that the gating of GJs, Ca^{2+}-dependent, could be mediated by calmodulin (CaM), an intermediate messenger protein that transduces

calcium signals by binding calcium ions and then modifying its interactions with various target proteins, among which there are connexins [150, 151].

Also an increase in intracellular pH decreases junctional conductance of gap junction channels and some connexins are markedly more sensitive to acidification than others, although it's not still clear whether H^+ acts directly on GJ channel [152-154].

An extensive way with which connexins are regulated regards several post-translational modifications. The C-terminal region of Cxs contains serine, tyrosine and threonine, residues that may be phosphorylated by different protein kinases. At least eight kinases, including protein kinase A, protein kinase C, mitogen-activated protein kinase (MAPK) and so many phosphatases control the phosphorylation/dephosphorylation of various connexins. Except for the not phosphorylable Cx26, these modifications in Cxs are associated with changes in gating, conductance, permeability, but this depends on specific connexin isoforms or cell type. However, other modifications have been identified such as hydroxylation, methylation, and acetylation. Other effects regard the assembly into GJ plaque, their biosynthesis or their proteolytic degradation, regulating in this way their half-life [155-158]. In addition, ubiquitination would seem an important mechanism which connexins are regulated; the most studied Cx43 for example, can be ubiquitinated at plasma membrane or at ER level. Recently it has been implicated also a SUMOylation for Cx43 [159-161].

There are chemical agents able to block the GJ communication, they belong to multiple class and structures. Some are lipophilic such as heptanol, octanol and halothane, glycyrrhetinic acid derivatives such as carbenoxolone (CBX), quinine derivatives and some others different for structure and action mechanism. These molecules have been used to study the functions of GJs in vitro and in vivo, but although many of these are used to treat common diseases, as quinine for malaria, they give a plethora of side effects, due sometimes by blocking other channels. The development of selective blockers will allow better understanding of GJs communication [162].

1.2.3 Pannexins

Pannexins belong to a new family of proteins, also able to form GJs. They have more similar homology with innexins than with connexins, so that initially it has been thought they belonged to the innexins family. Many later studies showed that Panxs were a distinct family of proteins. They do not share the strongly conserved residues of Inxs even if as these last possess two cysteine residues in extracellular loops, and in contrast to connexins have a larger intracellular loops (~68 aa versus ~30 aa).

1.2.3.1 Expression

In the genomes of human and rodent species, are coded three proteins, Panx1, Panx2 and Panx3. Their calculated molecular mass is respectively 48, 73 and 45 kDa. In human genome Panx1 and Panx3 gene are located on chromosome 11 and Panx2 on chromosome 22, while in rat genome the first two are located in chromosome 8 and Panx2 on chromosome 7 [163, 164].

Panx1 and Panx2 transcripts are found in many rodents tissue, as brain and spinal cord, eye, thyroid, prostate, kidney; in humans are found also in hearth, gonads and skeletal muscle (in which there is no presence in rodents).

Panx1 protein is expressed in numerous tissues in both human and rodents. Instead Panx2 is present in many tissues in rodents, but in humans is expressed only in the central nervous system (CNS). The expression of Panx3 seems confined to skin and cartilage cells, even if real-time PCR screening indicate that Panx3 mRNA is present also in kidney, spleen and brain [165, 166].

Pannexins have been found in large accumulation in ER and Golgi apparatus, suggesting that post-translational modifications and assembly in pannexons occur in the same way as demonstrated for connexins.

1.2.3.2 Functions and regulation

The first studies on this protein were made by Bruzzone et al. using an expression system in Xenopus oocytes, in which they discovered that the channel formed by pannexins, if activated by voltage, had given rise to passage of molecules. But they concluded that Panx1 alone was able to form homomeric channels, while Panx2 could form only heteromeric channels with Panx1 [167].

To date, increasing evidences show that also Panx2 alone is capable to form a functional homomeric channel. Indeed the heteromeric channels Panx1/Panx2 are unstable and disaggregate in few hours, this could explain because the heteromeric is present in systems as Xenopus oocytes or in HEK 293, but in mammalian cells and brain tissue the two proteins exist separately [168].

Another controversy regards the ability of Panxs to form or not gap junction channels, but recent studies revealed evidence that they may form GJs in vitro cells, but to date it has not been demonstrated in vertebrate.

As connexons, after activation pannexons open into large non-selective pores permeable to ions and small molecules up to 1 kDa and under a wide range of membrane depolarization. Panxs HCs differ from connexins HCs because they exhibit larger currents, faster kinetics of pore opening and a conductance of over 500 pS [167].

Different stimuli are able to open pannexons, among these mechanical stress, positive membrane potential, extracellular ATP, elevation in intracellular calcium, ischemic insult and inflammation, while acidification of cytoplasm cause the closing of the channels. The presence of mechanisms distinct from those of connexins, makes possible the co-expression of the two proteins types in the same cells [169].

The pannexons may play a role in generation of oscillatory and synchronization activity in brain. They can be involved in the propagation of calcium waves, to which normally have been implicated the GJs connexins-formed [170]. Panxs would use a mechanism based on release of adenosintriphosphate (ATP). This has been demonstrated in blood endothelium, where mechanical stress or ischemia open pannexons in endothelial cells and cause the extracellular release of ATP. The released ATP acts on purinergic receptors P2Y, whose activation has effect on phospholipase C, increasing the IP3 and causing the release of calcium from intercellular stores; this at the end cause a consequent release of other ATP that can propagate using classical way of GJs or via

pannexons. In this way, the propagation is ensured also without direct cell contact. This action probably is essential for the cells in normal tissue because Panxs channels, contrary to Cxs channels gated by cations as Ca^{2+} or Mg^{2+}, are able to open when external concentration of calcium is at physiological level.

So pannexons may propagate calcium waves and have a role in vasodilatation, inflammatory response and ischemic death of neurons [171-173].

Other evidences of calcium waves propagation are in erythrocytes, where there is expression only of Panx1 and not of Cx43 (the main Cx involved in calcium waves). In these the release of ATP occurs in response to ischemia and mechanical stress and after exhibit the same pathway observed in endothelium [174].

The Pannexins are the only ones, between the three families, to be extensively glycosylated. There is the presence of consensus sequence on the second extracellular loop and this modification seems important for intracellular trafficking and insertion in the cell membrane. Moreover, the glycosylation could prevent docking with other pannexons in adjacent cells [121, 175].

By analysis of amino acids sequence there are one phosphorylation site for Panx1 and multiple site for Panx2 and, as in connexins, these allow the activation/deactivation of the channels [176].

Another possible regulator of Panxs is the β-subunit of voltage-dependent potassium channel ($K_v\beta3$), a protein belonging to the family of regulatory beta-subunits of the voltage-dependent potassium channels. The co-expression of Panx1 and Kvb3 has been showed in principal neurons of the hippocampus and in Purkinje cells of the cerebellum. The mechanism would involve the binding of Kvβ3 to the carboxy-terminus of Panx1 and maybe in response to changes of the intracellular redox potential, it would control the inactivation mechanisms of Panx1 hemichannels [177].

1.2.4 Gap Junctions in nervous system

Electrical synapses are prevalent during the early phase of neurogenesis. Studies both in vitro and in vivo showed that gap junctions coupled all progenitor cells, neuroblasts and proliferating cells, in order to coordinate the neuronal communication. After about two weeks of postnatal development this communication is replaced by chemical synapses, but in mature neurons the expression of connexins is maintained and has a role in the coordination of neuronal activity and in mediation of synchrony and network oscillations [178-180]. The expression of different connexins depends by developmental stage, cell type and brain region. With electrophysiology, transgenic animals, cell imaging and freeze-fracture replica immunolabeling (FRIL) it has been possible examine the presence of these proteins in CNS vertebrates. Another medium available to study gap junctions is the dye coupling. This technique allows measuring the intercellular communication, through diffusion of a fluorescent dye, from one cell into neighbouring cells.

The first evidence of the GJs presence in the brain was in Schwann cells of peripheral nervous system. These cells proliferate after nerve development or after injury to promote the myelination; maybe GJs play a role in this process, distributing and synchronizing important signals to proliferation. Cx32 and Cx29, identified in Schwann cells, show the same distribution pattern at paranodal regions and in Schmidt-Lanterman incisures (small canals that interrupt the myelin sheath present in nerve cells and allowing the nourishment of the cells isolated from the myelin).

Cx32 is required for normal functions in peripheral nerve, as demonstrated with Cx32 knocked out (KO) animals or in Charcot-Marie Tooth disease. The role of Cx29 is not clear for controversial effects described in KO animal models [181-185].

Despite the detection of different Cxs in neurons (N), to date only Cx36 and Cx45 have been unequivocally identified in adult rat brain. With use of FRIL, gap junctions composed by Cx36 are detected in inferior olive, spinal cord, retina, olfactory bulb, visual cortex, suprachiasmatic nucleus, locus coeruleus, brainstem nuclei, cerebellum, thalamus, hypothalamus, basal forebrain, amygdala, pineal gland, hippocampus and cortex; whereas Cx45-based neuronal gap junctions have been found in the retina and olfactory bulb [186-190].

The expression of Cx36 is maximal in prenatal period, about at P7, but after shows a progressive decline in adult followed to decrease of dye coupling in cortex. Nevertheless the function of Cx36 in adult brain is considered fundamental because it would mediate the establishment of oscillatory networks between neuronal cells, as demonstrated in Cx36 knockout mice where there is an impairment of gamma oscillations in hippocampus [191]. The GABAergic interneurons are the population that mostly express Cx36, where it seems responsible for oscillatory behaviour. Some evidences support the presence of Cx36 also in dopaminergic neurons of substantia nigra and ventral tegmental area [192-194]. Single cell quantitative PCR indicate that Cx32 is expressed by interneurons in neocortex, although less abundantly than Cx36 [195].

In cortical astrocytes (A) have been detected Cx26, 30, 40, 43 and 45. But only Cx26, 30 and 43 have been confirmed at ultra structurally level in astroglial gap junctions. They show extensive co-localization, but while Cx26 and Cx30 can form heteromeric and heterotypic channels, they don't interact with Cx43 [196]. The expression of these three proteins is highly heterogeneous in CNS; there are some regions, as the subcortical area, in which are all equally expressed, while in cerebral cortex there are low levels of Cx26 and 30. The Cx30 is not detected in astrocytes of white matter regions as anterior commissure and internal capsule [197-199].

There are strong evidence that the main functional connexins in astrocytes is Cx43, because cultured astrocytes from Cx43-deficient mice don't form gap junctions even if some study show an equal contribution of Cx30 [200].

Even though there are functional evidences for neuron-glia gap junctions in vivo or in vitro, still has not be possible identify the probable candidates. Some studies show the importance of gap junctions in astrocytes to modulate the neurons , but some groups found no evidences about the communication via GJs through the two cell types at ultra structural level [199, 201].

Oligodendrocytes (O) are also in communication with GJs; in these have been detected Cx29, 32, 36, 45 and 47 [198, 202, 203]. Cx29 and Cx32 are the most expressed proteins and have been detected in soma and in myelinated fibers. It has suggested that Cx29 with Cx32 in adult brain may contribute to connexin-mediated communication between adjacent layers of uncompacted myelin [184].

FRIL data show that oligodendrocytes are extensively coupled only with astrocytes through Cx26, 30, 43 and 47 [204]; rather almost all gap junctions have been identified as O/A junctions, this maybe can serve to mediate an indirect communication with the others oligodendrocytes O/A/O such as in the proposed panglial syncytium. The panglial syncytium is a vast network of interconnected glial cells, astrocytes, oligodendrocytes, and ependymocytes, all extensively inter-linked by gap junctions. This structure in CNS provides widespread metabolic and osmotic support for neuronal somata, but it is particularly specialized for the ionic and osmotic homeostatic regulation of myelinated axons in white matter tracts [186].

Microglial cells (MC) express low levels of Cxs 36, 43 and 45, but after activation with cytokines the levels of Cx32 and 43 increase. In culture, electrical coupling between microglial cells and neurons is established via GJs formed by Cx36. This coupling is very important in neuropathology; indeed is known that microglia occurs rapidly in response to brain insults and its activation with release of pro-inflammatory cytokines or release of trophic factors can determine the death or the survival of neurons. So blocking of microglial GJs could decrease the inflammatory response, limiting neurodegeneration [205-207]. (**Figure 1.16**)

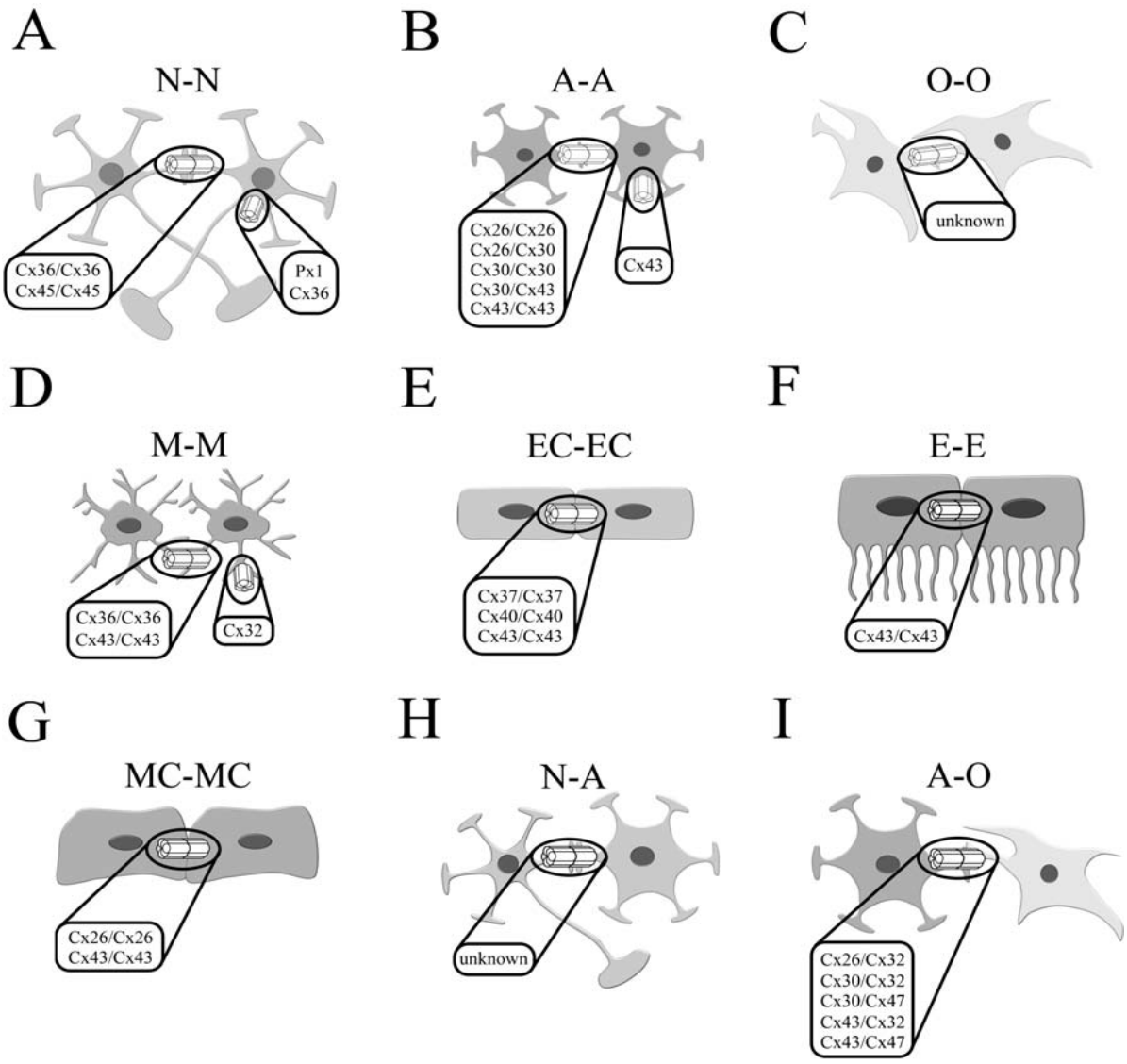

Figure 1.16 Distribution of gap junctional connexins in brain cells. The expression of the different proteins is the results of experiments performed in vivo and in vitro. In some cell type there is the presence of heterotypic gap junctions channel. Neuron (N), astrocyte (A), oligodendrocyte (O), microglia (M), endothelial cell (EC), ependimocyte (E) and meningeal cell (MC) are denoted in the pictures. (Adapted from Orellana et al., *Antioxid Redox Signal,* 2009 [208])

1.2.4.1 Pannexins in brain

Panx1 and Panx2 transcripts are detected in many regions in rodents CNS as retina, cortex, hippocampus, cerebellum, olfactory bulb, spinal cord. Panx1 is expressed primarily in neurons and maybe in oligodendrocytes. Panx2 seems to be brain-specific, but the two proteins are inversely regulated during the development of the rodents brain.

Panx1 show high levels of expression in the embryonic and young postnatal brain and decline considerably in the adult, whereas Panx2 mRNA expression is low in the prenatal brain but increase substantially during subsequent postnatal development with peaking at postnatal day 15 [209, 210].

Pannexins transcripts are particularly abundant in the adult cortex, in hippocampal and neocortical pyramidal cells but also in GABAergic interneurons, (to note the presence in both excitatory and inhibitory neurons, while Cx36 is present only into inhibitory interneurons), reticular thalamus, the inferior olive, magnocellular hypothalamic neurons, midbrain and brain stem motoneurons, Purkinje and Golgi cells in the cerebellum [209].

Panx1 protein is widely expressed in mammalian tissues; as regard brain is found in several regions including cortex, striatum, olfactory bulb, hippocampus, thalamus, inferior olive, inferior colliculus, amygdala, spinal cord, retina, and cerebellum. At the cellular level, Panx1 has been localized in different neuronal types, including olfactory bulb mitral cells, Purkinje cells, dopaminergic, cholinergic and glutamatergic neurons. In the cerebral cortex and hippocampus is localized in the postsynaptic cell membranes.

Panx1 is also detected in cultured astrocytes, immature oligodendrocytes and neurons, using immunofluorescence and Western blot analysis, but it has not been found in the cell surface of astrocytes [209-214].

Panx2 protein is expressed exclusively in the brain including the olfactory bulb, hippocampus, amygdala, superior colliculus, substantia nigra, cerebellum, hypothalamus and spinal cord. Its presence it has been confirmed in the majority of pyramidal cells and in GABAergic interneurons but as regard the expression in glial cells there are still contrasting opinions; under resting conditions, hippocampal astrocytes do not express Panx2 even if it appears in hippocampal astrocytes several hours after ischemia/reperfusion [209-212, 215].

The presence in brain becomes important because pannexins are present in all regions in which lack connexins but are alike coupled both metabolically and electrically. For example, the abundance of Panxs in Purkinje cells or in hippocampal pyramidal cells, that do not express Cx36 or Cx45, suggests that these are responsible for electric coupling and generation of high-frequency oscillations. Experiments in mice KO for Cx36 show a reduction in gamma frequency, while ultrafast oscillations are not modified. These data are consistent with the absence of Cx36, present only in hippocampal interneurons, while other channels may determine fast oscillations occurring in pyramidal cells [216-218]. However, the impact of Panxs channels on neuronal network synchronization remains to demonstrate.

It remains also unknown whether pannexins form functional GJCs and/or hemichannels. There are many controversial works regard the formation of GJs; in some systems as Xenopus oocytes or C6 glioma cells an over-expression of Panx1, but not Panx2, mediate intercellular coupling, while in other systems and other groups report no communication. In contrast, functional hemichannels formed by both Panx1 and 2 are demonstrated in different cellular system and in hippocampal neurons [211, 212, 219].

1.2.5 Electrical synchronization

Electrical synapses represent ontogenetically and phylogenetically a primitive form of intercellular communication; afterwards they have been replaced by chemical synapses in the CNS of vertebrates. However, they represent the principal form of communication during early development of mammalians CNS and in some less evolved organism as in crayfish cord giant fibers. Nevertheless in the adult mammalian CNS they are of great importance, indeed is thought that they have a role in the promotion of synchronous activity in different areas of the brain and in retina [220].

More and more evidences correlate electrical synapses with the establishment of oscillatory networks between neuronal cells. The neuronal synchronization occurs at different frequency bands, in hippocampus occurs through three types, theta θ oscillations (4-12 Hz), gamma γ waves (40-100 Hz) and high frequency oscillation (also named ripples) (~200 Hz). The gap junctions are involved in the generation of gamma and ripples oscillations [221].

The first demonstration of electrotonic coupling in neurons has been observed in pyramidal cells of hippocampus. Here, high-frequency oscillations (100-200 Hz), or ripples, are mediated by electrical coupling and occur via axo-axonal contact sites; these results are confirmed by the inhibition of spontaneous ripples with use of gap junction blockers, or by the maintaining of ripples in absence of chemical transmission. However, is not clear which proteins are capable to generate this synchronous oscillatory activity because the main player Cx36, is located only in interneurons, in fact in Cx36 KO mice the generation of ripples is not affected. Controversial study support the possible involvement of Cx45 or pannexins [222-225].

In hippocampus, gap junctions formed by Cx36 are present in GABAergic interneurons of CA1 and CA3 region, where they are important components of γ-frequency oscillation, even though in Cx36 deficient mice there is a residual gamma oscillations, suggesting the involvement of other proteins [189, 218, 224, 226]. An important electrical communication between GABAergic interneurons is demonstrated in various parts of the brain, as in the cerebral cortex, thalamus, striatum and cerebellum. These seem to be a fundamental feature for inhibitory circuits because they could facilitate synchronous spiking of interneurons providing in this way to the control of activity in neurons [227-230]. In the neocortex there are two networks of GABA neurons, FS (fast-spiking) cells

and LTS (low threshold cells) interconnected by GABA-mediated synapses; the FS cells are strongly interconnected by GABA-mediated synapses, while few chemical synapses are found among LTS cells. Studies using Cx36 deficient mice show the absence of electrical coupling or spikelet in LTS cells and a reduction in FS cells [218, 226].
Many others brain regions display an oscillatory activity mediated by gap junctions coupling as medium spiny neurons of striatum, locus coeruleus, inferior olive, spinal cord, hypothalamus and others [188].

1.2.6 Gap junctions and seizures

In epilepsy, many neurons are synchronized and there are several evidences that gap junctions coupling can mediate the generation of seizures and epileptiform activity.
Several works show that seizures, induced with 4-aminopyridine or kainic acid, are impaired using gap junctions blockers; while in Cx36 deficient mice the incidence of seizures is reduced [218, 225, 231].
The role of GJs in seizures has been demonstrated both in vitro and in human epilepsies, where are found increased levels of some mRNA connexins as Cx36, 32 and 43 and a more pronounced coupling between the cells to respect normal tissue. The presence of these connexins would suggest an increased coupling between neurons and astrocytes/oligodendrocytes in the epileptiform discharges [232, 233].
Gap junctions would seem crucial for the synchronization in the epileptic foci because their opening or blocking, respectively with trimethylamine and carbenoxolone, increased or decreased the duration of the seizures [234]. Interestingly specific blockers for Cx36 showed a reduction of the seizures only after ~30 discharges and considering the low levels of Cx36 mRNA in neocortex would suggest a rarely interconnection with GJs but after continued exposure to some factors this communication will increase [235].

1.2.7 Gap junctions and mesocorticolimbic system

The presence of GJs has been demonstrated with the presence of an electrotonic coupling or by dye marker transfer between brain cells.

Intercellular channels have been identified in Nucleus Accumbens (NAc), striatum, prefrontal cortex (PFC), hippocampus and moreover in these regions the dye transfer can be modulated by dopamine [236-241]. Particularly, several studies show the different action of agonists or antagonists in the two sections of NAc. The infusion of D1-like agonists in Nac core, causes a decrease in dye coupling and the effect is blocked by D1 or D2 antagonists; while in the NAc shell the administration of D2-like agonists increase dye transfer independently of the state of D1 activation [242, 243]. The mechanism by which DA modulates GJs is not known.

Also in ventral tegmental area (VTA) there is a wide network of GABAergic neurons coupled via Cx36 GJs, and they seem modulated by DA. Similarly, blocking Cx36 GJs increases inhibition of DAergic neurons, supporting a key role of GJs [192, 244].

Gap junctions can be also regulated by coupling with glutamate receptors, indeed in ischemia we assist to an increase of coupling in neurons and this is regulated by glutamate, via group II metabotropic glutamate receptors [245-247]. As the same way GJs can regulate the release of glutamate; indeed Cx43 glial hemichannels, under different stimuli, can release glutamate causing various effects in normal and pathological brain [248, 249].

The nucleus accumbens receives afferents from different limbic structures, so it must process the different input to absolve its role. It's possible that the different input contact distinct populations of neurons in NAc and each set could represent an ensemble coding capable to modulate the information [250, 251].

Several works showed that NAc neurons exhibited increase or decrease in firing rate in response to both natural (food, water) and not natural stimuli (as drugs of abuse). However, it would seem that natural stimuli activate distinct population of NAc neurons respect to those activated by drugs, even if is unknown the precise manner in which NAc neurons encode differently the two types of stimuli. Moreover, after protracted abstinence, the NAc neurons that encoded for cocaine behaviours were strongly increase; Cameron & Carelli (2012) have showed that normal reward processing is dysregulated and the encoding of drug related information is potentiated at the cost of

natural physiological rewards. They also postulate that a prolonged drug exposure and extended or repeated periods of abstinence could increase these effects, leading to the loss of control characteristic of the addicted state. Maybe gap junctions could mediate the synchronization of every ensemble coding [252-255].
Through electrical stimulation of hypothalamus, mimicking the behavioural positive reinforcement, and subsequent infusion of carbenoxolone (GJs blocker), the neuronal synchronization is reduced and so the stereotyped behaviours, as locomotor activity, indicating a role for GJs in self-stimulation reward [256].

1.2.8 Gap Junctions and cocaine abuse

Previous studies show changes in connexins expression after cocaine self-administration; this lead to support that change in neuronal gap junction communication may be responsible for the long-term alterations observed in addiction.
Following chronic cocaine SA (for 14 days) and 2 to 7 days of withdrawal, Bennett et al. show that the levels of Cx32 are decreased in NAc but the same decrease is not observed in dorsal striatum. This could be important because NAc is linked to the reinforcing effects of drug, while dorsal striatum is associated with motor behaviour. Other important changes are observed in hippocampus, where there is a decrease in Cx32 protein expression [257].
In another work, McCracken et al. show after exposure to cocaine for 10 days, changes in Cx36 protein expression in NAc, PFC and hippocampus. Interestingly the effects observed are different depending if the animals are sacrificed just after last cocaine injection or with 1 or 7 days of withdrawal; while in the first case there are no changes, after 7 days of abstinence is showed a very huge increase of Cx36 mRNA in NAc and a slight increase in PFC and hippocampus. This increase is not followed by the same increase in protein expression, indeed in NAc and PFC only a slight increase is present with 1 day of abstinence and nothing in hippocampus. Analysis of Cx32 and Cx43 in the same regions show no differences in both regions and at any time point [258].

2 Aim of the work

The aim of this study has been investigate the role of gap junctions in the mechanism of action of cocaine. Drug dependence is a huge social problem and cocaine has taken in the last years a prominent place among drug of abuse. Although the psychological effects and the risks are the same for all the users, it's clear that the phenomenon of addiction affects only a low, but significant, percentage of individuals. In this regard, many theories have been postulated. Some suppose a role for cocaine that cause neurobiological changes in brain rendering the individual addict; for some others the transition to addiction is a pathological response to the drug that cause several modifications in brain of addicted individuals, but it depends by specific characteristics of the individual, i.e. there would be people more susceptible to others.

The main goal in the study of addiction is to understand how the effects of a drug of abuse with a prolonged use, progressively lead to permanent molecular and cellular changes.

Nucleus accumbens is thought to be the area that mediates rewarding and reinforcing effects of cocaine and in this, distinct neuronal population seem operate and function as ensemble coding, that are set of neurons able to receive information and after analysis sort output to other regions. In addition, every ensemble coding seems to be synchronized to perform its function. In the NAc and in several other regions, as hippocampus or striatum, synchronization is mediated by electrical coupling directed by gap junctions. Already is known the importance of GJs in developing seizures in epilepsia, where increase in expression of some connexins genes have been found. In vitro and in vivo experiments, it is showed also, how GJs opener or blocker, respectively increase or reduces the duration of seizures.

The use of classic GJs blocker carbenoxolone, demonstrates to suppress the stereotyped behaviour in animals after induction of reward and reinforcement stimuli. Moreover, electrical coupling between brain cells is regulated by several neurotransmitters as dopamine and glutamate.

From above, the aim of this study has been identify the identity of gap junctions proteins involved in the mechanism of action of cocaine and furthermore see if there was an

involvement of neuronal GJs proteins or the ones present in glial cells. As the same time, verify the potential role of GJs in the transition to addiction.

In neurons, the only connexin certainly expressed is Cx36, but there are many evidences as regard the presence of Panx1. Instead in astrocytes, oligodendrocytes and microglia have been found Cx43, 32, 26, 30. For Panx2 controversial remains the exact localization, it has been found in neurons and under pathological conditions in astrocytes.

So connexins and pannexins mRNA and protein expression have been analyzed quantitavely in the nucleus accumbens, in medial prefrontal cortex and in ventral tegmental area, i.e. those brain regions of mesocorticolimbic system principally involved in the actions of cocaine abuse. To investigate the effect of cocaine we used animals exposed for different time to cocaine self-administration, to analyze the effects in the short and long-term. Moreover, to evaluate the differences between the instaneous effect of cocaine and that after one day of withdrawal, the animals were sacrificed after 40 minutes or 24 hours by the last cocaine injection.

A self-administration model has been used to discriminate the addiction-like behaviours, from those animals that instead kept a controlled use of the drug, and so verify if these proteins are involved in the transition to addiction.

Quantitative real-time PCR and immunoblotting have been used to analyze the mRNA expression and proteins level expression of connexins and pannexins.

3 Materials and Methods

3.1 Cocaine self-administration (SA) procedures

The protocol of cocaine self-administration and the animal model, have been carried out by Deroche-Gamonet et al. following the protocol already described [108, 109].

Subjects

Male Sprague-Dawley rats (280-300 g) were used. Rats were single housed under a 12 hr reverse dark/light cycle (on 20h00, off 8h00). Temperature (22 ± 1°C) and humidity (60 ± 5%) were also controlled. Animal care and use followed the directives of European Communities Council Directive (86/609/EEC).

Drugs

Cocaine HCl (Coopération Pharmaceutique Française, Bordeaux, France) was dissolved in 0.9% NaCl. Ketamine (Imalgene ®) and xylazine (Rompun®) were mixed for anesthesia. Gentamicine (Gentalline®) was dissolved in 0.9% NaCl.

Surgery

A silastic catheter (internal diameter = 0.28 mm; external diameter = 0.61 mm; dead volume = 12µl) was implanted in the right jugular vein under ketamine (100 mg/kg) + xylazine (1 mg/kg) anaesthesia. Rats were allowed to recover for 5 to 7 days after surgery. During the first 4 days following surgery, rats received an antibiotic treatment (gentamicine 1 mg/kg i.p.). After surgery, catheters were flushed daily with a saline solution containing unfractionated heparin (100 IU/ml).

Intravenous Self-Administration (SA) apparatus

The SA setup consisted of 48 SA chambers made of Plexiglas and metal (Imetronic, Pessac, France). Each chamber (40 cm long x 30 cm width x 52 cm high) was located within an opaque box equipped with exhaust fans that assured air renewal and masked background noise. Briefly, each rat was placed daily in a SA chamber and the intracardiac catheter connected to a pump-driven syringe (infusion speed: 20µl/sec). Two holes, located at opposite sides of the SA chamber placed at 5 cm from the grid floor, were used to record instrumental responding. A white house light at the top of the chamber allowed its complete illumination. A white cue light (1.8 cm in diameter) was located 9.5 cm above the active hole. A green cue light (1.8 cm in diameter) was located 10 cm right to the white cue light. A blue cue light (1.8 cm in diameter) was located on the wall opposite to the one containing the active hole at 33 cm from the floor on the left side. Experimental contingencies were controlled and data collected with PC Windows-compatible software.

Basal training protocol

The daily SA session was composed of three drug components (40 min each) separated by two 15 min periods during which responding in the active hole had no scheduled consequences (no drug periods). Drug periods were signaled by the blue cue light, while the no drug periods were signaled by illumination of the entire SA box and extinction of the blue cue light. During the drug periods, introduction of the rat's nose into one hole (active) turned on the white cue light located above it and then, 1 sec later, switched on the infusion pump. The cue light remained on for a total of 4 sec. Nose-pokes in the other hole (inactive) had no scheduled consequences. The self-infusion volume was 40 µL (2 sec infusion) and contained 0.8 mg/kg of cocaine. Each infusion was followed by a time-out period of 40 sec. During the first 6 days, a Fixed Ratio 3 (FR3) schedule of reinforcement was applied. Then, the FR was increased to 5 for the rest of the experiment. Criterion for acquisition of cocaine SA was defined by a stable number of self-infusions over at least three consecutive SA sessions (± 10%).

Addiction-like criteria

Cocaine use severity is assessed using three procedures resembling some of DSM-IV [30] criteria for substance dependence:
(i) persistence in drug seeking even if the drug is not available, measured by active responding during periods of signaled drug non-availability; (ii) high motivation for the drug, measured by a progressive ratio schedule of reinforcement and (iii) drug use despite negative consequences, measured by resistance to footshock-induced punishment during cocaine SA.

Persistence of drug seeking in the absence of cocaine. This test allows assessing the subject's difficulty to control drug seeking and drug use. It was assessed daily by measuring the responses in the active hole during the two no drug periods of the basal training protocol. Active responses during the two daily no drug periods were summed. For analysis, the mean responses over two to three consecutive sessions were used as dependent variables.
Progressive-ratio schedule. The ratio of responses per infusion was increased after each infusion according to the following progression (10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585), the last ratio completed, called “the breakpoint”, was used to measure motivation for cocaine. The session ceased after either 5 hours or when a period of 1 hour had elapsed since the previously earned infusion.
Resistance to punishment: During this session rats were placed for 40 min in the SA chamber. The blue cue light signaling drug availability was on. The schedule was the following: the first response led to the illumination of the green cue light signaling the presence of the shock. After 3 additional responses, rats received an electric footshock (0.2 mA, 1 sec), and after the 5th response, rats received both an electric footshock (0.2 mA, 1 sec) and a cocaine infusion (0.8 mg/kg), associated with the corresponding conditioned stimulus (white cue light). Then the green cue light was turned off. The schedule reinitiated at the end of the time-out period, i.e. 40 sec after the infusion. If, within a minute, rats did not complete response requirements leading to shock and shock plus infusion respectively, the green cue light turned off and the sequence was reinitiated, i.e. the following response turned on the green cue light. The number of active responses earned during this test was expressed in percentage of the number of

active responses performed, over the same period of time, during the basal training session conducted the day before.

Establishment of Addict-like and non Addict-like groups

The rats were classified based on their scores for each of the three addiction-like tests and considering a rat positive for an addiction-like criterion when its score for this behaviour was in the 35% highest percentile of the distribution. Four groups of rats are isolated depending on the number of positive criteria met (0crit, 1crit, 2crit and 3crit). 0 and 3 criteria rats are defined as non Addict-like and Addict-like animals respectively. 1crit and 2crit rats either represent specific stable cocaine "use-related troubles" or intermediate steps toward addiction.
The scores in the three addiction-like behaviours were linearly related to the number of addiction-like criteria met.

Addiction score

It was calculated as the algebraic sum of standardized scores of each of the three addiction-like criteria. Standardization consisted in subtracting the mean of the group to each individual score and then dividing this number by the standard deviation. This procedure yields scores which have a mean of 0 and a standard deviation of 1. The addiction score is thus distributed along a scale from -3 to 3.

Evaluation of vulnerability to addiction-like behaviour in early cocaine use

The animal model developed allowed also to evaluate the vulnerability to addiction during an early cocaine use (after 15 to 20 days of SA). Indeed, starting from sessions 11-13, addict rats showed a tendency to a progressive increase in response during the last part of the non-drug periods. Then, analyzing several variables characterizing responding during the early non-drug periods were individuated early behavioural features that allow predicting transition in a reliable and consistent manner in early cocaine use.

The variables analyzed were the number of active responses measured at early or late minutes of no-drug periods (for example during the first 6 minutes or the last 9 minutes of no drug periods). Rats were then ranked for these four variables. Rats with a score in the 20th highest percentile of the population were defined as positive for this predictive criterion. Rats with 4 positive criteria were defined Addiction Vulnerable rats, whilst rats with 4 negative criteria were called Addiction Resistant rats.

Groups of animals analyzed

For expression of connexins and pannexins genes and proteins have been tested different groups of animals. Three groups of rats were subjected to cocaine self-administration for 7, 18 and 72 days. The groups of rats treated for 7 and 18 days were ulteriorly subdivided in two independent subgroups, the first subgroup were animals sacrificed after 40 minutes by the last cocaine injection and the second group with animals sacrificed after 24 hours by the last cocaine injection. The groups of rats treated for 72 days were analyzed only after 40 minutes and only for western blot analysis. Each group was formed also by animals Controls; these were left undisturbed in the animal house for the same time of the others. In order to obtain the same time point within groups the start of the SA sessions of each animal was scheduled appropriately.
With 7 days of SA the rats were indicated as cocJ7.
At 18 days of cocaine SA the animals, depending behavioural features observed during no-drug periods were divided in vulnerable (predicted 3criteria) and resistant to addiction (predicted 0 criteria) rats.
At 72 days of cocaine SA the animals were classified as 3 criteria (addict) or 0 criteria (non-addict) depending by their addiction score.

Brain regions dissection

Rats were anesthetized with a mix of ketamine (100mg/kg) and xylazine (1mg/kg), followed by decapitation. Brains were rapidly removed and placed into an ice-cold dissection plate and areas of interest were hand-dissected, then frozen on dry ice and stored in centrifuge tubes at -80°C.

3.2 Quantitative real-time PCR

Samples

Three structure have been analyzed, nucleus accumbens (NAc), medial prefrontal cortex (mPFC), ventral tegmental area (VTA) on 4 groups of rats

Every group was constituted by:

7days SA/40 minutes: n=15 (controls n=8, CocJ7/40' n=7)

18days SA/40 minutes: n=23 (controls n=8; pred 0crit n=9; pred 3crit n=6)

7days SA/24 hours: n=14 (controls n=7, CocJ7/24h n=7)

18days SA/24hours: n=32 (controls n=10; pred 0crit n=10; pred 3crit n=12)

RNA extraction and PCR procedures

RNA was extracted with TRIzol® in according with manufacturer's protocol (Invitrogen, Carlsblad, CA, USA). After extraction with chloroforme and ethanol precipitation, RNA samples were treated with a DNase inactivation reagent kit (Ambion® TURBO DNA-free™ DNase; Ambion, Austin, TX, USA). To evaluate the purity of RNA, the ratio of absorptions at 260 nm vs 280 nm (for contamination by other nucleic acids and protein) and ratio 230 nm vs 260 nm (for contamination by organic compounds), were assessed with spectrophotometer (Eppendorf AG, Hamburg, Germany).

The RNA quality was assessed by capillary electrophoresis, using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA with RIN (RNA integrity number) > 8.5] was used for the study.

cDNA was synthesized from 2 µg total RNA with Reverse Transcriptase SuperScript™ III (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, in presence of random primer and Oligo(dT)18 primer (Fermentas, Waltham, Massachusetts, USA). The RT-PCR amplification was carried out: 10 minutes at 25°C, 1 hour at 50°C and 15 minutes at 70°c, using a PTC-200 Biorad Thermal Cycler (BioRad, Hercules, California, USA).

Quantitative analysis with real-time PCR

Primers were designed using Primer Express Software v3.0 (Applied Biosystems, Foster City, CA, USA) and were purchased by Eurogentec (Seraing, Belgium). Each primer was tested to evaluate efficiency and specificity (range 91-103%). The primers sequences were the following:

Gene	Forward	Reverse
Cx43	F: 5'- CCCCGACGACAACCAGAAT - 3'	R: 5'- TTGGTCCACGATGGCTAATG - 3'
Cx36	F: 5'- GGAGGATATTTTTCCCCCTTCA - 3'	R: 5'- GAAAAAGAAAAGCTGGCATGCT - 3'
Cx26	F: 5'- CTTAGCATTGTCATTCTGCCTGAT - 3'	R: 5'- GAAGTGTCCCCTGTTGGATTTG - 3'
Cx32	F: 5'- CATGCTGTCAGGTACCCCACTT - 3'	R: 5'- CCTCCTCCACTCTGCTTGCT - 3'
Cx30	F: 5'- GGACGGTTTGGTGAGCTAGTG - 3'	R: 5'- GCAAACGGCGATGTATGTTGT - 3'
Cx45	F: 5'- TGGGAGATGCTTCATCTAGGGTT - 3'	R: 5'- TCATCAAGTTCCCTCCTTTTACTG - 3'
Panx1	F: 5'- AGGCAGGAGAACGGGATTTT - 3'	R: 5'- GTTCCGTGTTCTGCCCTCAT - 3'
Panx2	F: 5'- GTGAGTGGCCTAAGCAGACGT - 3'	R: 5'- ACTCGGGCAGGTGCTTGTAC - 3'

Table 3.1 Sequence of primers used for qPCR. For each gene, are indicated the forward and reverse sequence.

Several housekeeping genes were tested to choose the optimal reference gene in each group and structure for subsequent quantitative analysis. Two reference genes have been selected for every experiment among which showing more stability in our PCR conditions.

Group	Structure	Reference genes	
7days SA/40 minutes	NAc	**Sdha**	**Gapdh**
	mPFC	**Sdha**	**Gapdh**
	VTA	**Sdha**	**Gapdh**
18days SA/40 minutes	NAc	**Sdha**	**Gapdh**
	mPFC	**Sdha**	**Gapdh**
	VTA	**Tuba4a**	**Gapdh**
7days SA/24 hours	NAc	**Eef1a1**	**Sdha**
	mPFC	**Actb**	**Gapdh**
	VTA	**Actb**	**Eef1a1**
18days SA/24hours	NAc	**Sdha**	**Actb**
	mPFC	**Actb**	**Eef1a1**
	VTA	**Tuba4a**	**Sdha**

Table 3.2 Reference genes chosen for each structure in the several groups. **Eef1a** (eukaryotic translation elongation factor 1 alpha 1); **Gapdh** (glyceraldehyde-3-phosphate dehydrogenase); **Actb** (actin, beta); **Sdha** (succinate dehydrogenase complex, subunit A, flavoprotein (Fp)); **Tuba4a (**tubulin, alpha 4A).

Real time comparative PCR was performed on a LightCycler® 480 Real-Time PCR System (Roche, Germany) using 4 ng of cDNAs as templates and LightCycler® 480 DNA SYBR Green I Master Mix (Roche, Germany) according to the manufacturer's instructions. The RT-PCR amplification of primers was carried out with a denaturation step at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 61° for 30 s, primer extension at 72°C for 1 min and final extension at 72°C for 5 min.

Each sample was analyzed in duplicate. For each sample, relative expression of target genes was calculated based on real-time PCR efficiencies (E) and the threshold cycle (Ct) difference (Δ) of an treated sample versus a control ($\Delta Ct_{control-sample}$) and expressed relative to the reference genes chosen, in according to the $2^{-\Delta\Delta Ct}$ method.

3.3 Immunoblotting

Samples:

Three structures have been analyzed, nucleus accumbens (NAc), medial prefrontal cortex (mPFC), and ventral tegmental area (VTA) on 5 groups of rats:

7days SA/40 minutes: n=15 (controls n=8, CocJ7/40' n=7)

18days SA/40 minutes: n=23 (controls n=8; pred 0crit n=9; pred 3crit n=6)

72Days SA/40 minutes: n=31 (controls n=10; 0crit n=11; 3crit n=10)

7days SA/24 hours: n=18 (controls n=9, CocJ7/24h n=9)

18days SA/24hours: n=32 (controls n=10; pred 0crit n=10; pred 3crit n=12)

Protein extraction: Protein extracts from rat nucleus accumbens (NAc), medial prefrontal cortex (mPFC) and ventral tegmental area (VTA) were prepared with a homogenizing protocol using the Precellys 24 (Bertin Technologies, France). The tissues were homogenized with ceramic CK14 beads (Bertin Technologies, France) in RIPA buffer (Radioimmunoprecipitation assay buffer), containing DTT, protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA) at 5000 rpm 2x30 sec + 10 sec break. After centrifugation at 10000 rpm for 10 minutes the supernatant was kept and stored at -80 °C. Proteins concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting analysis: The proteins (20-50 µg) were denatured in Laemmli sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mm Tris, pH 6.8, 0.008% bromophenol blue) for 5 min at 95 °C and then were separated by SDS-PAGE on 4-15% gradient gels (BioRad, Hercules, California, USA) using a Mini-Protean II apparatus (Bio-Rad Laboratories) according to standard protocols. The proteins were separated at 15mA/gel in Tris/glycine/SDS migration buffer and transferred to PVDF membranes (Immobilon P, Millipore, USA). The transfer was performed overnight at 4 °C, in Tris/glycine/methanol 20% buffer, at 15 V. To evaluate the correct proteins transfer the membranes were stained with reversible red Ponceau S (Sigma, St. Louis, MO, USA). After decoloration the membranes were then blocked for 1h in a milk solution (5% dry powdered nonfat milk, TBS, Tween 20 0.1%) or in a BSA solution (5% BSA lyophilized powder, TBS, Tween 20 0.1%) at room temperature and incubated overnight at 4 °C in a milk or BSA solution containing the primary antibody. The membranes were washed (2 × 7 min) with TBS/Tween 20 0.1% and incubated for 1 h at room temperature in a milk or BSA solution containing the HRP-conjugated secondary antibody. The membranes were again washed three times for 5 min with TBS/Tween 20 0.1%, and once in TBS alone. The signal was then revealed using ECL Luminata Forte Western HRP substrate (Millipore, USA) according manufacturers' instructions and the signal was detected with Biomax-MR films (Eastman Kodak, Rochester, NY, USA).

The proteins were revealed using the following antibodies:

Primary Antibody		**Dilution**	**Secondary antibody**		**Dilution**
Anti-Cx36	AbCam ab86408	0,2ug/ml	**Anti -Rabbit**	Cell Signaling 7074	1/5000
Anti-Cx43	AbCam ab11370	1/40000	**Anti -Rabbit**	Cell Signaling 7074	1/40000
Anti-Cx32	AbCam PAB19101	0,5ug/ml	**Anti -Mouse**	Cell Signaling 7076	1/5000
Anti-Cx26	AbCam ab65969	1,5 ug/ml	**Anti -Rabbit**	Cell Signaling 7074	1/4000
Anti-Panx1	Santa Cruz sc-49695	1/200	**Anti -Goat**	Sigma A5420	1/25000
Anti-Panx2	Santa Cruz sc-133880	1/500	**Anti -Rabbit**	Cell Signaling 7074	1/5000

Table 3.3 Antibodies used for western blot analysis. For each primary antibody is indicated the relative secondary antibody and the dilution used.

Analysis of the results

The bands in western blot were quantified by densitometry using a GS-800 scanner (in transmission mode) and the associated Quantity One software (Bio-Rad). The X-ray films were scanned and the optical density (OD) of each pixel was measured. OD of each band was corrected by subtracting the background OD. Identical quantities (µg) of proteins were loaded on the gels in each experiment. The Neuronal Class III β-tubulin (βIII-tubulin) monoclonal antibody (MMS-435P; 1/1000 dilution) used as a loading control was purchased from Eurogentec (Seraing, Belgium).

Data were presented and analyzed as adjusted band volume (adjusted volume OD) and/or percentage of basal values, ±SEM.

3.4 Statistics

Results are expressed as mRNA fold change expression or protein levels expression relative to control value and are shown as mean ± S.E.M. The differences of each group with the group of controls were assessed with Student t-test. Significance is set at $P<0.05$ and is represented with symbol #.

To evaluate group differences in the expression, the values of each sample in a group were normalized with the average of controls values, and after the groups were analyzed with one-way ANOVA. The Newman-Keuls *post hoc* test was used for pair wise comparison of means in the different groups. Significance was set at $P<0.05$ and is represented with symbol *.

Statistical tests were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and Statistica 10.0© (StatSoft Inc, Tulsa, OK, USA).

4 Results

The main goal of this work has been evaluate the effects of cocaine on the expression of connexins and pannexins genes and protein levels. We wanted to investigate the modifications after acute and after prolonged self-administration of cocaine. Moreover we divided the different groups of animals in two subgroups, 40 minutes and 24 hours, depending by the time elapsed by last injection at the moment of sacrifice. Another goal of this work was establish if there were different regulations between animal showed addiction-like behaviour and non-addict animals.

4.1 Effects of cocaine self-administration on Nucleus Accumbens gap junctions

4.1.1 Effect on Cx36 in Nucleus Accumbens

There were no significant differences in the Cx36 mRNA expression at 40 minutes between the groups treated for 7 days and the two subgroups (pred 0 crit and pred 3 crit) treated for 18 days respect to the controls. Also the protein expression showed no variations after 7 or 18 days of treatment but in the addict-like group at 72 days there was an increase of 1.32±0.14% to respect the controls ($P<0.05$). Instead the slight tendency to increase shown by the group of non-addict rats at 72 days was negligible

The experimental groups at 40 minutes differed in the protein expression of Cx36 after cocaine SA; group effect ANOVA: $F(4,31)=3.90$, $P<0.05$; *post hoc*: *P<0,05 for 3crit J72 vs cocJ7 and 3crit J72 vs pred 0crit.

After 24 hours by the last cocaine injection, there were no mRNA expression differences in both the groups at 7 and 18 days. The protein expression was instead increased significantly after 18 days of SA; pred 0crit 1.18±0.05%, $P<0.05$; pred 3crit 1.25±0.18%, $P<0.05$ and there was a group effect, ANOVA $F(2,23)=7.24$, $P<0.01$; *post hoc*: **P<0,01 for pred 3crit vs cocJ7 and *P<0,05 for pred 0crit vs cocJ7. (**Figure 4.1**)

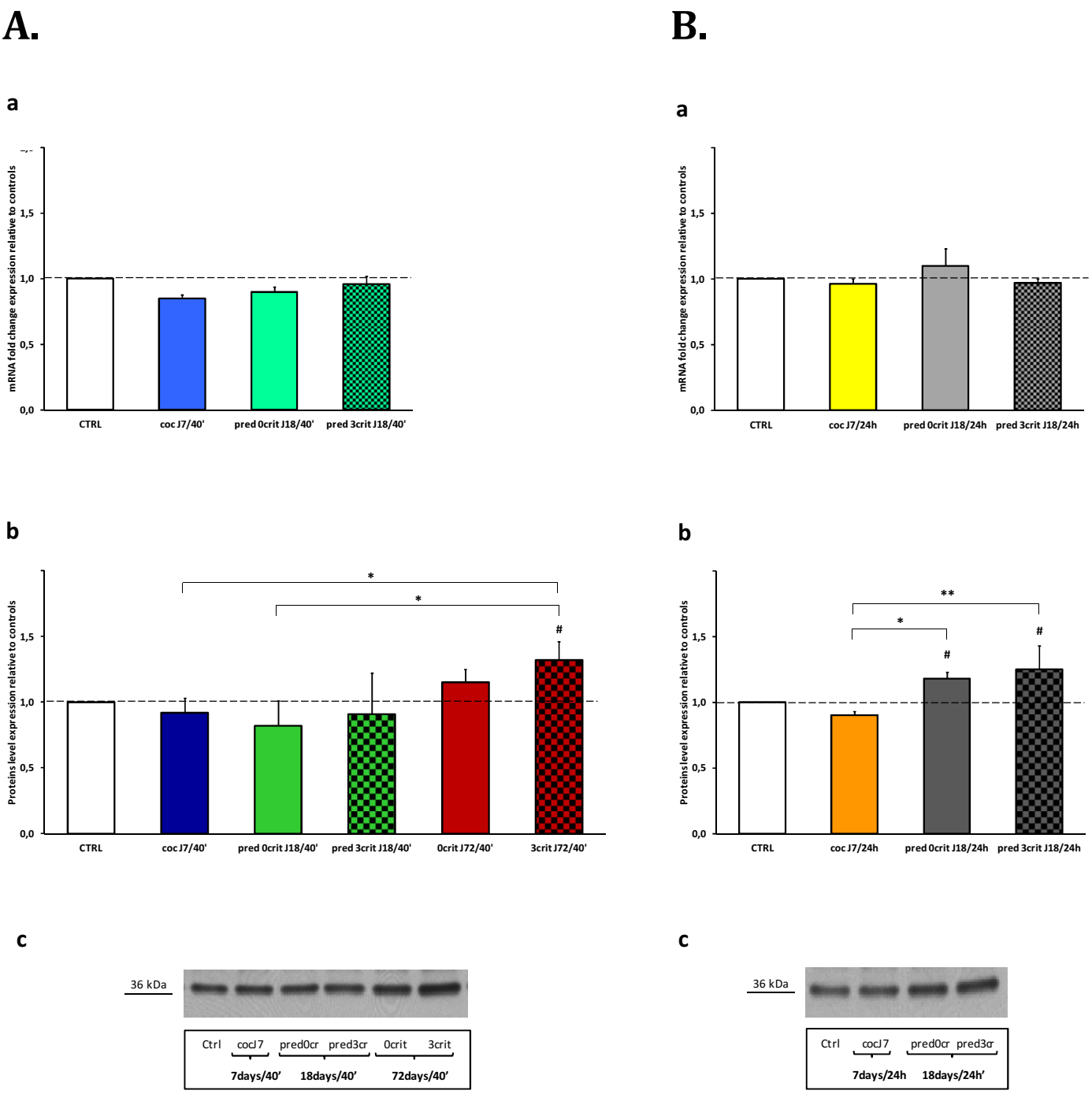

Figure 4.1 Effect of cocaine self-administration on Cx36 in NAc. **A:** In samples collected after 40' by last injection of cocaine, there were not changes in mRNA expression in all groups; the Cx36 protein was significantly increased after 72 days of SA in the groups of 3crit (1.32±0.14% relative to controls) and this group differed from others (ANOVA, group effect $F(4,31)=3.90$ $P<0.05$; post hoc: *P<0,05 for 3crit J72 vs cocJ7 and 3crit J72 vs pred 0crit). **B:** after 24 hours by last cocaine infusion there were no changes in mRNA expression. Protein levels were increased in both groups at 18 days of SA (pred 0crit 1.18±0.05%, pred 3crit 1.25±0.18%, $P<0.05$) and showed a group affect (ANOVA, $F(4,31)=3.90$ $P<0.05$; post hoc: *P<0,05 for 3crit J72 vs cocJ7 and 3crit J72 vs pred 0crit). **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.1.2 Effect on Cx43 in Nucleus Accumbens

After 40' the mRNA expression of Cx43 did not show changes, both the groups subjected to 7 and 18 days of SA. On the contrary the protein expression showed significant changes: after 7 days of treatment there was a decrease of 0.70±0.22%, $P<0.05$; at 18 days both the groups of resistant and vulnerable rats showed a decrease of 0.57±0.10% and 0.58±0.11% respectively (t-test: pred 0crit $P<0.001$; pred 3crit $P<0.01$). Interestingly after 72 days of cocaine SA, the 0crit group restored the protein expression to level comparable to controls, but the 3crit animals showed a reduction of 0.64±0.06% with a $P<0.05$. There was also a group affect, as analyzed with ANOVA $F(4,30)=4.55$ $P<0.01$; *post hoc*: *P<0,05 for 0crit J72 vs cocJ7, pred 0crit, pred 3crit and 3crit J72.

After 24 hours there were still no changes in the mRNA expression in all the groups analyzed. Moreover there were no variations in the protein expression after 7 days. After 18 days there was a slight reduction in Cx43 expression in predicted 3 criteria group 0.81±0.14%, $P<0.05$.

The levels of protein expression, decreased at any time point in the animals with drug on board were restored after one days of abstinence, even though the groups of vulnerable animals at 18 days/24 hours started to show the reduction that they would stably maintained. (**Figure 4.2**)

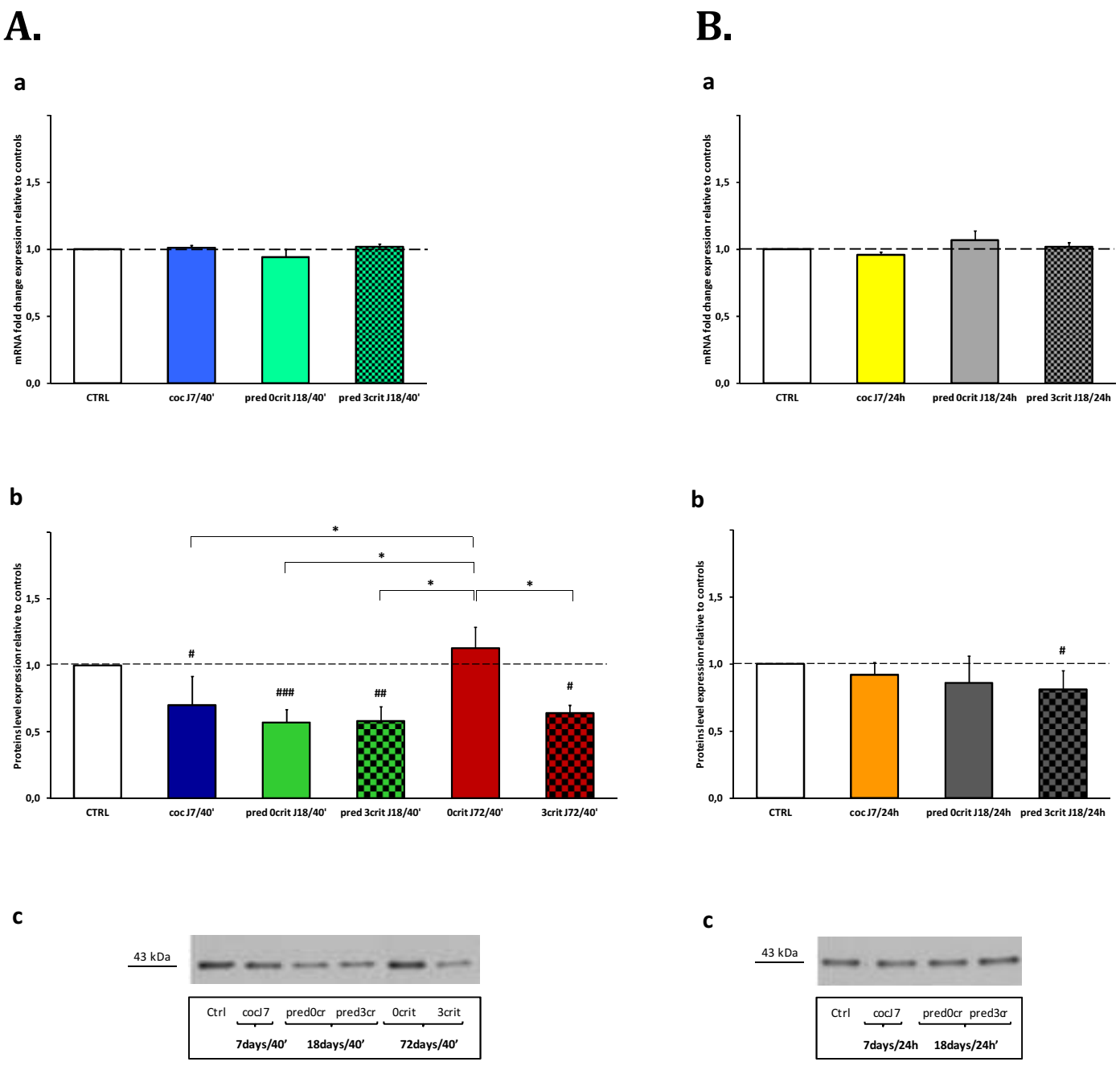

Figure 4.2 Effect of cocaine self administration on Cx43 in Nac. **A:** 7 or 18 days of SA did not influence expression of Cx43 mRNA in samples at 40' while the protein showed a reduction at any time point; after 7 days of SA there was significant decrease in Cx43 protein (0.70±0.22% P<0.05); after 18 days both pred 0crit and pred 3crit showed the same level of decrease, 0.57±0.1% and P<0.001 for pred 0crit and 0.58±0.11% P<0.01 for pred 3crit. After 72 days, 0crit restored levels than to controls 1.13±0.16%, while 3 crit animals exhibited low levels of Cx43 protein (0.64±0.06% P<0.05). The groups differed accordingly ANOVA : F(4,30)=4.55 P<0.01; *post hoc*: *P<0.05 for 0crit 72J/40' vs all the other 4 groups cocJ7, pred 0crit J18, pred 3crit J18 and 3crit J72. **B:** mRNA expression was not modified in the samples collected after 24 hours in both groups at 7 and 18 days. 24 hours of abstinence restored the normal levels of protein at 7 days, but after 18 days a small decrease was detected in the predicted 3 criteria group (0.81±0.14% P<0.05). **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.1.3 Effect on Cx32 in Nucleus Accumbens

After 7 days of SA, at 40' the Cx32 mRNA expression showed a decrease, that was weak 0.87±0.02% for resistant animals, and slightly more pronounced 0.80±0.03%, for vulnerable animals, in the groups treated for 18 days. Both reductions in the two groups were significant $P<0.05$ for pred 0crit and $P<0.01$ for pred 3crit.

The Cx32 protein expression, instead was significantly reduced after 18 days of cocaine SA (0.47±0.24% $P<0.001$ for pred 0crit and 0.60±0.19% $P<0.01$ for pred 3crit). This decrease was restored to control levels after 72 days, at least for 0crit group while 3crit group showed moreover an increase 1.51±0.37%, $P<0.01$. The five groups differed in accord with analysis of variance (ANOVA, $F(4,28)=26.26$ $P<0.001$; *post hoc*: ***P<0,01 for 3crit J72 vs cocJ7, pred 0crit, pred 3crit and for 0crit J72 vs pred 0crit, pred 3crit; **P<0,01 for cocJ7 vs 0crit J72; *P<0,05 for 3crit 72J vs 0crit 72J and for cocJ7 vs pred 0crit).

After 24 hours, Cx32 mRNA expression displayed no modifications with the exception of a slight increase in pred 0 crit at 18 days (1.17±0.08% $P<0.05$), significant also respect to the other groups of rats cocaine-treated (ANOVA: $F(2,24)=7.48$ $P<0.01$; *post hoc*: **P<0,01 for pred 0crit vs cocJ7 and pred 0crit vs pred 3crit). Instead the Cx32 protein expression was not significantly changed, because of the high standard deviation found in the several samples. (**Figure 4.3**)

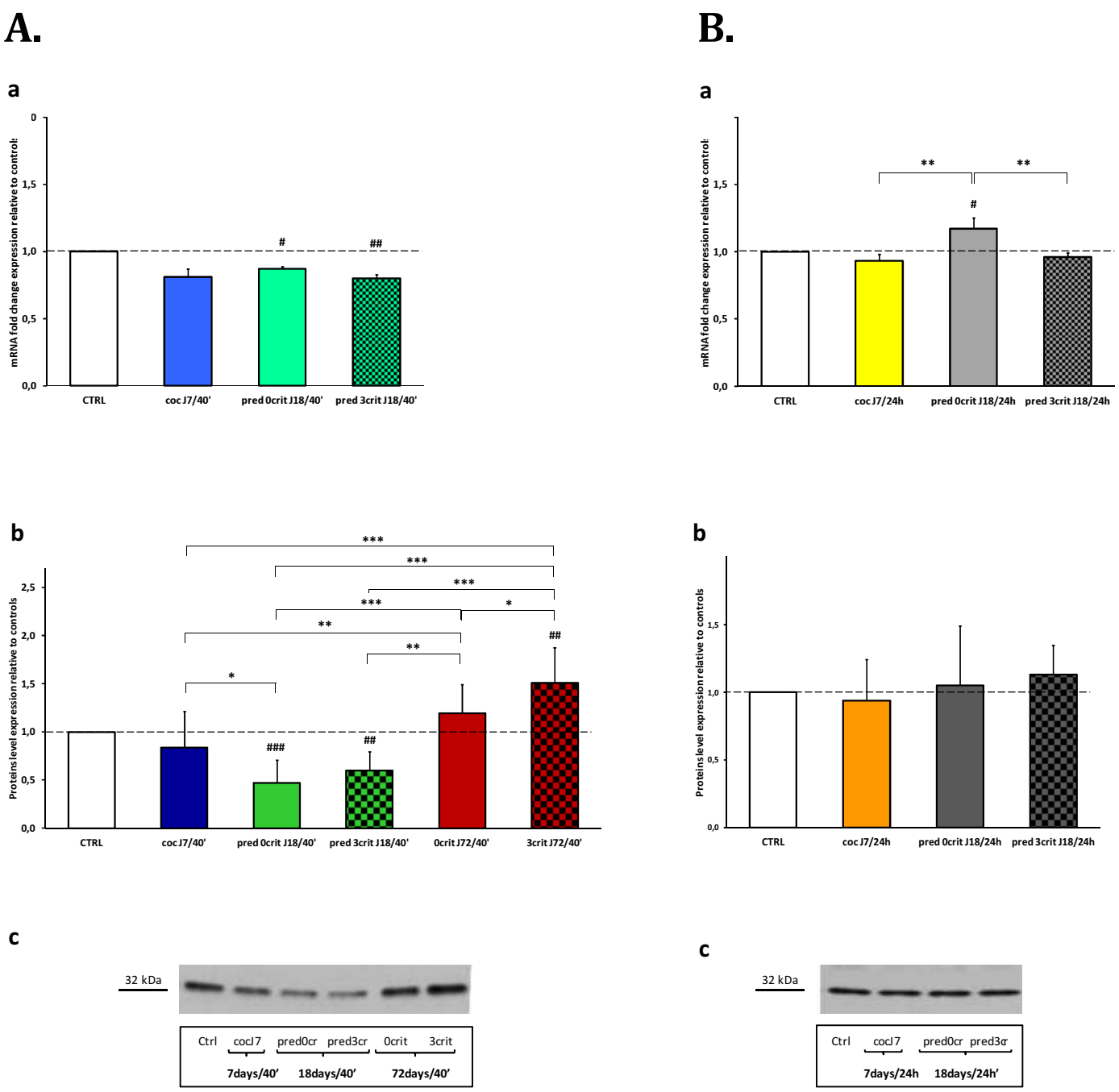

Figure 4.3 Effect of cocaine self administration on Cx32 in NAc. **A:** Slight decrease at 18 days of SA in both predicted 0 and pred 3criteria (0.87±0.02 P<0.05 for pred 0crit and 80±3 P<0.01 for pred 3crit). In protein expression this decrease is reinforced, pred 0crit 0.47±0.24% with P<0.001 and pred 3crit 0.60±0.19% P<0.01. 72 days of self-administration caused an increase in Cx32 expression, a weak, not significant, increase for 0 crit and an increase of 1.51±0.37% in 3 crit group with a t-test P<0.01 compared to controls. The ANOVA showed strong differences between groups, F(4,28)=26.26 P<0.001; *post hoc*: ***P<0,01 for 3crit J72 vs cocJ7, pred 0crit, pred 3crit and for 0crit J72 vs pred 0crit, pred 3crit; **P<0,01 for cocJ7 vs 0crit J72; *P<0,05 for 3crit 72J vs 0crit 72J and for cocJ7 vs pred 0crit. **B:** cocJ7 and pred 3crit at 18 days revealed no differences in Cx32 gene expression, only a weak increase in pred 0 crit 1.17±0.08% P<0.01, confirmed moreover by ANOVA for effect group, F(2,24)=7.48 P<0.01 *post hoc*: **P<0,01 for pred 0crit vs cocJ7 and pred 0crit vs pred 3crit. The proteins at 24 hours showed no significant differences. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.1.4 Effect on Panx1 in Nucleus Accumbens

At 40' the Panx1 mRNA expression was not modified in NAc. Only a weak increase in cocJ7 of 1.15±0.04% with P<0.05 was present and gave also a group effect to respect the group of pred 3crit at 18 days F(2,19)=4.53 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 3crit. The protein expression was not modified in the several groups with exception for the reduction in 3crit 72J group, 0.75±0.07% P<0.05. Also in this case was possible make a distinction between the effect of prolonged cocaine exposure in the group of addict-animals to respect the non-addict, not subjected to decrease in protein expression; The groups differed accordingly ANOVA, F(4,30)=4.32 P<0.01; *post hoc*: *P<0,05 for 3crit72J vs pred 0crit and pred 3crit.

At 24 hours there were no modifications both in mRNA and in protein expression, in all the groups analyzed. (**Figure 4.4**)

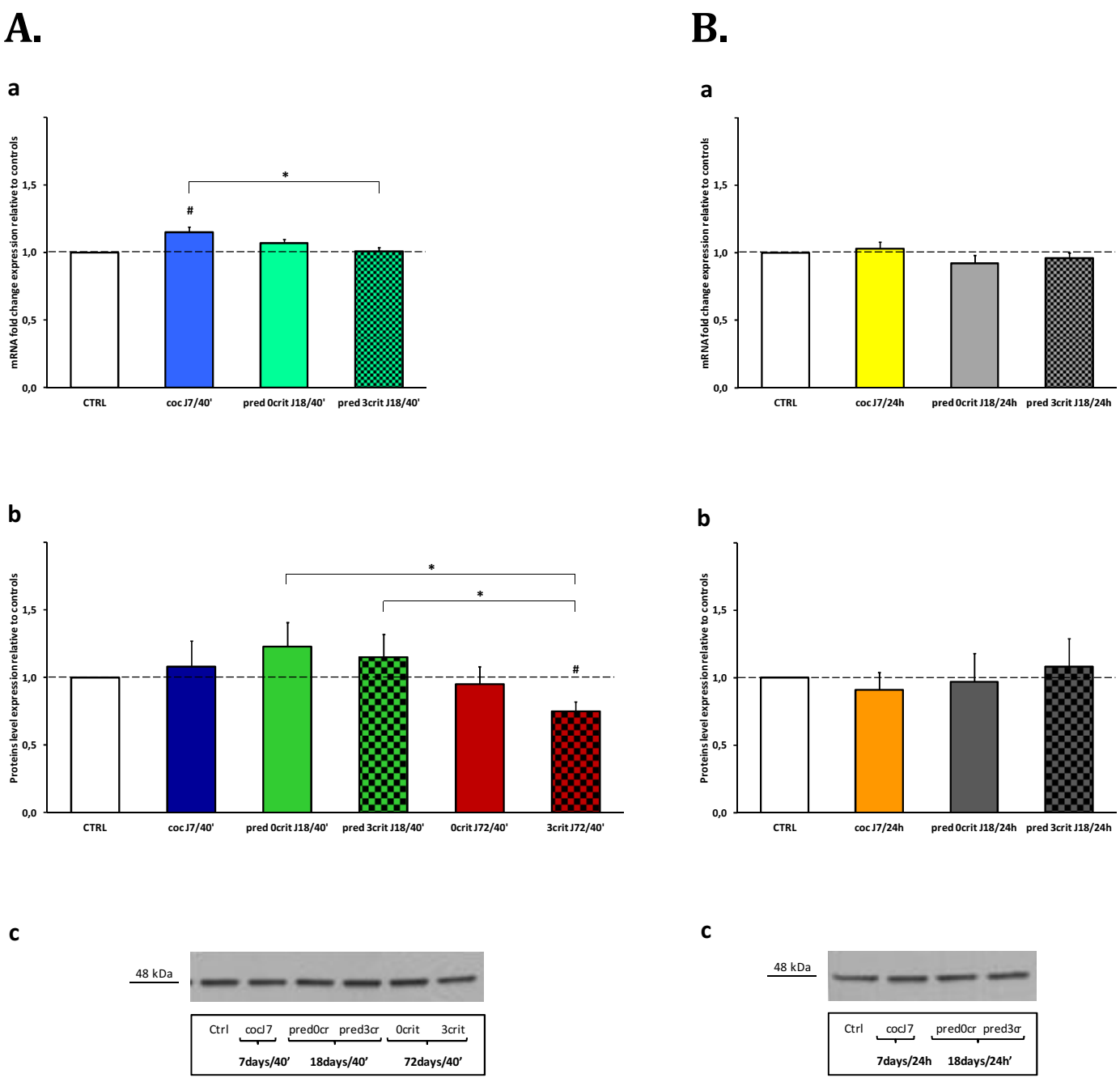

Figure 4.4 Effect of cocaine self administration on Panx1 in NAc. **A:** the Panx1 mRNA showed a weak increase after 7 days of SA 1.15±0.04% P<0.05 and a group effect characterized by ANOVA F(2,19)=4.53 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 3crit. For protein expression there was a decrease at 72 days in addicted animals 0.75±0.07% P<0.05, while the other groups displayed no changes or slight non significant changes. The groups differed accordingly ANOVA, F(4,30)=4.32 P<0.01; *post hoc*: *P<0,05 for 3crit72J vs pred 0crit and vs pred 3crit. **B:** Panx1 mRNA and protein were not modified in the groups of samples collected after 24 hours. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.1.5 Effect on Panx2 in Nucleus Accumbens

Cocaine SA for 7 days at 40' by last injection, caused a high significant increase in mRNA expression 2.10±0.09%, P<0.001, but these levels were restored to control levels after 18 days in both vulnerable and resistant animals. ANOVA for these groups was F(2,14)=11.91 P<0.001; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit.

Intriguingly, the huge increase observed at the first week of treatment was not accompanied by an increase of protein expression that instead decreased 0.71±0.12% P<0.05. The expression of Panx2 decreased ulteriorly in both groups at 18 days (pred 0crit 0.55±0.10 P<0.01, pred 3crit 0.57±0.17% P<0.05) while after 72 days the expression was totally changed, the 0crit group went back to control levels but 3crit group showed an increase 1.33±0.19% P<0.05. The differences between groups were confirmed by analysis of variance (ANOVA F(4,31)=15.35, P<0.001; post hoc: ***P<0,01 for 3crit J72 vs cocJ7, pred 0crit, pred 3crit; **P<0,01 for 0crit J72 vs pred 0crit, pred 3crit; *P<0,05 for 3crit 72J vs 0crit 72J and for 0 crit J72 vs cocJ7).

After one day of abstinence the mRNA expression levels tended to reach normal levels, indeed in group of cocJ7 there was an increase of 1.48±0.02% P<0.05. After 18 days of SA the two groups had both normal levels of mRNA; also in this case it is possible differentiate the group at 7 days with those at 18 days: ANOVA F(2,21)=3.89 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit.

The Panx2 protein expression showed no relevant alterations at 24 hours.

(**Figure 4.5**)

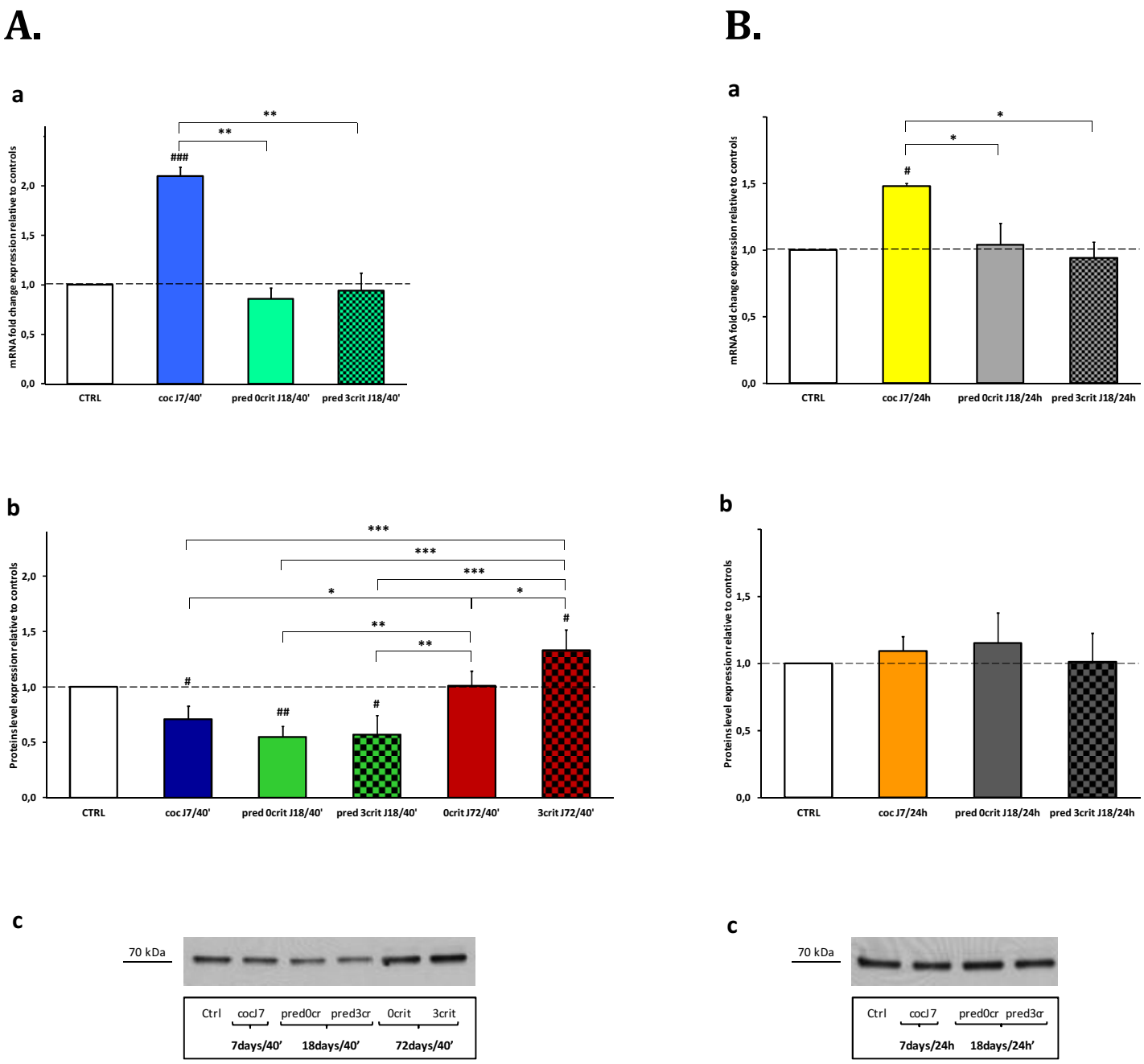

Figure 4.5 Effect of cocaine self administration on Panx2 in NAc. **A:** The mRNA expression after 7 days of cocaine SA at 40' showed a huge increase 2.10±0.09%, P<0.001. At 18 days the levels returned to control levels in predicted addict and non-addict animals. ANOVA was F(2,14)=11.91 P<0.001; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit. The Panx2 protein expression showed a different profile: 7 days of cocaine SA caused a decrease 0.71±0.12% P<0.05. The expression of Panx2 decreased ulteriorly in both groups at 18 days (pred 0crit 0.55±0.10 P<0.01, pred 3crit 0.57±0.17% P<0.05). After 72 days the 0crit group returned to normal levels while 3crit group showed a significant increase 1.33±0.19% P<0.05. These differences were confirmed by analysis of variance (ANOVA F(4,31)=15.35 P<0.001; *post hoc*: ***P<0,01 for 3crit J72 vs cocJ7, pred 0crit, pred 3crit; **P<0,01 for 0crit J72 vs pred 0crit, pred 3crit; *P<0,05 for 3crit 72J vs 0crit 72J and for 0 crit J72 vs cocJ7. **B:** After 24 hours the effect on Panx 2 genes were attenuated; in cocJ7 group there was an increase 1.48±0.02% P<0.05 but after 18 days the two groups had both normal levels of mRNA. The groups differed accordingly (ANOVA F(2,21)=3.89 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit). The Panx2 protein expression at 24 hours showed no modifications. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.1.6 Effects of cocaine SA on Nucleus Accumbens in other genes

In nucleus accumbens, we analyzed also the mRNA expression of Cx26, Cx30 and Cx45.
It has been analyzed also the Cx26 protein expression, but this was undetectable with western-blot technique in this area.
Analysis of cx26 mRNA expression in NAc revealed no relevant differences. only the group of predicted 0crit at 18j/40' showed a weak increase, 1.37±0.12%, that was not significant respect to the controls group but there was a group effect, accordingly with ANOVA $F(2,17)=3.91$ $p<0.05$; post hoc: *$p<0.05$ for pred 0crit vs cocj70,41.
The groups analyzed at 24 hours showed no alterations except of a very slight, not significant, tendency to decrease in pred 3 crit group.

In cx30 mRNA expression there were no appreciable variations at 40' but after 24 hours the group of resistant to addiction animals, exhibited an increase of 1.26±0.11% $p<0.05$. This increase gave also a discrete group effect by revealed with ANOVA $F(2,22)=4.57$ $p<0.05$; post hoc: *$p<0,05$ for pred 0crit vs cocj7 and pred 0crit vs pred 3crit.

The Cx45 gene, showed no modifications in expression in all the groups analyzed.
(**Figure 4.6**)

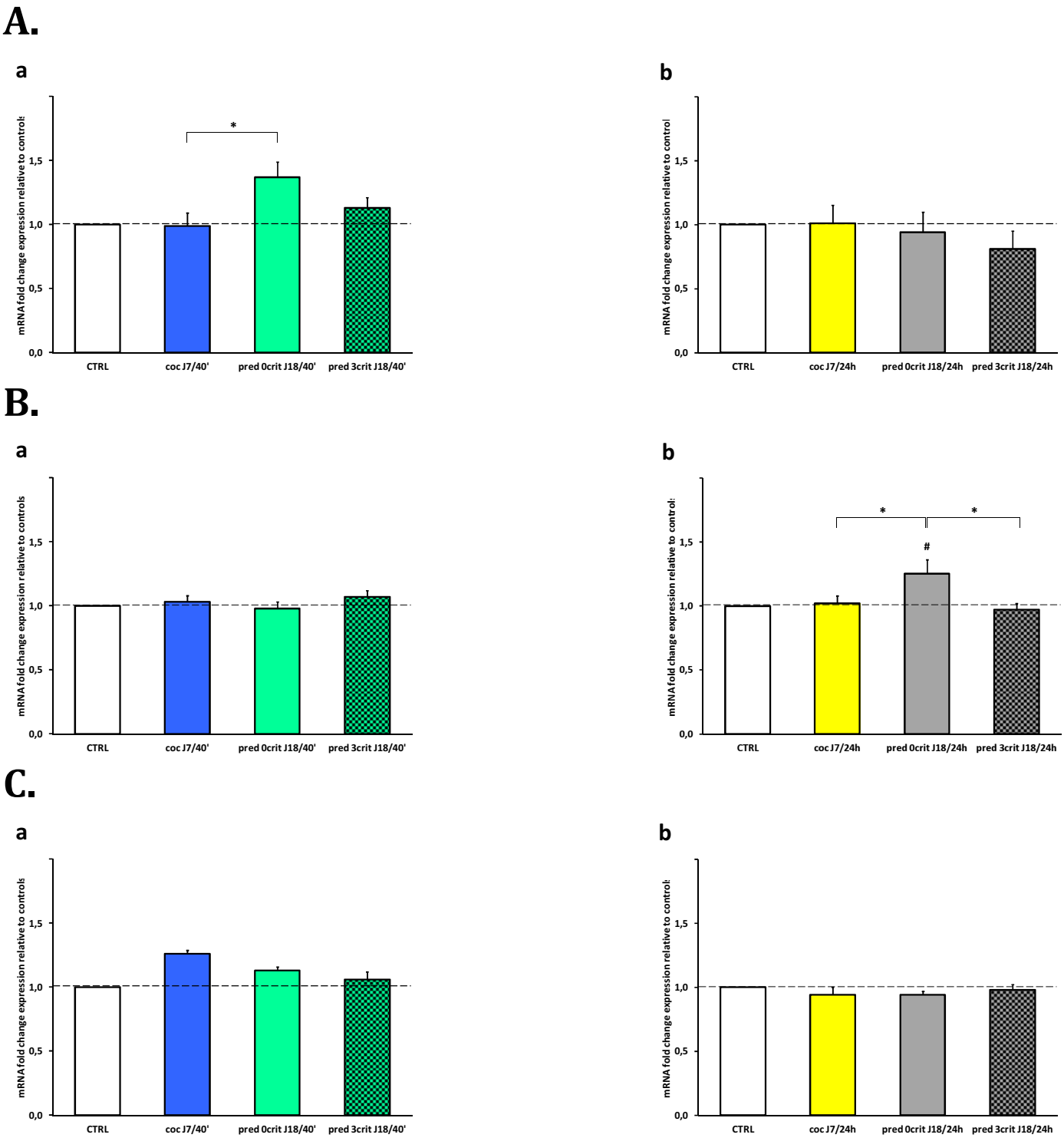

Figure 4.6 Effects of cocaine self-administration on other genes in NAc. **A:** Cocaine SA had no effect on Cx26 gene at 7 days of SA both at 40' and 24h. At 18 days no particular alterations were present, with exception of pred 0crit 18J/40' for which there was an, not significant, increase 1.37±0.12%; the groups differed accordingly ANOVA $F(2,17)=3.91$ $p<0.05$; post hoc: *$p<0.05$ for pred 0crit vs cocj7. **B:** Cx30 was not modified by cocaine SA both at 7 and 18 days of SA. Only in pred 0crit j18/24h there was an increase 1.26±0.11% $p<0.05$. This gave also a group effect (ANOVA $F(2,22)=4.57$ $p<0.05$; *post hoc*: *$p<0,05$ for pred 0crit vs cocj7 and pred 0crit vs pred 3crit). **C:** cocaine SA had no impact on Cx45 mRNA expression in NAc. **a:** Representative results for quantitative PCR at 40'. **b:** Representative results for quantitative PCR at 24h. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.2 Effects of cocaine SA in medial prefrontal cortex gap junctions

4.2.1 Effect on Cx36 in medial prefrontal cortex

The analysis of Cx36 in medial prefrontal cortex showed no changes both at 40' and 24h for mRNA and protein expression levels.
(Figure 4.7)

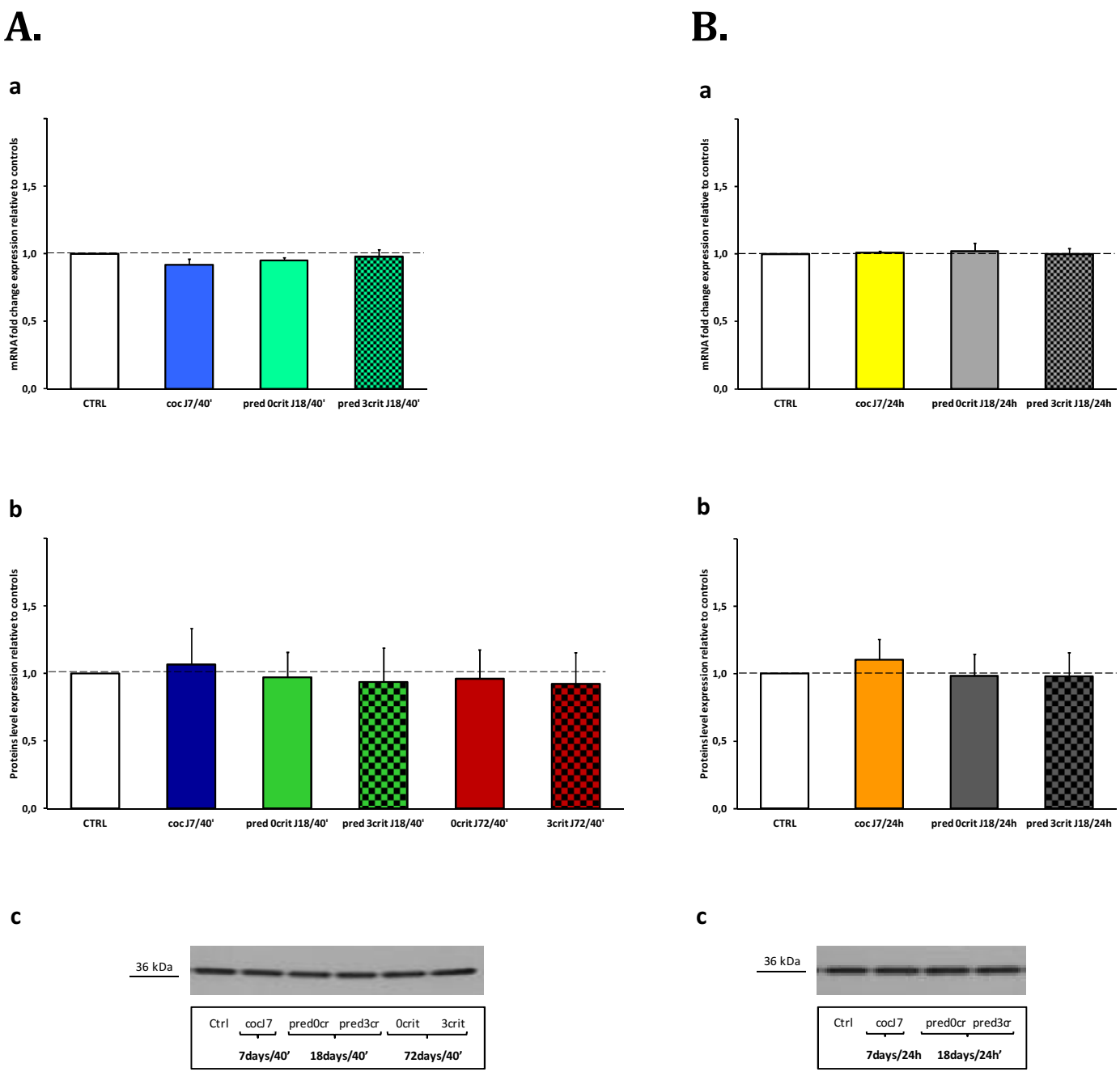

Figure 4.7 Effect of cocaine self-administration on Cx36 in mPFC. **A:** the self-administration of cocaine produced no alterations in all the groups analyzed both for mRNA and protein expression of Cx36 in rats with drug on board. **B:** Identical situation in animals analyzed at 24 hours. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.2.2 Effect on Cx43 in medial prefrontal cortex

In the nucleus accumbens Cx43 showed no differences as regard the mRNA expression respect to the controls, but protein levels were differentially regulated.

There were no changes in mRNA expression after 7 or 18 days of SA at 40 minutes.

The protein expression showed instead some differences: 7 days of SA were not sufficient to give rise modifications, even though it seemed to be a tendency to increase. 18 days of SA caused a decrease in protein expression, mostly for the predicted 3 criteria group, in which there was a reduction of 0.54±0.31%, $P<0.05$. A prolonged exposure instead showed a different behaviour to respect the same protein analyzed in NAc; the 0 crit group had protein levels comparable to controls (even if the SEM was high) but the 3 crit group showed an increase in Cx43 expression of 1.40±0.34% $P<0.01$. The groups differed accordingly ANOVA $F(4,31)=6.79$ $P<0.001$; *post hoc*: ***P<0,001 for 3crit J72 vs pred 3crit; **P<0,01 for 3crit J72 vs pred 0crit and for pred 3crit vs cocJ7; *P<0,05 for pred 3crit vs 0crit J72.

One day of abstinence caused no modifications in Cx43 gene expression, but the treatment for 18 days caused a reduction in both vulnerable and resistant to addiction animal groups, pred 0crit 0.80±0.22% $P<0.05$, pred 3crit 0.80±0.17% $P<0.05$.

(Figure 4.8)

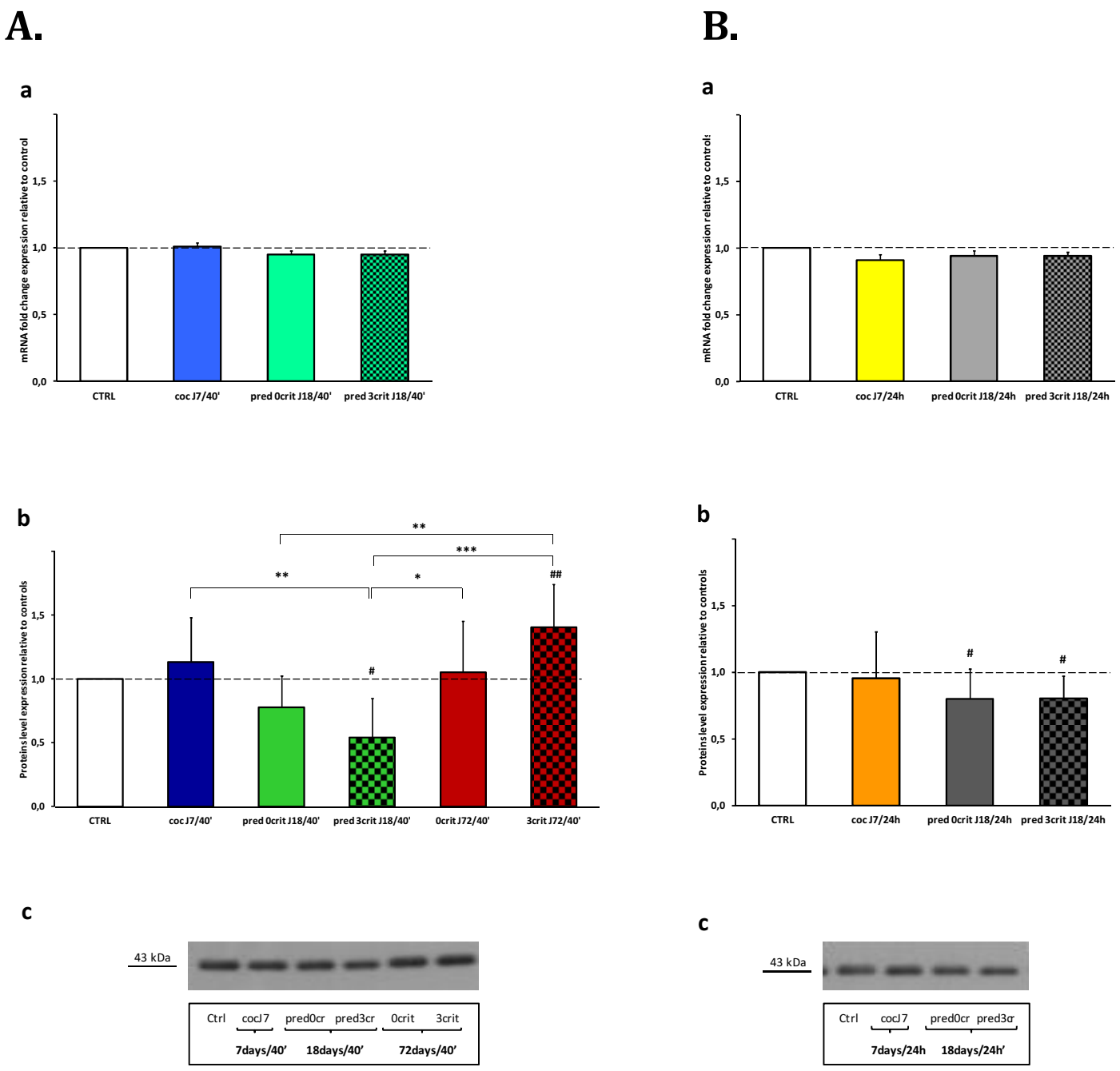

Figure 4.8 Effect of cocaine self-administration on Cx43 in mPFC. **A:** the mRNA expression of Cx43 at 40' was not subjected to modifications. The expression levels of Cx43 protein evidenced a reduction at 18 days, of about -20% in pred 0 criteria group, while for the predicted 3 criteria group there was a significant reduction 0.54±0.31%, P<0.05. 72 Days of SA caused no changes in non-addict animals while the 3 criteria group showed a relevant increase 1.40±0.34% P<0.01. The groups differed accordingly analysis of variance, ANOVA F(4,31)=6.79 P<0.001; *post hoc*: ***P<0,001 for 3crit J72 vs pred 3crit; **P<0,01 for 3crit J72 vs pred 0crit and for pred 3crit vs cocJ7; *P<0,05 for pred 3crit vs 0crit J72. **B:** the groups analyzed at 24 hours displayed no changes for mRNA, instead the protein expression remained decreased in both groups at 18 days , pred 0crit 0.80±0.22% P<0.05, pred 3crit 0.80±0.17% P<0.05. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.2.3 Effect on Cx32 in medial prefrontal cortex

One week of SA produced a relevant increase in Cx32 gene expression, 1.68±0.19% P<0.05, that decreased during the time. We found also a weak, no significant decrease in pred 0 criteria and a level similar to controls for pred 3 crit. A group effect was evidenced by ANOVA F(2,16)=8.09 P<0.019*; post hoc*: **P<0,01 for cocJ7 vs pred 0crit.

This increase in mRNA was not accompanied by an increase in Cx32 protein, indeed at any time point the protein expression was not modified. In all groups analyzed there was a high standard deviation, maybe due to the problems in the manual dissection of the different areas.

24 hours of abstinence to cocaine, were instead sufficient to give a decrease of Cx32 mRNA. There was a tendency to low levels although only pred 0 criteria showed a significant reduction, 0.65±0.10% respect to the control groups and P<0.05.

The protein expression in predicted 3 criteria group at 24h, showed a reduction 0.72±0.30%, P<0.05, while coc J7 and pred 0crit at 18 days were not modified. This decrease give also a group effect, ANOVA: F(2,17)=4.59 P<0.05; *post hoc*: *P<0,05 for pred 3crit vs pred 0crit and cocJ7. (**Figure 4.9**)

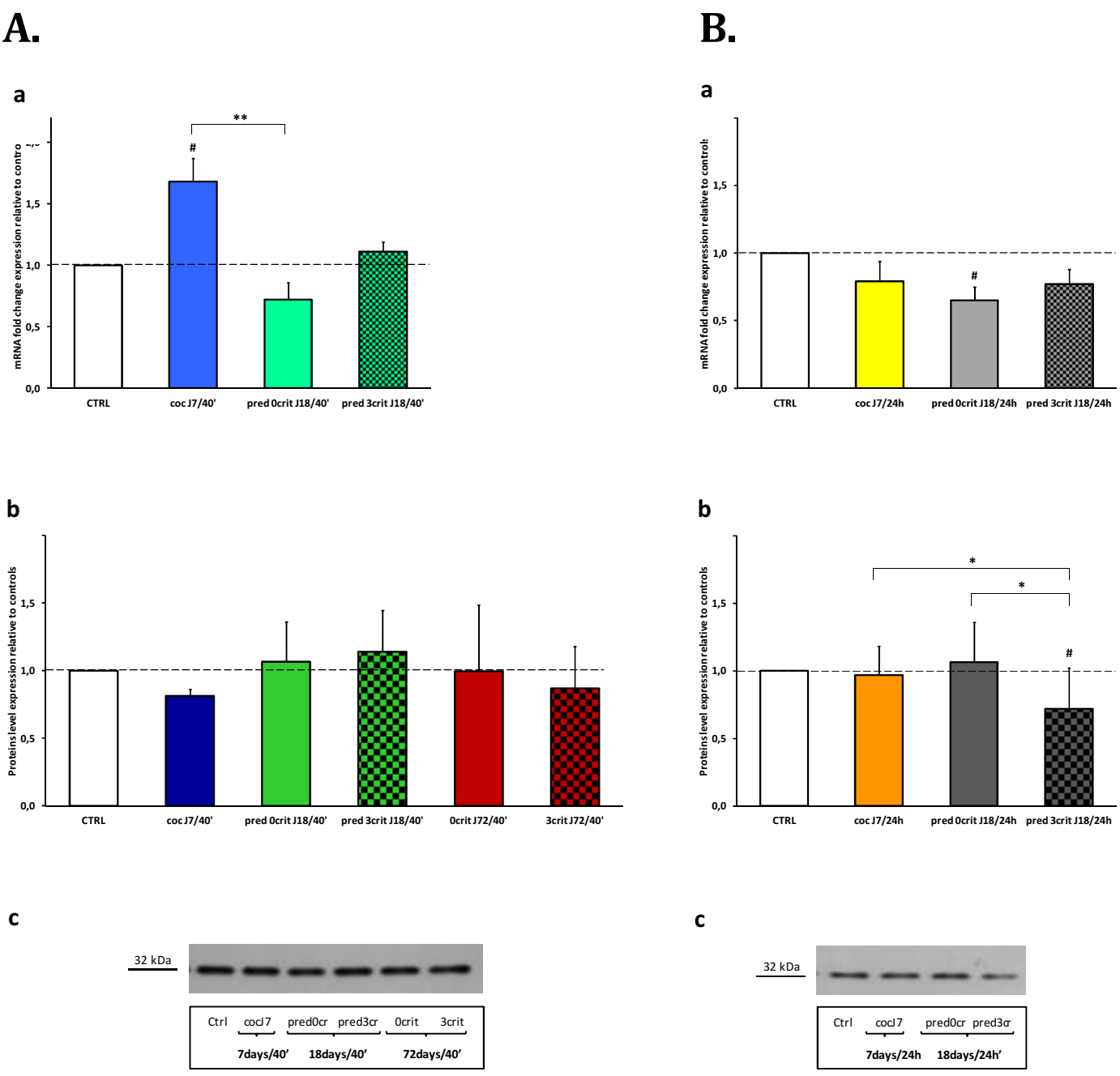

Figure 4.9 Effect of cocaine self-administration on Cx32 in mPFC. **A:** the mRNA of Cx32 was increased of 1.68±0.19% in rats treated for 7 days at 40' (P<0.05). After 18 days the levels were downsized to levels comparable to controls. Analysis of variance showed an effect group F(2,16)=8.09 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit. The Cx32 protein expression, depending by the high SEM found, rendered not possible a clear profile; the different groups however showed not particular tendency to increase or decrease. **B:** One day of withdrawal gave a reduction in mRNA expression in all the three groups but only pred 0crit showed a decrease statistically relevant 0.65±0.10, P<0.05. In predicted 3 criteria group protein expression was subjected to down-regulation 0.72±0.30%, P<0.05, while coc J7 and pred 0crit at 18 days were not modified. The three groups differed accordingly ANOVA, F(2,17)=4.59 P<0.05, ; *post hoc*: *P<0,05 for pred 3crit vs pred 0crit and vs cocJ7. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.2.4 Effect on Panx1 in medial prefrontal cortex

Panx1 mRNA expression, exhibited no differences both at 7 and 18 days of cocaine self-administration in the animals with drug on board.

Instead the Panx1 protein showed several alterations; 7 days of exposure to cocaine there were not changes but during the time, the levels were reduced. Only pred 0crit exhibited an increase 1.36±0.33 $P<0.05$, while both pred 3 crit and the groups at 72 days of SA showed a decrease in the protein levels: non-addict rats 0.74±0.25%, addicted 0.70±0.24%, for both $P<0.05$. This determined an effect groups revealed by analysis of variance, ANOVA $F(4,32)=11.83$ $P<0.001$; *post hoc*: ***P<0,05 for pred 0crit vs pred 3crit, 0crit J72, 3crit J72; **P<0,01 for pred 0crit vs cocJ7; *P<0,05 for cocJ7 vs pred 3crit, 3crit J72.

The samples collected with 24 hours of abstinence, showed no modifications (with a slight exception for cocJ7/24h group in which Panx1 mRNA was weakly, but significantly, reduced 0.86±0.02% $P<0.05$ and this gave also a group effect to respect the pred 0crit at 24h, $F(2,26)=4.00$ $P<0.05$; *post hoc*: *P<0,05 for cocJ7 vs pred 0crit.

The Cx32 protein was unchanged in all three groups of animals in which the mPFC area was collected after 24 hours by the last cocaine injection. (**Figure 4.10**)

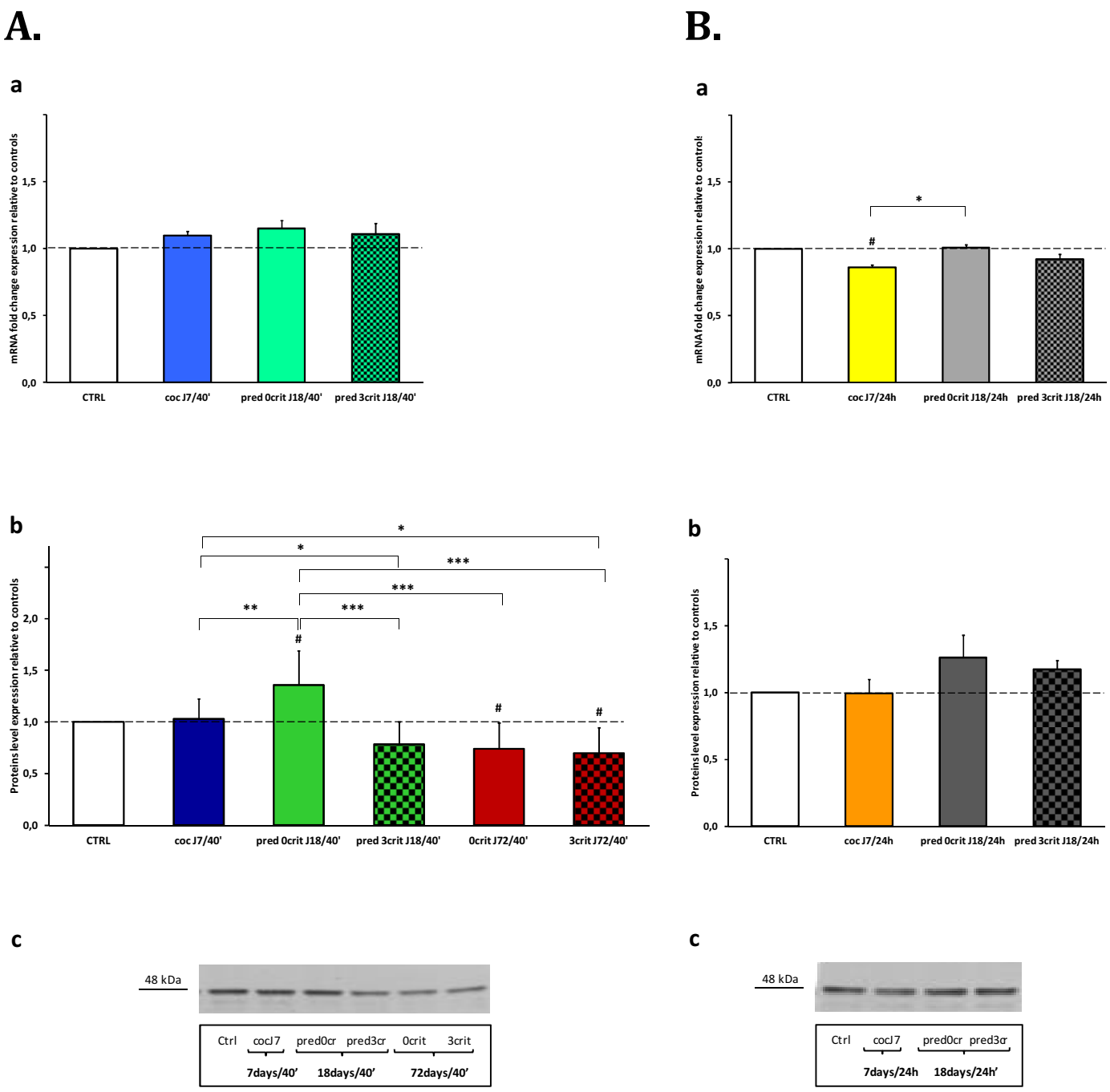

Figure 4.10 Effect of cocaine self-administration on Panx1 in mPFC. **A:** 7 or 18 days of cocaine SA had no effects on Panx1 gene expression. All groups were unmodified respect the controls. Pannexin 1 protein was diminished during the time of cocaine use. In predicted 0crit samples the protein levels were increased 1.36±0.33 P<0.05 After 72 days of SA, 0 crit animals had reduced levels 0.74±0.25 P<0.05%, such as the 3 criteria group 0.70±0.24%, for both P<0.05. The groups differed accordingly statistical analysis of variance (ANOVA: F(4,32)=11.83 P<0.001; *post hoc*: ***P<0,05 for pred 0crit vs pred 3crit, 0crit J72, 3crit J72; **P<0,01 for pred 0crit vs cocJ7; *P<0,05 for cocJ7 vs pred 3crit and vs 3crit J72. **B:** Samples analyzed at 24 hours by last cocaine infusion showed no relevant modifications at any time point both for gene and protein expression. Unique exception was the slight effect of reduction in CocJ7 group of 0.86±0.02% P<0.05 and a group effect revealed by ANOVA, F(2,26)=4.00 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 0crit. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.2.5 Effect on Panx2 in medial prefrontal cortex

The gene expression of Panx2, was strongly increased after one week of SA, 2.09±0.07% P<0.001. 18 days of cocaine exposition were sufficient to restored normal levels of Panx2 mRNA, indeed there were no differences in the Ct values of predicted 0 and 3 criteria animals analyzed; ANOVA for mRNA expression in 40 minutes groups was F(2,14)=13.49 P<0.001; *post hoc*: ***P<0,001 for cocJ7 vs pred 0crit and cocJ7 vs pred.
At the same time the mRNA levels in cocJ7 at 24 hours were reduced than those at 40', but however higher than normal 1.64±0.06% P<0.01. The levels were not modified at 18 days/24 hours in both groups.
In Panx2 protein expression more appreciable changes were observed after 72 days of SA. There was a weak tendency to decrease in cocJ7 and pred 3 crit at 18 days but was not significant; instead 0crit group showed small reduction 0.84±0.13% P<0.05 while the 3 crit groups exhibited a very significant reduction 0.65±0.27 P<0.01. The ANOVA was F(4,29)=3.11 P<0.05; *post hoc*: *P<0,05 for pred 0crit vs 3crit J72.
At 24 hours also the predicted 3 crit group showed a reduction 0.72±0.21 P<0.05. The slight increase in pred 0crit gave rise a group effect, ANOVA F(2,18)=6.13 P<0.01; *post hoc*:*P<0,05 for pred 0crit vs pred 3crit and vs cocJ7. (**Figure 4.11**)

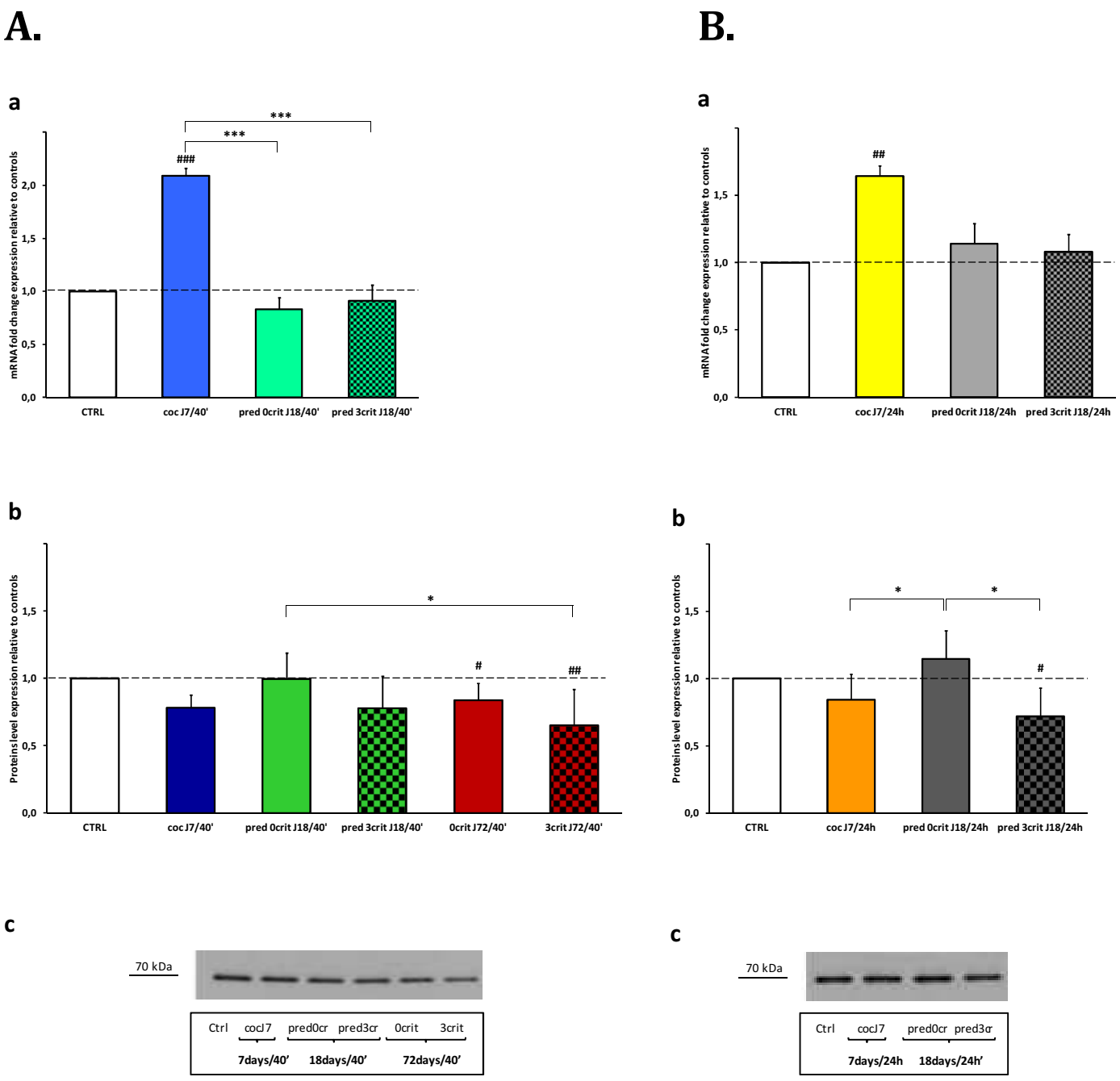

Figure 4.11 Effect of cocaine SA on Panx2 in mPFC. **A:** after 1 week of SA, group of cocJ7 showed an increase of 2.09±0.07% P<0.001 while 18 days caused no variations. The groups differed accordingly ANOVA, F(2,14)=13.49 P<0.001; *post hoc*: ***P<0,001 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit. The protein levels were slightly, but not significantly, reduced at 7 days and in predicted vulnerable animals. At 72 days of cocaine SA non-addict animals had a decrease in Panx2 protein of 0.84±0.13% P<0.05 while the animals showing addiction-like behavior had higher reduction 0.65±0.27 P<0.01. The analysis of variance was F(4,29)=3.11 P<0.05, *post hoc*: *P<0,05 for pred 0crit vs 3crit J72. **B:** one week of SA and 24h of abstinence presented still high levels 1.64±0.06% P<0.01, but proceeding with cocaine assumption the levels were totally restored. In withdrawal animals a reduction in Panx2 protein was present in vulnerable to addiction animals 0.65±0.27 P<0.01 and there was a group effect given by weak, not significant, increase in pred 0crit, ANOVA F(2,18)=6.13 P<0.01; *post hoc*: *P<0,05 for pred 0crit vs pred 3crit and vs cocJ7. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.2.6 Effect of cocaine SA on other genes in medial prefrontal cortex

In medial PFC the analysis of Cx26 gene during cocaine SA, showed a strong effect after 18 days and 40' in the 0crit group, indeed in this last there was an increase of 2.36±0.54% P<0.05. In addition, a weak tendency to decrease, but not significant, was found in cocJ7/40' group. The three groups differed depending on analysis of ANOVA F(2,17)=8.95 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit and *P<0,05 for pred 0crit vs pred 3crit. In contrast all the three groups analyzed after 24 hours by the last cocaine injection were decreased respect to the controls, cocJ7 0.68±0.08% P<0.05; pred 0crit 18J 0.62±0.12% P<0.05; pred 3crit 18J 0.46±0.12% P<0.05.

Analysis of Cx30 revealed no modifications in mRNA expression in all the analyzed samples.

The identical situation was found in expression of Cx45 gene, in which only a weak decrease in cocJ7/24hours, 0.80±0.05% P<0.01, gave a group effect in the animals subjected to abstinence (ANOVA F(2,26)=4,93 P<0,05; post hoc: *P<0,05 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit).

As in the NAc the Cx26 was undetectable in this region.
(**Figure 4.12**)

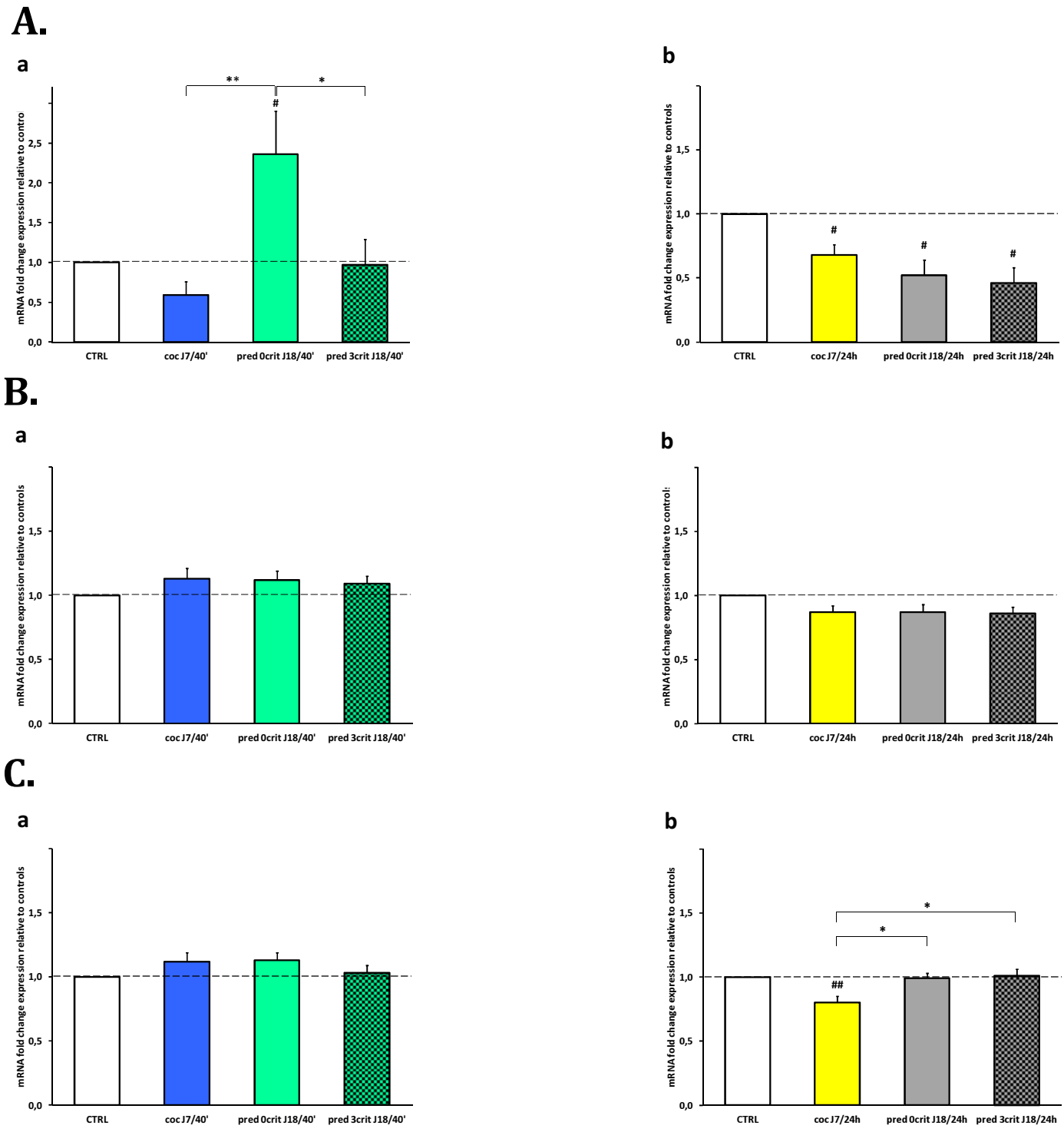

Figure 4.12 Effects of cocaine self-administration on other genes in mPFC. **A:** Analysis of Cx26 gene, showed a strong effect after 18 days and 40' in the 0crit group with an increase of 2.36±0.54% P<0.05. The three groups differed, depending on ANOVA F(2,17)=8.95 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit and *P<0,05 for pred 0crit vs pred 3crit. The three groups at 24 hours showed all a decrease, cocJ7 0.68±0.08% P<0.05; pred 0crit 18J 0.62±0.12% P<0.05; pred 3crit 18J 0.46±0.12% P<0.05. **B:** Analysis of Cx30 revealed no modifications in mRNA expression in all analyzed groups. **C:** Cx45 gene showed a slight decrease in cocJ7/24hours, 0.80±0.05% P<0.01; a group effect was present in the groups at 24h (ANOVA F(2,26)=4.93 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit). **a:** Representative results for quantitative PCR at 40'. **b:** Representative results for quantitative PCR at 24h. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.3 Effects of cocaine SA on ventral tegmental area gap junctions

The VTA is the first brain region involved in the cocaine effect. This in fact stimulates the dopaminergic transmission and then the communications with the other parts of mesocorticolimbic system.

4.3.1 Effect on Cx36 in ventral tegmental area

Cx36 mRNA expression was not influenced by cocaine SA at 7 or 18 days. Moreover also with an abstinence of 24 hours there were no changes in gene expression.

Similarly, the protein was not influenced; there was only a low increase in pred 3crit 18J/40' group 1.27±0.19% $P<0.05$

But nothing else variations in the other groups. (**Figure 4.13**)

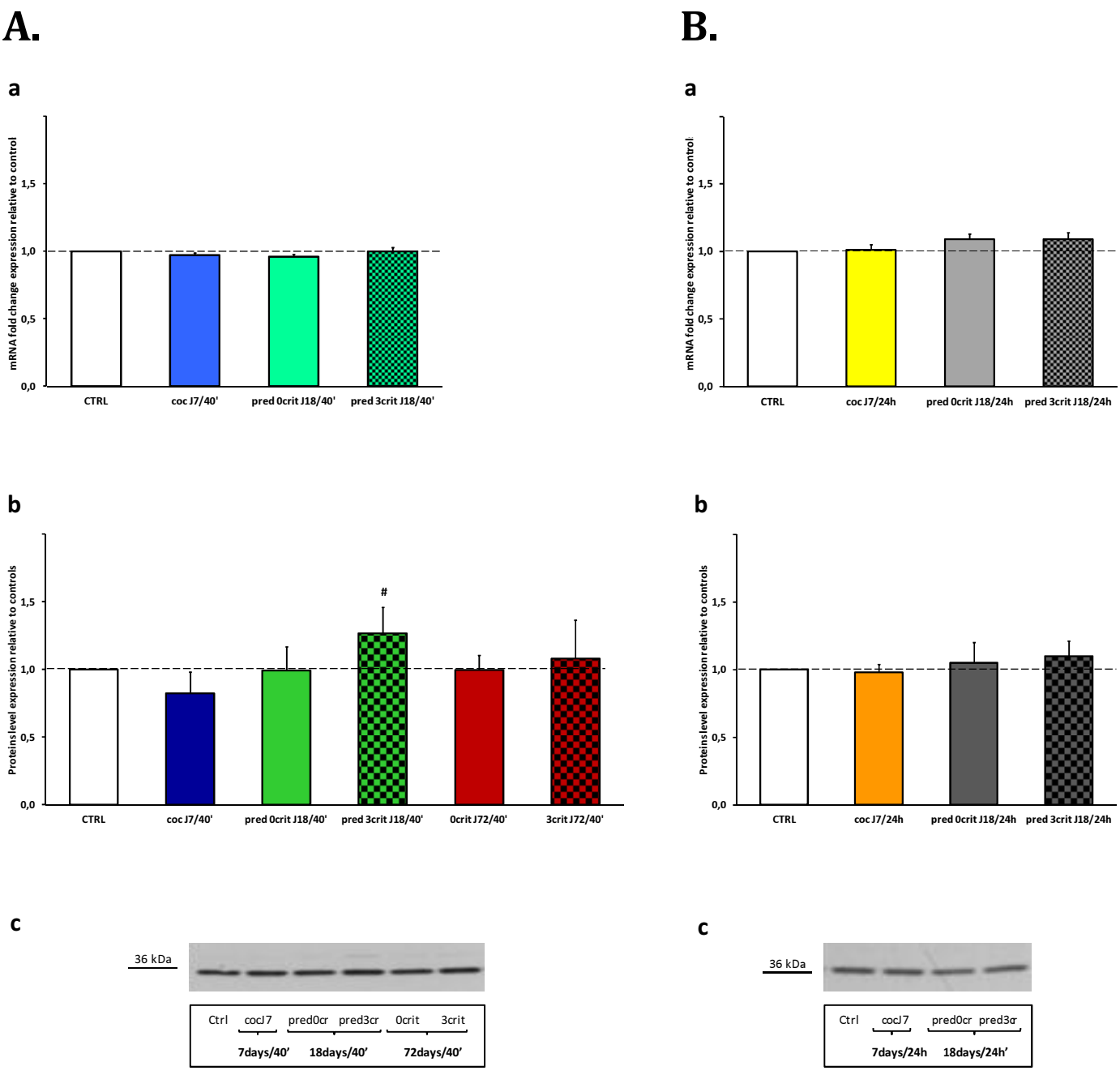

Figure 4.13 Effects of cocaine self-administration on Cx36 in VTA. **A:** Cx36 mRNA expression was not changed in the three groups at 40 minutes. Similarly the protein expression was not subjected to relevant variations, with exception of pred 3crit 18J/40' group, 1.27±0.19% P<0.05. **B:** The groups analyzed at 24 hours showed the same levels of Cx36 mRNA and protein expression respect to the controls. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.3.2 Effect on Cx43 in ventral tegmental area

The Cx43 gene was weakly regulated, a small reduction in mRNA levels was revealed in the group of animals treated for 7 days 0.84±0.02% P<0.01, but the levels were normal in animals treated for 18 days. This weak decrease gave also a group affect as evidenced by ANOVA F(2,18)=9.13 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit.

Instead for Cx43 protein was found a general decrease of the expression in all groups. The rats treated for 7 days showed a decrease of 0.72±0.11% P<0.05; after 18 days of treatment, the groups had also a decrease in levels expression but not significant. Addict and non-addict animals had both low levels of Cx43 expression, 0crit 0.64±0.10% P<0.05 and 3crit 0.63±0.13% P<0.05.

In the groups at 24 hours there were no appreciable changes (even though a more slight reduction in pred 0crit give a statistical relevance with analysis of variance F(2,25)=5.42 P<0.05; *post hoc*: *P<0,05 for pred 0crit vs pred 3crit.

The Cx43 protein at 24 hours were not altered, indeed showed levels comparable to those of controls. (**Figure 4.14**)

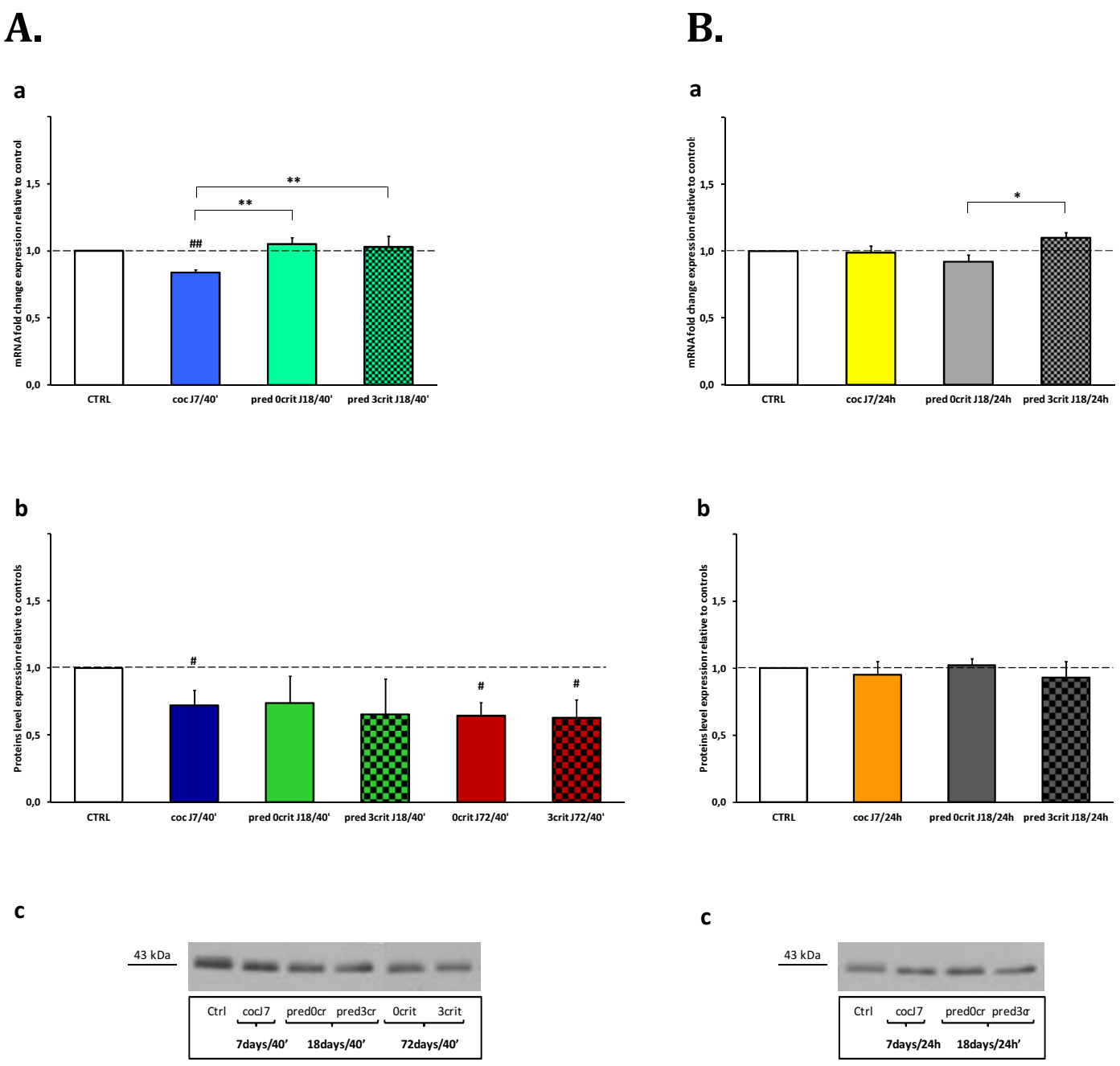

Figure 4.14 Effects of cocaine self-administration on Cx43 in VTA. **A:** Cx43 mRNA was diminished in the group of animals treated for 7 days 0.84±0.02% P<0.01, while the levels were normal in groups at 18 days. A group affect was evidenced by ANOVA F(2,18)=9.13 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit. Cx43 protein expression showed low levels in all groups. Groups of rats treated for 7 days showed a decrease of 0.72±0.11% P<0.05, the groups at 18 days of treatment had also a not significant decrease in levels expression. Both the groups subjected to 72 days of cocaine SA were decreased, 0crit 0.64±0.10% P<0.05 and 3crit 0.63±0.13% P<0.05. **B:** In the groups at 24 hours, there were no significant variations, even though analysis of variance revealed a group effect, F(2,25)=5.42 P<0.05; *post hoc*: *P<0,05 for pred 0crit vs pred 3crit. The protein at 24 hours were not altered respect the levels in rats of control. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.3.3 Effect on Cx32 in ventral tegmental area

Cx32 gene expression was not modified in all the groups analyzed. All the Ct values of several samples were completely comparable to the Ct values of the controls. It was evident that the drug assumption had no effects on this gene, either after 40' than after 24 hours of abstinence. Interestingly the protein showed instead a different regulation, Initially cocaine diminished the expression of Cx32, indeed cocJ7 at 40 minutes had 0.74±0.12 $P<0.05$, but following a more long exposure there was a difference; the resistant to addiction group of animals had normal levels of Cx32 protein but the group of vulnerable animals showed a significant increase 1.63±0.37% $P<0.05$. At 72 days of cocaine exposition both the groups presented the same levels of protein than control levels. The five groups differed accordingly analysis of variance $F(4,30)=6.42$ $P<0.001$; *post hoc*: ***P<0,001 for pred 3crit J18 vs pred ocrit and vs cocJ7; **P<0,01 for pred 3crit vs 0crit and vs 3crit.

At 24 hours the levels of the Cx32 protein were normal in all groups. (**Figure 4.15**)

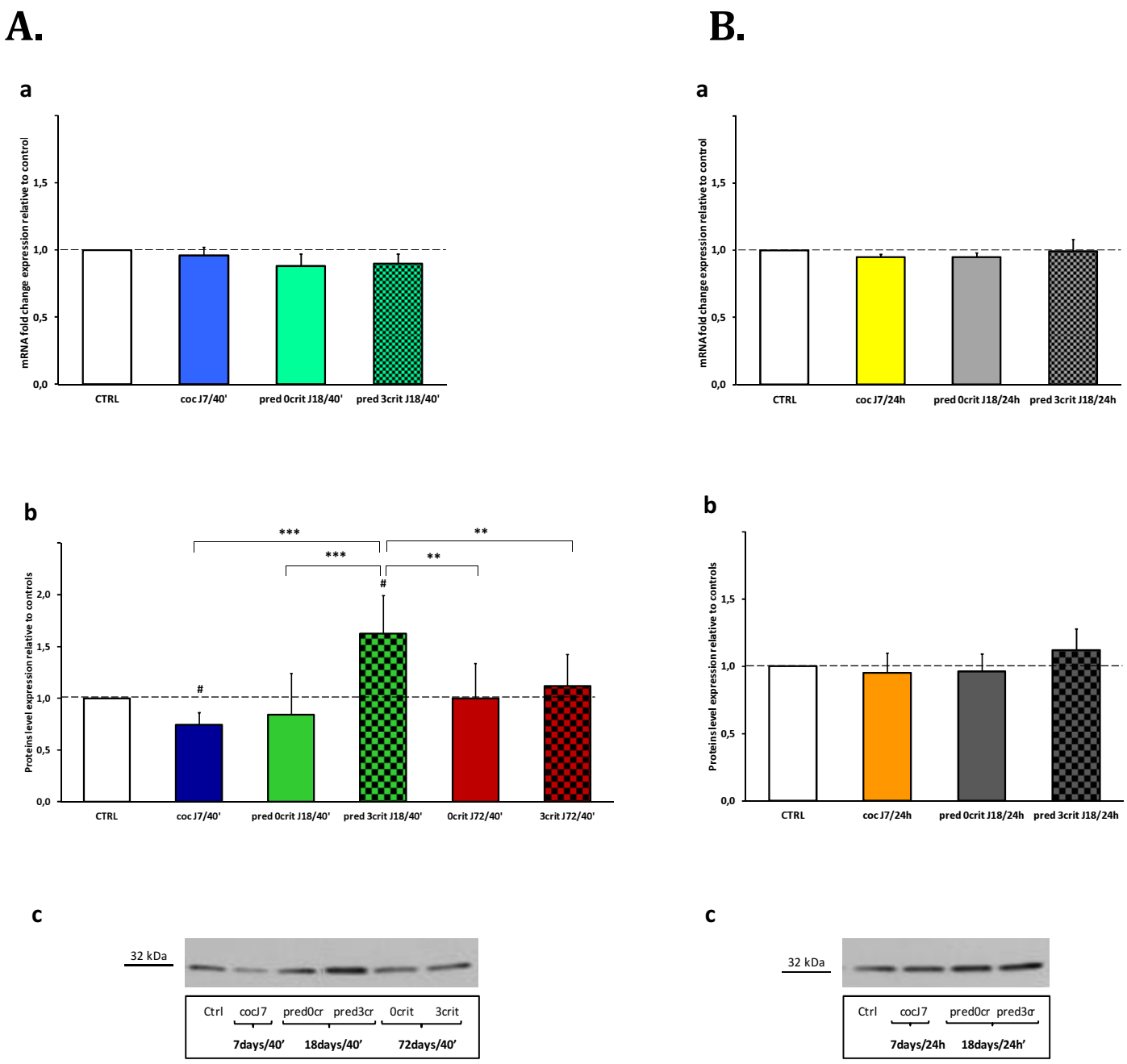

Figure 4.15 Effects of cocaine self-administration on Cx32 in VTA. **A:** Cx32 mRNA expression was normal in all groups analyzed. Protein expression revealed a decrease 0.74±0.12 P<0.05 in cocJ7 group at 40 minutes; 18 days of exposure showed differences between pred 0crit and pred 3crit groups, only this last had a significant increase 1.63±0.37% P<0.05. At 72 days both the groups presented levels comparable to controls. The five groups differed accordingly analysis of variance F(4,30)=6.42 P<0.001; *post hoc*: ***P<0,001 for pred 3crit J18 vs pred ocrit and vs cocJ7; **P<0,01 for pred 3crit vs 0crit and vs 3crit. **B:** Both mRNA and protein expression of Cx32 in VTA after 24 hours were not subjected to any effect. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.3.4 Effect on Panx1 in ventral tegmental area

Panx1 mRNA was not modified in rats analyzed with drug on board while with 24 hours of abstinence we assisted to a small decrease only in cocJ7 group 0.87±0.03% P<0.05 and a modest increase in vulnerable group at 18 days 1.29±0.08% P<0.05. ANOVA for these groups was F(2,25)=8.13 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 3crit and *P<0,05 for pred 0crit vs pred 3crit.

The protein at 40 minutes was not modified, but seemed to be a general tendency to a diminution, as observed also in Cx43 protein, but only the group of cocJ7 showed a significant decrease 0.71±0.14 P<0.05. As for this protein, 24 hours of abstinence did not modify the levels or restored the slight differences observed in proteins at 40 minutes. (**Figure 4.1**)

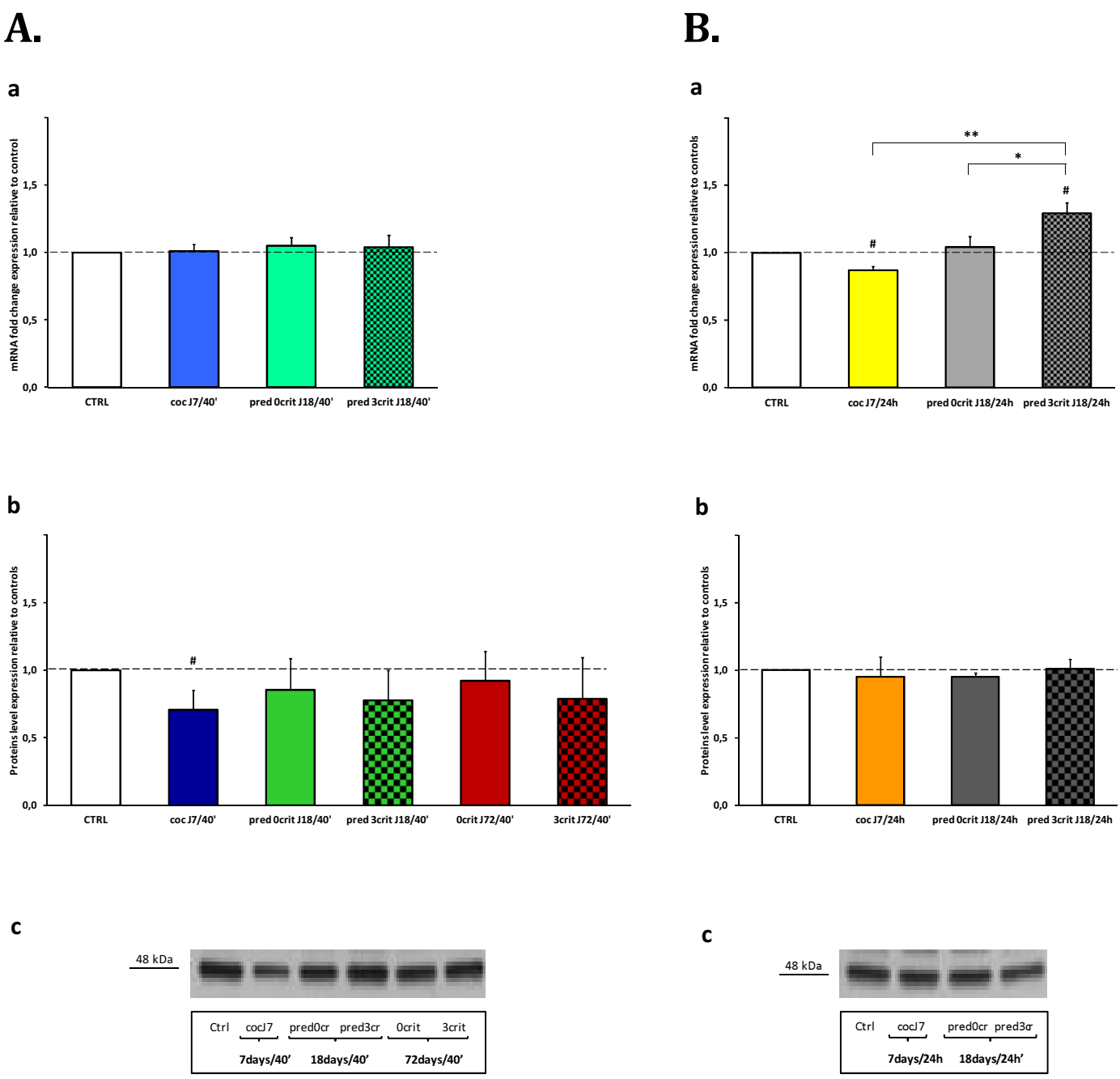

Figure 4.16 Effects of cocaine self-administration on Panx1 in VTA. **A:** Panx1 mRNA levels were no modified in regions analyzed after 40 minutes. The protein at 40 minutes showed a general tendency to a decrease but only cocJ7 group showed a significant decrease 0.71±0.14 P<0.05. **B:** 24 hours of abstinence caused a weak decrease in cocJ7 group of 0.87±0.03% P<0.05 and a modest increase in vulnerable group at 18 days 1.29±0.08%. The groups analyzed differed according to ANOVA F(2,25)=8.13 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 3crit and *P<0,05 for pred 0crit vs pred 3crit. Protein levels expression measured at 24 hours were no altered. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.3.5 Effect on Panx2 in ventral tegmental area

Even in this structure, the profile for Panx2 mRNA was exactly the same of that evidenced in nucleus accumbens and medial prefrontal cortex.

There was a huge increase at the first week of treatment 2.46±0.02% P<0.001 that went back to normal levels with 18 days of SA. The groups differed accordingly ANOVA F(2,13)=10,02 P<0,01; post hoc: **P<0,01 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit

24 hours of no-cocaine administration allowed to Panx2 mRNA to restore levels, indeed cocJ7/24h was increased of 1.42±0.06% P<0.05 while groups at 18 days had levels comparable to control group. Despite this strong increase in mRNA expression, the Panx2 protein was not subjected to the same regulation; indeed the analysis of the protein revealed no modifications at any time point analyzed. (**Figure 4.17**)

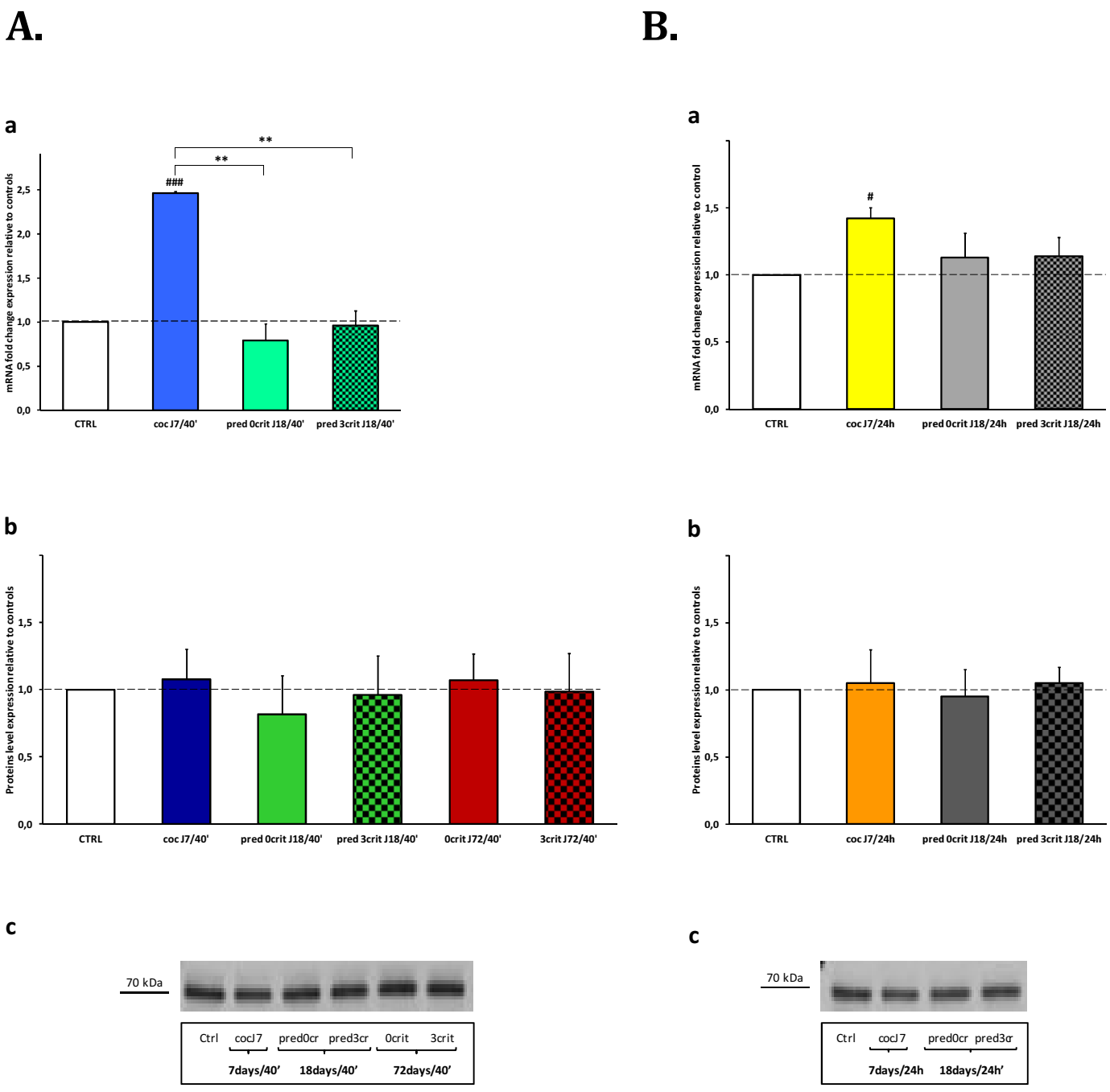

Figure 4.17 Effect of cocaine self-administration on Panx2 in VTA. A: mRNA expression of Panx2 after 7 days of SA showed a huge increase 2.46±0.02% P<0.001. At 18 days of SA the levels returned equal those of control groups. A group effect was revealed by ANOVA F(2,13)=10.02 P<0.01; post hoc: **P<0,01 for cocJ7 vs pred 0crit and cocJ7 vs pred 3crit. The protein expression analyzed in all groups at 40' was not modified. B: animals treated for 7 days and analyzed after 24 hours showed an increase of 1.42±0.06% P<0.05 while the other groups showed no differences. Also at 24 hours the Panx2 protein was not altered. **a:** Representative results for quantitative PCR. **b:** Representative results for WB analysis. **c:** Representative western blot of three independent experiment. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.3.6 Effects of cocaine SA on other genes in ventral tegmental area

In VTA, equally to the other two structures analyzed, with western-blot technique was not possible reveal the Cx26 protein.

Analysis with real-time PCR evidenced a decrease in mRNA expression of Cx26 in cocJ7 group analyzed at 40 minutes of 0.71±0.03 $P<0.05$, while at 18 days the levels were restored equal to controls mRNA levels. An effect group was present, indeed ANOVA gave $F(2,17)=9.25$ $P<0.01$; *post hoc*: **P<0,01 for coc J7 vs pred 0crit and cocJ7 vs pred 3crit. At 24 hours there were no relevant modifications but a slight increase in predicted 3crit gave a group effect , ANOVA $F(2,25)=4.43$ $P<0.05$; *post hoc*: *P<0,05 for cocJ7 vs pred 3crit.

Cx30 mRNA expression exhibited no differences in the groups at 40'. A weak increase was present in pred 3crit group at 24 hours 1.19±0.04 $P<0.05$ and analysis of variance between the three groups revealed an effect group $F(2,25)=6.15$ $P<0.01$; *post hoc*: **P<0,01 for cocJ7 vs pred 3crit and *P<0,05 for cocJ7 vs pred 3crit.

Also Cx45 gene showed no relevant alterations. A slight increase in both groups treated for 18 days and sacrificed at 40 minutes evidenced a group effect if compared to groups of animals treated for 7 days ANOVA $F(2,18)=4.46$ $P<0.05$; *post hoc*: *P<0,05 for coc J7 vs pred 0crit and cocJ7 vs pred 3crit. Instead at 24 h was found only a slight, but significant increase in pred 3crit group 1.19±0.07 $P<0.05$. (**Figure 4.18**)

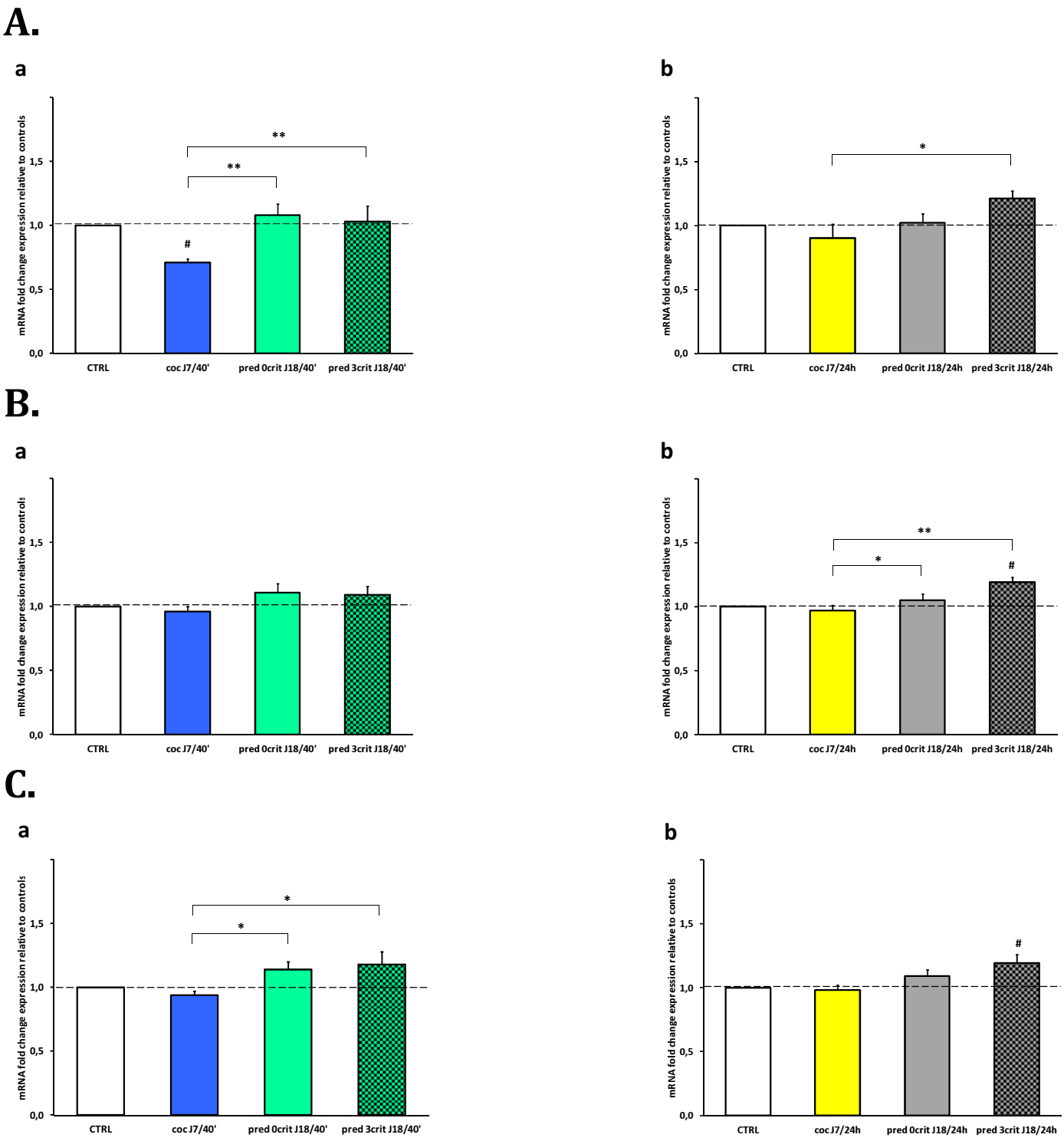

Figure 4.18 Effect of cocaine SA on other genes in VTA. **A:** Cx26 mRNA expression was diminished in cocJ7/40', 0.71±0.03 P<0.05 while at 18 days the levels were restored. An group effect was present, ANOVA F(2,17)=9.25 P<0.01; *post hoc*: **P<0,01 for coc J7 vs pred 0crit and cocJ7 vs pred 3crit. At 24h an increase in pred 3crit gave a group effect F(2,25)=4.43 P<0.05; *post hoc*: *P<0,05 for cocJ7 vs pred 3crit. **B:** Cx30 mRNA expression showed no differences at 40'. A weak increase of 1.19±0.04 P<0.05, was present in pred 3crit group at 24 hours. The groups differed accordingly analysis of variance, F(2,25)=6.15 P<0.01; *post hoc*: **P<0,01 for cocJ7 vs pred 3crit and *P<0,05 for cocJ7 vs pred 3crit. **C:** Cx45 gene showed no relevant alterations. A slight increase in the two groups treated for 18 days/40' evidenced a group effect (ANOVA F(2,18)=4.46 P<0.05; *post hoc*: *P<0,05 for coc J7 vs pred 0crit and cocJ7 vs pred 3crit). At 24 h was found only a slight increase in pred 3crit group 1.19±0.07 P<0.05. **a:** Representative results for quantitative PCR at 40'. **b:** Representative results for quantitative PCR at 24h. *Results are expressed as mRNA fold change expression and protein levels expression relative to control values and are shown as means ± SEM. Each group is composed by n=6-12 animals. In all graphs, statistical analysis was Student t-test (#) significant P<0.05 and one-way ANOVA with Newman-Keuls post hoc test (*) significant P<0.05.*

4.4 Summary of relevant modifications in NAc gap junctions

Nucleus accumbens is the regions mostly involved in the rewarding and reinforcing effects of cocaine following the activation of dopaminergic system in VTA. After acute cocaine administration there is a huge increase of DA in NAc shell but blocking the administration, the DA levels return to basal levels. With a prolonged exposure to cocaine, there is instead depletion of DA and glutamate. In NAc has been demonstrated the presence of gap junctions and their regulation by dopamine, depending by DA receptorial types and area interested. We analyzed the whole NAc and we observed a decreased expression of the principal connexins and pannexins, indeed Cx43, Cx32 and Panx2 at 7 and 18 days of cocaine SA showed low protein expression levels respect to the animal controls. But with a prolonged exposure the differences in expression of connexins and pannexins, between addict and non-addict animals became more pronounced. Indeed, we observed for Cx36 that just to 3 weeks of SA did not affect the protein expression, but with 72 days of SA there were increased levels only in 3 criteria animals while non-addicted animals had normal levels of this protein.

Cx32 and Panx2 exhibited the same profile; after a general decrease in the expression at 18 days, in which both groups vulnerable and resistant to addiction rats, exhibited the same diminution in the protein levels, 72 days of cocaine exposition allowed to 0 criteria animals restored normal protein levels while in addicted rats region there were raised levels. The Cx43 showed instead low levels in addicted animals, in accord to those observed at 18 days of SA but interestingly also in this case was possible to observe a difference with non addict animals that restored protein levels comparable to controls.

At 24 hours of withdrawal, instead we observed a tendency to restore normal protein levels to respect the changes observed at 40 minutes. An exception was present in Cx36 protein expression, in which we observed an increase, although weak, in both predicted 0 and 3 criteria at 18 days of SA. Low levels were already stable in vulnerable animals for Cx43 at 18 days. In contrast, for Panx2 we found increased protein levels only in 0criteria at 18 days/24 hours. (**Table 4.1**)

		Effects of cocaine SA on NAc GJs at 40'				
		7 Days SA	18 Days SA		72 Days SA	
		Coc J7/40'	pred 0crit 18J/40'	pred 3crit 18J/40'	0cri 72J/40'	3crit 72J/40'
Cx36	RNA					
	Prot					↑ 1.32±0.14% P<0,05
Cx43	RNA					
	Prot	↓ 0.70±0.22% P<0,05	↓ 0.57±0.10% P<0,001	↓ 0.58±0.11% P<0,01		↓ 0.64±0.06% P<0.05
Cx32	RNA		↓ 0.87±0.02% P<0,05	↓ 0.80±0.03% P<0,01		
	Prot		↓ 0.47±0.24% P<0.001	↓ 0.60±0.19% P<0.01		↑ 1.51±0.37% P<0.01
Panx1	RNA	↑ 1.15±0.04% P<0.05				
	Prot					0.75±0.07% P<0.05
Panx2	RNA	↑ 2.10±0.09% P<0.001				
	Prot	↓ 0.71±0.12% P<0.05	↓ 0.55±0.10 P<0.01	↓ 0.57±0.17% P<0.05		↑ 1.33±0.19% P<0.05

		Effects of cocaine SA on NAc GJs at 24h		
		7 Days SA	18 Days SA	
		Coc J7/24h	pred 0crit 18J/24h	pred 3crit 18J/24h
Cx36	RNA			
	Prot		↑ 1.18±0.05%, P<0.05	↑ 1.25±0.18%, P<0.05
Cx43	RNA			
	Prot			↓ 0.81±0.14%, P<0.05
Cx32	RNA		↑ 1.17±0.08% P<0.05	
	Prot			
Panx1	RNA			
	Prot			
Panx2	RNA	↑ 1.48±0.02% P<0.05		
	Prot			

Table 4.1 Summary of relevant modifications in NAc gap junctions. The table on the top summarizes the changes revealed in samples at 40 minutes. Table in bottom summarizes the alterations in samples collected after 24 hours by the last cocaine injection. Summary of relevant modifications in medial prefrontal cortex gap junctions

4.5 Summary of relevant modifications in mPFC gap junctions

Medial prefrontal cortex is the region involved in higher-order cognition and emotion. In addiction, it has implicated with salience attribution and inhibitory control, and also in compulsive behaviours, as craving and compulsive drug intake. It receives DAergic stimulation from VTA and sends glutamatergic projections to NAc.

We observed in this area, a different trend than that observed in NAc. Here, Cx36 was completely not influenced; prolonged exposure or withdrawal had no effects on protein levels and mRNA expression.

Cx32 at 7 days and 40 minutes showed a relevant increase in mRNA expression, but this was not followed by the same increase in protein. We observed similarly no changed levels at any time point analyzed, with only exception of vulnerable animals at 18 days and 24 hours of abstinence, in which there was a decrease of about 30% in the protein expression levels.

Cx43 was subjected to a diminution in protein expression at 18 days at 40' of about 50% only in predicted 3 criteria animal while at 24 hours there were still low levels but the diminution was more slight (-20%) and it was observed in both two groups. A prolonged exposure instead, differently from what happened in NAc, caused in the addicted animals at 40' an increase while the non-addicted maintained levels equal to controls.

The two pannexins showed instead a same profile; at 40 minutes no relevant modifications were observed at 7 and 18 days of SA in proteins expression, but after chronic cocaine exposition, both 0 and 3 criteria groups showed low protein levels. Only for Panx2, the diminution in protein expression was also present in vulnerable animals at 18 days and 24 hours of withdrawal.

Even in this region we observed the same trend as regard the mRNA expression of Panx2, a huge increase at 7 days, in animals analyzed with drug on board that was relatively maintained also at 24 hours, but nothing variations was observed in the others time point. (**Table 4.2**)

		Effects of cocaine SA on mPFC GJs at 40'				
		7 Days SA	18 Days SA		72 Days SA	
		Coc J7/40'	pred 0crit 18J/40'	pred 3crit 18J/40'	0cri 72J/40'	3crit 72J/40'
Cx36	RNA					
	Prot					
Cx43	RNA					
	Prot			↓ 0.54±0.31% P<0.05		↑ 1.40±0.34% P<0.01
Cx32	RNA	↑ 1.68±0.19% P<0.05				
	Prot					
Panx1	RNA					
	Prot		↑ 1.36±0.33 P<0.05		↓ 0.74±0.25% P<0.05	↓ 0.70±0.24% P<0.05
Panx2	RNA	↑ 2.09±0.07% P<0.001				
	Prot				↓ 0.84±0.13% P<0.05	↓ 0.65±0.27 P<0.01

		Effects of cocaine SA on mPFC GJs at 24h		
		7 Days SA	18 Days SA	
		Coc J7/24h	pred 0crit 18J/24h	pred 3crit 18J/24h
Cx36	RNA			
	Prot			
Cx43	RNA			
	Prot		↓ 0.80±0.22% P<0.05	↓ 0.80±0.17% P<0.05
Cx32	RNA		↓ 0.65±0.10% P<0.05	
	Prot			↓ 0.72±0.30% P<0.05
Panx1	RNA	↓ 0.86±0.02% P<0.05		
	Prot			
Panx2	RNA	↑ 1.64±0.06% P<0.01		
	Prot			↓ 0.72±0.21 P<0.05

Table 4.2 Summary of relevant modifications in mPFC gap junctions. The table on the top summarizes the changes revealed in samples at 40 minutes. Table in bottom summarizes the alterations in samples collected after 24 hours by the last cocaine injection.

4.6 Summary of relevant modifications in VTA gap junctions

Ventral tegmental area is a part of midbrain constituted by 60% of DAergic neurons; inhibitory GABAergic and excitatory Glutamatergic neurons regulate these. This is the first region to be affected by cocaine; in VTA cocaine causes increase of dopamine and the consequent increased Daergic transmission.

In this area, we observed the smallest effects in connexins and pannexins after cocaine self-administration.

For first however, Panx2 mRNA showed identical profile to that observed in accumbens and prefrontal cortex. As in NAc, the first week of cocaine treatment caused a decrease in the proteins expression of Cx43, Cx32 and Panx1.

The more evident changes were observed in Cx43 and Cx32 protein, analyzed after 40 minutes by last cocaine injection. Cx32 was slightly diminished at the first week of treatment but intriguingly a relevant increase was evident in predicted 3 criteria at 18 days of SA, but nothing else variations was seen with prolonged exposure in all groups analyzed. The Cx43 showed a general decrease in protein expression at any time point analyzed and moreover was not possible discriminate between addicted and non-addicted animals at 72 days because was present the same level of reduction in protein expression. Others changes were revealed as weak increase in Cx36 only in predicted 3 criteria at 18 days/40' or increase in Panx2 in the same group but analyzed at 24 hours. (**Table 4.3**)

		Effects of cocaine SA on VTA GJs at 40'				
		7 Days SA	18 Days SA		72 Days SA	
		Coc J7/40'	pred 0crit 18J/40'	pred 3crit 18J/40'	0cri 72J/40'	3crit 72J/40'
Cx36	RNA					
	Prot			↑ 1.27±0.19% P<0.05		
Cx43	RNA	↓ 0.84±0.02% P<0.01				
	Prot	↓ 0.72±0.11% P<0.05			↓ 0.64±0.10% P<0.05	↓ 0.63±0.13% P<0.05
Cx32	RNA					
	Prot	↓ 0.74±0.12 P<0.05		↑ 1.63±0.37% P<0.05		
Panx1	RNA					
	Prot	↓ 0.71±0.14 P<0.05				
Panx2	RNA	↑ 2.46±0.02% P<0.001				
	Prot					

		Effects of cocaine SA on VTA GJs at 24h		
		7 Days SA	18 Days SA	
		Coc J7/24h	pred 0crit 18J/24h	pred 3crit 18J/24h
Cx36	RNA			
	Prot			
Cx43	RNA			
	Prot			
Cx32	RNA			
	Prot			
Panx1	RNA	↓ 0.87±0.03% P<0.05		↑ 1.29±0.08% P<0.05
	Prot			
Panx2	RNA	↑ 1.42±0.06% P<0.05		
	Prot			

Table 4.3 Summary of relevant modifications in VTA gap junctions. The table on the top summarizes the changes revealed in samples at 40 minutes. Table in bottom summarizes the alterations in samples collected after 24 hours by the last cocaine injection.

5 Discussion

Cocaine abuse causes alterations in brain cells and in synaptic transmission. Many modifications have been found under acute use of cocaine and many more after prolonged exposure. Interestingly, cocaine use can give dependency only in a small percentage of individuals. Many theories have been postulated to explain how, the progressive use of cocaine would lead to permanent changes in brain and in particular in mesocorticolimbic system [31-34].

Modifications in transcriptional factors expression, changes in chromatin and DNA structure, induction of non-coding sRNA, but also down- or up-regulation of several receptors, alterations in intracellular pathways, are all effects observed in CNS [43, 44]. Moreover have been demonstrated alterations in synaptic plasticity in addicted individuals, suggesting a different susceptibility in individuals. This is in contrast to traditional point of view that hypothesize a principal role of cocaine to cause neurobiological and behavioural changes rendering an individual addicted [97, 110].

The presence of intercellular gap junctions has been extensively demonstrated in nucleus accumbens, prefrontal cortex and ventral tegmental area. GJs can be modulated by several neurotransmitters as dopamine or glutamate, indeed has been showed changes in the coupling of the cells depending on administration of specific agonists or antagonists [189, 233-244]. Few works demonstrated an alteration in gap junctional communication after cocaine self-administration. In particular Bennett [257] and McCracken [258], showed modifications in Cx36 and Cx32 mRNA and proteins expression after several days of withdrawal. However, in these works, the animals were subjected only to 10 or 14 days of SA but with a longer availability of the drug during the day and with an infusion of cocaine of 1.5 mg/Kg weight (double respect to that used in our animal model).

For this work has been used an experimental animal model subjected to daily SA session composed of three drug components (40 min each) separated by two no drug periods of 15 min. The self-infusion volume was 40 µL (2 sec infusion) and contained 0.8 mg/kg of cocaine. Moreover, this model allows to discriminate addiction-like behaviour in rats, similar to those observed in human. Indeed with a prolonged exposure (72 days), was

possible to distinguish, depending of some parameters as difficulty in stopping drug intake, high motivation and the continued use of cocaine despite negative consequences, animals showed an addiction-like behaviour (termed also 3 criteria positive) from animals maintaining a controlled use of the substance (termed 0 criteria positive). This distinction could be also made after 18 days, even if at this time point we speak of predictive 0 criteria (or resistant to addiction) and predictive 3 criteria (or vulnerable to addiction), because still there is only an indication of the future state progression.

So with the work we wanted analyze the expression of connexins and pannexins for their potential involvement in the effects of cocaine in mesocorticolimbic system and moreover to analyze the probable role in the transition to addiction.

With this analysis we wanted discriminate between:

- the effects observed in the short and long term of cocaine SA (7, 18 and 72 days);
- the effects on expression between animals with drug on board and with one day of abstinence (after 40 minutes or 24 hours by the last cocaine injection)
- the effects on addicted and non-addicted animals.

5.1 General considerations

An evidence of considerable interest is the different regulation of mRNA and protein expression of GJs observed in these experiments. In fact, mRNA expression is not subjected to changes, with some exceptions as Panx2, Cx26, Cx32, while proteins expression is more modified in the experiments analyzed.

This indicates us three things:

i. Cocaine does not induce changes in genes expression of Cx36, Cx43 and Panx1 in the structures analyzed. Neither doses in acute nor in chronic, nor abstinence cause changes in the mRNA of these. Cx32 and Cx26 show some modifications in some groups of individuals but without a clear relationship between exposure times. Panx2 mRNA instead is modified by early cocaine use, showing a huge increase with 7 days of SA and normal levels with prolonged exposition to cocaine.

ii. The proteins expression is subjected to more modifications. This is an indication that expression of connexins and pannexins is tightly regulated by the cellular and extracellular environment. Many works show as these proteins are regulated by a plethora of substances. So at any time connexins and pannexins proteins are subjected to modifications by the different events occurring in brain cells as a result of alterations cocaine-induced, rather than be regulated by the cell at transcriptional level.

iii. The regulation of GJs or HCs is made at the translational level or post-translational rather than at the transcriptional level. Indeed, multiple works show changes in proteins without changes in mRNA or vice versa, sometimes with a trend completely opposite between the two levels [246, 258-262].

GJs biosynthesis and assembly are tightly regulated, indeed these have a half-life of only few minutes or hours. Moreover the regulation can occur at different levels; they are continously assembly and degraded and the post-transdutional modifications have a strong role in these processes. This rapid process is probably fundamental for a quick adaptation of these cells to mutated physiological or environmental conditions.

5.2 Neuronal synchronization

Cx36 is the principal connexins found in neurons. Gap junctions formed by Cx36 have been found extensively in almost whole brain and the population that mostly express this connexin are GABAergic interneurons, even if there are evidences regard its presence in DAergic neurons [189-192]. The function of Cx36 is considered fundamental for the brain, because this mediates the establishment of oscillatory networks between neurons; indeed genetic ablation of Cx36 gene, causes reduced synchronization between interneurons, impaired cerebellar motor learning, reduced γ- and high frequency oscillation in hippocampus and impairment of learning and memory. In particular, the electrical interconnection between GABAergic interneurons is a fundamental feature, because it facilitates the synchronization and then the activity of inhibitory circuits [218, 222, 224-226].

Also normal synaptic plasticity is impaired in absence of Cx36, indeed in hippocampus Cx36 KO mice show an impairment of learning and memory, probably due to reduction of LTP; an effect observed also in visual cortex [263, 264].
Another important role ascribed to Cx36, is the involvement in the seizures in epylepsy. Indeed increased levels of Cx36, but also of Cx43 and Cx32 mRNAs are found in seizures model and the administration of GJs blockers reduces the epyleptiform activity [234].

In NAc we found no modifications in Cx36 mRNA, but after prolonged exposure to cocaine, there was a weak increase in protein expression in both groups at 18 days of SA and 24 hours of abstinence, while after 72 days of SA only the addict animals showed an increase of 32% in the protein expression.
For contrast in the others two structures, mPFC and VTA, the Cx36 did not show any modification, neither at mRNA level nor at protein level. This indicates that in these structures the neuronal communication via Cx36 GJs is not modified, even though there might be modifications at post-translational level regulating the conductance or permeability.
The increase of Cx36 protein expression found in NAc of addicted animals may be instead the result of an increased neuronal synchronization. It has been postulated that neurons in NAc, may function as distinct set of neurons, termed ensemble coding, having each a specific function. Indeed is showed as drugs of abuse stimulate specific ensemble coding, but different from those activated by natural stimuli, even if the different populations have not been identified [250-253]. Moreover, GJs blocker carbenoxolone reduces neuronal synchronization and then the stereotyped behaviours in a positive reinforcement model [256].
The increase found at 18 days and 24 hours but not at 40 minutes, may indicate that an increased synchronization in cells population in NAc could be established as an effect of withdrawal. It has been found that Cx36 GJs coupling is increased by activation of group II mGluRs, following increase of glutamate induced by brain injury [246]. In fact, after prolonged cocaine exposition, withdrawal leads to a reduced glutamate levels; however in animals showing a cocaine-induced reinstatement drug-seeking behaviour, occurs instead an elevation in NAc glutamate levels, due to glutamatergic stimulation of NAc by PFC [77-79]. So, after 18 days of SA, could be present high glutamate levels in both vulnerable and resistant to addiction animals, causing then the increase of Cx36 protein

observed. But proceeding with drug exposure, the modifications in glutamatergic transmission and the differences between addict and non-addict rats would become more strong, and the increase of glutamate would occur only in 3 criteria rats, i.e. those showing a cocaine-induced reinstatement drug-seeking behaviour. In this way the increase of Cx36 protein, become a permanent alteration only in addicted animals. It is also possible that this increase in Cx36 expression reinforces specifically the synchronization in the ensemble coding related with rewarding and reinforcing effects of cocaine.

Cx45 is the other connexin localized at neuronal levels, even though this has a main role during brain development while in adult brain seems have a marginal function. Similarly to Cx36 mRNA expression also the mRNA expression of Cx45 is not modified by cocaine in the three structure analyzed.

5.3 Cocaine and inflammation

Inflammation and oxidative stress occur during cocaine use. Several studies show increased expression of genes, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-8 (IL-8), nuclear factor-κ B (NF-κB) and interferon-regulating genes, consistent with activation of inflammatory and immune responses [265]. The inflammatory response also causes increase of oxidative stress and may trigger neurotoxic condition [266].

Microglia is the first system to be activated after brain injury caused by several acute insults, as hypoxia, glutamate, LPS and cytokines [208, 267]. Activated microglia, releases large amounts of glutamate, that causes neurotoxicity by NMDA receptor signaling, and pro-inflammatory cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, which also promote neuronal damage [207].

In activated microglia the passage of neurotoxic compounds, as glutamate, occurs throughout HCs or GJs. Indeed, blocking GJs with carbenoxolone (CBX), cause reduction of neurotoxic effects, decreasing of inflammatory response and limited neurodegeneration. TNF-α released by microglia has a double role; it induces neurodegeneration through the silencing of cell survival signals and caspase dependent

cascades, even if direct neurotoxicity seems counterbalanced by the activation of neuroprotective factors, including expression of MAPK and NF-κB. Nevertheless, TNF-α also stimulates extensively microglial glutamate release, in an autocrine manner by up-regulating glutaminase, to cause excitoneurotoxicity. Glutamate originating from microglia, is released principally through the Cx32 GJs [207].

Beyond the direct effect of microglial cells on neurons with the release of neurotoxic substances, they can also act indirectly, promoting astroglial activation.

Astrocytes can be activated by various inflammatory mediators, but are particularly sensitive to pro-inflammatory cytokines released by microglia. Astrocytes are highly coupled via GJs, forming in this way a wide network able to cooperate and to exert effects on neurons. One of their main roles is transfer glutamine via Cx43 GJs to neurons (that use this to synthesize glutamate and GABA). Beyond this, astrocytes release several gliotransmitters as glutamate, GABA, ATP, adenosine, D-Serine, and there are demonstrations that many pass through GJs. Another important role for astrocytes is the propagation of calcium waves; indeed they can propagate calcium signals through transfer of IP3 or ATP via Cx43 GJs. Several studies showed also as, during inflammation or other brain injuries, the astrocytes enhance the communication via HCs and reduced that via GJs. It seems that in this way they may exert an opposite action, passing from neurotrophic actions with GJs to a neurotoxic action, because through HCs would be mediate the passage of signals death [208, 267].

Cx43 is the main functional connexin present in astrocytes. In these cells are present also Cx26 and Cx30 but it has been reported that these are expressed at very low levels and that mice deficient for Cx43 are unable to form gap junctions. So the contribute of Cx26 and Cx30 is minimum [197-199].

Cx32 instead is one of the most expressed connexin in oligodendrocytes and in interneurons, even though at lower levels than Cx36. Cx32 together with Cx43, Cx30 and Cx26, form also the extensive communication between oligodendrocytes and astrocytes to form panglial syncitium [184, 186, 202-204].

Cx32 and Cx43 are expressed in microglial cells. After activation induced by inflammation, these cells show increased levels of Cx32 and Cx43 [205-207].

In our work found an initial decrease in Cx43 protein expression at 7 or 18 days of SA in NAc. But, prolonged exposure had different effects; indeed non-addict animals restored normal levels while the addict ones maintained low levels of Cx43 protein. In addition,

24 hours of withdrawal caused at 18 days of SA, a diminution of Cx43 protein levels in predicted addict animals.

Some works show as TNF-α and IL-1β reduce the total levels of Cx43 in glial cells by increasing internalization and degradation. The same cytokines induce activation of p38 MAPK in astrocytes, which in turn can induce the expression of NOS and then increase NO production. Also this oxidant compound seems participate in the reduction of Cx43 protein, even if the mechanism is unknown [268, 269] .

The down-regulation of Cx43 protein, observed both in NAc and VTA could be due therefore to the inflammatory environment rich in cytokines. However, while in VTA the effect of reduction seems due to a cocaine effect, because was found in both groups of animals, in NAc the effect seem specific for addiction. It could be interesting to know why non-addict animals showed normal Cx43 levels in NAc. In addition, changes in dopamine levels may have consequences on decreasing of Cx43, indeed the action of agonists and antagonists for DA receptors have effects on dye coupling in NAc, although it depends by the interested NAc area and receptorial type [233-240].

In mPFC**,** Cx43 protein exhibited an opposite trend after prolonged cocaine exposure respect to NAc. The non-addict maintained equally normal protein expression level while addict showed an increase. During withdrawal at 18 days instead, there were diminished levels in both groups analyzed. The increased Cx43 levels may be due to an effect mediated by CNTF. Indeed after brain injury the astrocytes release ciliary neurotrophic factor (CNTF, a member of Il-6 family) that is a potent survival factor for neurons, oligodendrocytes, microglia and may be relevant in reducing tissue destruction during inflammatory attacks. It has been showed that CNTF infusion within its soluble receptors CNTFRα cause an increase of Cx43 protein of 70%, probably mediated by JAK/STAT pathway [270]. In addition cocaine exposure, causes activation of JAK/STAT pathway, rendering the system more responsiveness to action of CNTF [271].

Furthermore, three putative CNTF-response elements have been found in the promoter region of Cx43. Therefore, it would be to verify if between addict and non-addict animals there are different levels of CNTF or however differences in the activation of JAK/STAT pathway.

The Cx32 showed in NAc an initial decrease in both mRNA and protein expression, but after 72 days of SA there was an increase in protein expression of 50% only in addict animals, while non-addict rats showed normal protein levels. This increase may be due

to activation of microglia and release of TNF-α. It has been demonstrated that glutamate is released from activated microglia through Cx32 gap junctions, even more significantly than by glutamate transporters such as excitatory amino acids transporter (EAAT). As the same time, TNF-α released from microglia, enhances the surface expression of Cx32 hemichannels [207]. Then, the increase of Cx32 may be the consequence of the persistent inflammatory state cocaine-induced and it may enhance the release of neurotoxic compounds from microglia. Why this increase occurs only in addict is at the moment unknown, but maybe is caused by different cellular and extracellular conditions in addict and non-addict rats.

An identical situation to that observed in our work, is showed by studies on global ischemia. Indeed, it has been found an up-regulation of Cx36 and Cx32 proteins in hippocampal GABAergic inhibitory interneurons after ischemia and these changes occur in the absence of detectable changes in the corresponding mRNAs. Furthermore, Cx32-null mice exhibit enhanced vulnerability to the effects of ischemia.

These evidences are consistent with a role for Cx32 GJs in neuroprotection of hippocampal interneurons against ischemia-induced cell death. Indeed, while astrocytic GJs seem to be important for the propagation and amplification of injury, the GJs between interneurons may promote inhibitory transmission and thereby may afford protection against neuronal injury after global ischemia [261].

Therefore, it would be interesting observe in NAc where this Cx32 increasing occur, to establish if it has neuroprotective or neurotoxic function. However, this is a specific effect for rats showing an addiction-like behaviour.

In mPFC, 7 days of cocaine SA caused an increase of ~ 70% in Cx32 mRNA expression in rats sacrificed after 40 minutes, even if this was not followed by modifications in protein expression at any time point analyzed. Only at 18 days of SA and 24 hours of withdrawal there was a decrease in Cx32 expression in predicted 3 criteria animals. Also this decrease may be explained as a consequence of inflammation. Indeed studies on endothelial cells culture, treated with pro-inflammatory substances as TNF-α, show as a decrease of Cx32 protein is related with an aggravation of inflammation and raising the levels of pro-inflammatory cytokines, while the over-expression of Cx32 exerts the opposite effect [272].

Then, it seems that TNF-α can exert an opposite effect on Cx32, maybe for the involvement of other proteins or pathways, differently activated by this factor in the different regions and also in the different cell types.
Numerous evidences show also an increase of Cx36 protein expression following neuronal injury as ischemia, trauma, inflammation. In these conditions Cx36 GJs would be responsible of neuronal death, indeed their presence amplify the NMDAR-mediated excitotoxicity. As the same time, the activation of mGlu2/3 receptors, caused by release injury-mediated of glutamate, has been found induce synthesis of new neuronal GJs that spread the neuronal death [246].

5.4 Synaptic plasticity

In physiological conditions, astroglial network communication is crucial for precise synaptic information processing and in fact, a potential role is to contribute to synaptic plasticity. Astrocytes become reactive during pathological states and exposure to drugs of abuse such as cocaine lead to reactive astrocytosis and altered glial fibrillary (GFAP) expression. Dysfunction of astroglial networks over time instead, should result in an alteration of normal processes regulating synaptic functions.
Astrocytes act as recycler for many NTs (glutamate with enzyme glutamine synthetase); they exhibit biochemical excitability that is manifest as Ca^{2+} oscillations able to propagate from an astrocyte to another. The elevation of Ca^{2+} is also necessary for the release of chemical transmitters, occurring between astrocytes, neurons and microglia; indeed various substances cross through astrocytic gap junction channels, as neurotransmitters and ions, important to regulate basal synaptic activity [273].
Astrocytes, beyond glutamate, release D-serine. This acts as NMDAR co-agonist and in several cerebral regions D-Ser is the endogenous ligand for the glycine-binding site of the NMDA receptors. In this way in some regions the availability of astrocytic D-Ser is critical for controlling NMDA receptors activity and as a consequence determines whether stimuli that induce synaptic plasticity lead to long-term potentiation or long-term depression [274].

Given the strong involvement of astrocytes in regulate some brain function, this highlight the possibility that they can mediate or modulate the plastic events occurring in response to drugs of abuse.

Kasanetz et al. [109] have showed a suppression of LTD NMDAR-mediated in NAc after 18 days of SA in both vulnerable and resistant animals, while after a prolonged exposure to cocaine, the suppression of LTD occurred only in addict animals. Non-addict animals instead in the same condition restore the ability of LTD induction.

The analysis of Cx43 protein expression in NAc follows the same profile of LTD regulation observed by Kasanetz et al. in NAc. Then, if the expression of Cx43 is low, also the gliotransmission is impaired because GJs formed by Cx43 are main channels used for the release of gliotransmitters. In this way, the glutamate and D-Serine levels decrease and probably this is the cause of failure of activation NMDA receptors, suppressing the ability of synapses to induce LTD. Indeed, Cx43 was decreased at 18 days in all cocaine-treated individuals, such as suppression of LTD. But, after 72 days of SA, interestingly the non-addict individuals restored both normal levels of Cx43 protein and LTD induction while addict-like rats showed still diminution of Cx43 expression and suppressed LTD. It has been postulate that only addict individuals exhibit this form of anaplasticity, i.e. the inability of brain to adapt and change in response to synaptic alterations. Maybe this is due to permanent modifications in the normal astrocytic pathway, causing alterations in release of gliotransmitters through GJs.

5.5 Pannexins

A most interesting aspect in this work was the profile of pannexins genes and proteins expression. Panx2 mRNA expression in all the three structures analyzed was subjected to the same regulation, indeed we observed a huge increase at the first week of treatment, of about 110% in NAc and mPFC and of 146% in VTA in samples analyzed with drug on board. Animals underwent to the same time of SA, but analyzed after 24 hours by the last cocaine injection still showed high levels of Panx2 mRNA 48-62% more than levels found in controls. With a prolonged exposure to cocaine the levels of mRNA

Panx2 returned to normal levels both at 18 or 72 days of SA and indifferently in rats analyzed with drug on board or with 24 hours of abstinence.

The protein expression instead did not exhibit the same profile but there were different regulation depending by the structure and by the time of exposition to cocaine. Interestingly, it was possible to observe a general diminution of protein expression in NAc and in mPFC but while in mPFC low levels were present at 72 days indiscriminately in addicted and non-addicted animals, on the NAc was present an increase of Panx2 protein only in addicted animals.

Panx1 instead, seemed less influenced by the cocaine action. In NAc, nothing effect was present in mRNA expression and the protein expression was slightly decreased only in addicted animals at 72 days /40 minutes. In medial PFC this diminution in protein levels was exhibited in both 0 and 3 criteria rats as yet observed for Panx2. In VTA, a decrease was present at 7 days and 40' and an increase in predicted 3 criteria animals at 18 days and one day of withdrawal.

Little is known about these proteins. Surely, many evidences demonstrate that they act as HCs, and about the possibility to form GJs there are contrasting results. In addition, the right localization is controversy: Panx1 has been found exclusively in neurons but other works describe it in glia. Also Panx2 seems present in neurons even if the transcript is also identified in glial cells and the proteins appear in hippocampal astrocytes after ischemia/reperfusion [215].

Pannexin1 has been implicated in propagation of calcium waves; they in fact release ATP that, by binding with purinergic receptor (in particular P2X7), stimulates IP_3 and then increases Ca^{2+}. This increase causes release of other ATP, which can continue the cycle [167].

Several studies show a huge increase in Panx1 mRNA expression following treatment with IFN, LPS and TNF-α. It has been demonstrated that in macrophages and astrocytes cultures, Panx1 linked to P2X7 has a role in the inflammatory state. Indeed P2X7, activated by ATP, causes opening of large pore (pannexons) throughout may pass ATP and calcium. The interaction between Panx1 and P2X7 seems moreover essential for activation of caspase-1, (incorporated into large protein complexes termed inflammasomes), and then for processing pro-IL-1β. Indeed knock-down of pannexin-1 attenuates the release of IL-1 β from stimulated macrophages [169, 174, 275, 276]. It has been proposed also a link between astrocytic GJs channel and pannexons in neurons.

Initially, under brain insults, the activation of microglia causes release of pro-inflammatory cytokines (TNF-α/IL-1β), which increase astroglial hemichannel activity. Then, astrocytes release glutamate and ATP via Cx43 hemichannels, which activate opening of Panx1 hemichannels in neurons, acting respectively on NMDAR and purinergic receptors. ATP released, as a result of Panx1 hemichannel opening, could contribute in the progression and activation of intracellular neurotoxic cascades, for their contribute to intracellular $Ca2^{+}$ overload [267].

Now it's possible that Panx2, less known than Panx1, can absolve the same role of the homologue, and this may explain the huge increase of Panx2 mRNA expression observed in all the structures after 1 week of SA, such as observed with increase of Panx1 mRNA following pro-inflammatory stimuli. It is strange as in these structures the Panx1 gene is not affected, but maybe in different sites the functions and the regulations of the two pannexins are interchangeable. But this increase in the mRNA expression is differently regulated by the cell, indeed we didn't find the same regulation at protein levels.

In NAc we observed that the increase of Panx2 protein level occurs only after prolonged exposure and only in addict-animals; while at the first weeks of treatment there was a general decrease in the expression followed by a restore of normal level in non-addict.

Then, we observed an increase on NAc of addict animals in three proteins, Cx36 normally present in neurons, Cx32 also found in interneurons and Panx2 expressed in neurons; So, it's possible that this increase in the communication, is due to an increased cellular synchronization, mediating the rewarding-linked effects of cocaine.

In mPFC, there was a decrease in the protein expression of Panx1 and Panx2 in both groups after prolonged cocaine exposure.

Astrocytes, activated by microglial cytokines, are stimulated to release several molecules, which modulate neuronal excitability and synaptic strenght. They have a role in LTP that depend by the secretion of D-serine, which in turns bind to glycine-site in NMDAR and carry out their role in LTP. Astrocytes also release TNF-α, known as enhancer of synaptic strenght because it increases the surface expression of AMPAR.

It has been found in hippocampus that a critical factor for the LTP HFS-induced seems to depend by activation of microglial P2X7 receptors with ATP released by astrocytes, stimulating IL-1 production. This microglial IL-1, together with TNF-α can facilitate NMDAR activation and increasing AMPAR expression in neurons [277].

This occurs in physiological conditions, but an increase of IL-1, IL-6 and TNF-α can instead impair the induction and maintenance of LTP, maybe due to an increase in SOD activity and ROS levels, along the activation of the MAP kinase cascade, stress-related MAPK c-jun JNK and p38 MAPK and activation of NF-kB [277].

In contrast it is found that in hippocampus the p38 MAPK pathway mediates the LTD mGluR-dependent, indeed inhibition of p38 MAPK decreases LTD and have no consequences in hippocampal LTP [278].

Blocking of P2X7 receptors has been shown prevent the release of IL-1β and TNF-α, but the activation of P2X7 receptors by ATP, is linked with the increase of p38 MAPK phosphorylation [279].

We found a decrease in both pannexins after prolonged exposure, even if Panx1 expression was decreased at the same level in addict and non-addict, while Panx2 showed lower levels in addict than in non-addict. If pannexons and P2X7 are in relationship to mediate processing of IL-1β, likely a decrease in pannexins expression is accompanied by a decrease of purinergic receptors. This phenomenon prevents the release of IL-1β and TNF-α, then inhibits the activation of p38MAPK necessary for LTD mGluR-mediated, that is showed to be suppressed in addict animals [110]. This mechanism may contribute to the loss of plasticity observed in addicted.

5.6 Conclusions

Our work shows the modifications occurring in connexins and pannexins with cocaine SA. Several modifications occur at early cocaine use and many more after prolonged exposure. Moreover some proteins are differently regulated depending by addiction-like behaviour in animals.

- NAc and mPFC are the regions in which the connexins and pannexins are mostly modified at early and prolonged cocaine exposure, while in VTA there are fewer alterations, principally observed during early cocaine exposition. These modifications would be linked with the main role of NAc and mPFC in the aspects cocaine-related regarding the reward and the phenomenon of learning.
- The withdrawal restores the alterations in the proteins to normal levels, occurring at 7 and 18 days of SA.
- The protein most influenced is Cx43, the main astrocytic connexin, with modifications occurring in all the analyzed structures. This leads to suppose a strong involvement of astrocytes in the mesocorticolimbic system after cocaine use.
- The increased expression of neuronal Cx36 in NAc, but also of Cx32, presumably present in interneurons, and Panx2, could be linked to an increased neuronal synchronization.
- The effects are different depending on exposure time and the specific protein type. Remarkable points are the differences observed in these proteins between addict and non-addict animals, supporting a potential role in the transition to addiction.

Hence, it would be interesting clarify the exact role of these proteins in the cocaine effects, i.e. if the modifications occurring in GJs proteins actively participate to cause alterations in brain with modifications at molecular or at synaptic level leading to addiction.

Further investigation with KO models will be essential for better understanding the role played by these proteins and could give us indications about their use as target/marker of addiction pathology.

6 Abbreviations

0crit	0 criteria
3crit	3 criteria
5-HT	5-Hydroxytryptamine
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)
AP-1	activator protein-1
Arc	activity-regulated cytoskeletal associated protein
ATP	adenosintriphosphate
BDNF	brain-derived neurotrophic factor
CAMKII	Ca^{2+}/calmodulin-dependent protein kinases II
cAMP	cyclic adenosine monophosphate
CBX	carbenoxolon
Cdk5	cyclin-dependent kinase 5
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cAMP response element binding protein
Cx	connexin
Cys	cysteine
DA	dopamine
DARPP-32	dopamine and cyclic AMP-regulated phosphoprotein, Mr 32kDa
DAT	dopamine active transporter
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Edition

eCB	endocannabinoids
ER	endoplasmic reticulum
FRIL	freeze-fracture replica immunolabeling
GJ	gap junction
Glut	glutamate
HC	hemichannel
HFS	high frequency stimulation
IFN	interferon
IL-1β	interleukin-1β
Inx	innexin
KO	knock-out
LFS	low frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MEF2	multiple myocite-specific enhancer factor 2
mGluR	metabotropic receptors
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
NE	norepinephrine
NF-κB	nuclear factor-κB
NMDA	N-methyl-D-aspartate
NT	neurotransmitter
Panx	pannexin

PFC	prefrontal cortex
PKA	protein kinase A
PP-1	protein phosphatase-1
PP2A	protein phosphatase-2A
RNA	ribonucleic acid
SA	self-administration
TNF-α	tumor necrosis factor-α
VTA	ventral tegmental area

7 References

1. Brownlow, H.A. and J. Pappachan, *Pathophysiology of cocaine abuse.* Eur J Anaesthesiol, 2002. **19**(6): p. 395-414.
2. Karch, S.B., *Cocaine: history, use, abuse.* J R Soc Med, 1999. **92**(8): p. 393-7.
3. Carrera, M.R., M.M. Meijler, and K.D. Janda, *Cocaine pharmacology and current pharmacotherapies for its abuse.* Bioorg Med Chem, 2004. **12**(19): p. 5019-30.
4. Goldstein, R.A., C. DesLauriers, and A.M. Burda, *Cocaine: history, social implications, and toxicity--a review.* Dis Mon, 2009. **55**(1): p. 6-38.
5. Fleming, J.A., R. Byck, and P.G. Barash, *Pharmacology and therapeutic applications of cocaine.* Anesthesiology, 1990. **73**(3): p. 518-31.
6. Jentzen, J., *Medical complications of cocaine abuse.* Am J Clin Pathol, 1993. **100**(5): p. 475-6.
7. Frishman, W.H., et al., *Cardiovascular manifestations of substance abuse part 1: cocaine.* Heart Dis, 2003. **5**(3): p. 187-201.
8. Rump, A.F., M. Theisohn, and W. Klaus, *The pathophysiology of cocaine cardiotoxicity.* Forensic Sci Int, 1995. **71**(2): p. 103-15.
9. Baldwin, G.C., et al., *Evidence of chronic damage to the pulmonary microcirculation in habitual users of alkaloidal ("crack") cocaine.* Chest, 2002. **121**(4): p. 1231-8.
10. Restrepo, C.S., et al., *Pulmonary complications from cocaine and cocaine-based substances: imaging manifestations.* Radiographics, 2007. **27**(4): p. 941-56.
11. Terra Filho, M., et al., *Pulmonary alterations in cocaine users.* Sao Paulo Med J, 2004. **122**(1): p. 26-31.
12. Roy, A., *Characteristics of cocaine-dependent patients who attempt suicide.* Am J Psychiatry, 2001. **158**(8): p. 1215-9.
13. Harris, D. and S.L. Batki, *Stimulant psychosis: symptom profile and acute clinical course.* Am J Addict, 2000. **9**(1): p. 28-37.
14. Herning, R.I., et al., *The regulation of cerebral blood flow during intravenous cocaine administration in cocaine abusers.* Ann N Y Acad Sci, 1999. **890**: p. 489-94.
15. Koppel, B.S., L. Samkoff, and M. Daras, *Relation of cocaine use to seizures and epilepsy.* Epilepsia, 1996. **37**(9): p. 875-8.
16. Konkol, R.J., et al., *Seizures induced by the cocaine metabolite benzoylecgonine in rats.* Epilepsia, 1992. **33**(3): p. 420-7.
17. Brady, K.T., et al., *Cocaine-induced psychosis.* J Clin Psychiatry, 1991. **52**(12): p. 509-12.
18. Green, R.M., et al., *Multiple intracerebral hemorrhages after smoking "crack" cocaine.* Stroke, 1990. **21**(6): p. 957-62.
19. Cocores, J.A., et al., *Sexual dysfunction in abusers of cocaine and alcohol.* Am J Drug Alcohol Abuse, 1988. **14**(2): p. 169-73.
20. Brown, D.N., M.J. Rosenholtz, and J.B. Marshall, *Ischemic colitis related to cocaine abuse.* Am J Gastroenterol, 1994. **89**(9): p. 1558-61.
21. Van Thiel, D.H. and J.A. Perper, *Gastrointestinal complications of cocaine abuse.* Recent Dev Alcohol, 1992. **10**: p. 331-4.
22. Gitman, M.D. and P.C. Singhal, *Cocaine-induced renal disease.* Expert Opin Drug Saf, 2004. **3**(5): p. 441-8.
23. Roth, D., et al., *Acute rhabdomyolysis associated with cocaine intoxication.* N Engl J Med, 1988. **319**(11): p. 673-7.
24. Frank, R.A., et al., *Cocaine euphoria, dysphoria, and tolerance assessed using drug-induced changes in brain-stimulation reward.* Pharmacol Biochem Behav, 1992. **42**(4): p. 771-9.
25. Hammer, R.P., Jr., Y. Egilmez, and M.W. Emmett-Oglesby, *Neural mechanisms of tolerance to the effects of cocaine.* Behav Brain Res, 1997. **84**(1-2): p. 225-39.
26. Nestler, E.J., *Cellular responses to chronic treatment with drugs of abuse.* Crit Rev Neurobiol, 1993. **7**(1): p. 23-39.
27. Sinha, R., D. Catapano, and S. O'Malley, *Stress-induced craving and stress response in cocaine dependent individuals.* Psychopharmacology (Berl), 1999. **142**(4): p. 343-51.
28. Jaffe, J.H., et al., *Cocaine-induced cocaine craving.* Psychopharmacology (Berl), 1989. **97**(1): p. 59-64.

29. de Wit, H. and J. Stewart, *Reinstatement of cocaine-reinforced responding in the rat.* Psychopharmacology (Berl), 1981. **75**(2): p. 134-43.
30. . Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR®). 2000: American Psychiatric Association.
31. Koob, G.F. and M. Le Moal, *Drug abuse: hedonic homeostatic dysregulation.* Science, 1997. **278**(5335): p. 52-8.
32. Robinson, T.E. and K.C. Berridge, *Addiction.* Annu Rev Psychol, 2003. **54**: p. 25-53.
33. Hyman, S.E., R.C. Malenka, and E.J. Nestler, *Neural mechanisms of addiction: the role of reward-related learning and memory.* Annu Rev Neurosci, 2006. **29**: p. 565-98.
34. Belin, D., et al., *High impulsivity predicts the switch to compulsive cocaine-taking.* Science, 2008. **320**(5881): p. 1352-5.
35. Prakash, A. and G. Das, *Cocaine and the nervous system.* Int J Clin Pharmacol Ther Toxicol, 1993. **31**(12): p. 575-81.
36. Quinn, D.I., A. Wodak, and R.O. Day, *Pharmacokinetic and pharmacodynamic principles of illicit drug use and treatment of illicit drug users.* Clin Pharmacokinet, 1997. **33**(5): p. 344-400.
37. Ritz, M.C., E.J. Cone, and M.J. Kuhar, *Cocaine inhibition of ligand binding at dopamine, norepinephrine and serotonin transporters: a structure-activity study.* Life Sci, 1990. **46**(9): p. 635-45.
38. Schmitt, K.C. and M.E. Reith, *Regulation of the dopamine transporter: aspects relevant to psychostimulant drugs of abuse.* Ann N Y Acad Sci, 2010. **1187**: p. 316-40.
39. Hyman, S.E. and R.C. Malenka, *Addiction and the brain: the neurobiology of compulsion and its persistence.* Nat Rev Neurosci, 2001. **2**(10): p. 695-703.
40. Kalivas, P.W. and K. McFarland, *Brain circuitry and the reinstatement of cocaine-seeking behavior.* Psychopharmacology (Berl), 2003. **168**(1-2): p. 44-56.
41. Koob, G.F., P.P. Sanna, and F.E. Bloom, *Neuroscience of addiction.* Neuron, 1998. **21**(3): p. 467-76.
42. Nestler, E.J., *Molecular neurobiology of addiction.* Am J Addict, 2001. **10**(3): p. 201-17.
43. LaPlant, Q. and E.J. Nestler, *CRACKing the histone code: cocaine's effects on chromatin structure and function.* Horm Behav, 2011. **59**(3): p. 321-30.
44. Robison, A.J. and E.J. Nestler, *Transcriptional and epigenetic mechanisms of addiction.* Nat Rev Neurosci, 2011. **12**(11): p. 623-37.
45. Bibb, J.A., et al., *Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5.* Nature, 2001. **410**(6826): p. 376-80.
46. White, F.J. and D.C. Cooper, *The vicious cyclin of addiction.* Nat Med, 2001. **7**(4): p. 416-7.
47. Vialou, V., et al., *Serum response factor and cAMP response element binding protein are both required for cocaine induction of DeltaFosB.* J Neurosci, 2012. **32**(22): p. 7577-84.
48. Larson, E.B., et al., *Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats.* J Neurosci, 2011. **31**(45): p. 16447-57.
49. Edwards, S., et al., *Region-specific tolerance to cocaine-regulated cAMP-dependent protein phosphorylation following chronic self-administration.* Eur J Neurosci, 2007. **25**(7): p. 2201-13.
50. Barrot, M., et al., *CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11435-40.
51. Pluzarev, O. and S.C. Pandey, *Modulation of CREB expression and phosphorylation in the rat nucleus accumbens during nicotine exposure and withdrawal.* J Neurosci Res, 2004. **77**(6): p. 884-91.
52. Colby, C.R., et al., *Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine.* J Neurosci, 2003. **23**(6): p. 2488-93.
53. Kelz, M.B., et al., *Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine.* Nature, 1999. **401**(6750): p. 272-6.
54. Larson, E.B., et al., *Striatal regulation of DeltaFosB, FosB, and cFos during cocaine self-administration and withdrawal.* J Neurochem, 2010. **115**(1): p. 112-22.
55. Nestler, E.J., *Review. Transcriptional mechanisms of addiction: role of DeltaFosB.* Philos Trans R Soc Lond B Biol Sci, 2008. **363**(1507): p. 3245-55.
56. Wallace, D.L., et al., *The influence of DeltaFosB in the nucleus accumbens on natural reward-related behavior.* J Neurosci, 2008. **28**(41): p. 10272-7.
57. Norrholm, S.D., et al., *Cocaine-induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of cyclin-dependent kinase-5.* Neuroscience, 2003. **116**(1): p. 19-22.
58. Russo, S.J., et al., *The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens.* Trends Neurosci, 2010. **33**(6): p. 267-76.

59. Wolf, M.E. and C.R. Ferrario, *AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine.* Neurosci Biobehav Rev, 2010. **35**(2): p. 185-211.
60. McClung, C.A. and E.J. Nestler, *Regulation of gene expression and cocaine reward by CREB and DeltaFosB.* Nat Neurosci, 2003. **6**(11): p. 1208-15.
61. Bramham, C.R., et al., *The Arc of synaptic memory.* Exp Brain Res, 2010. **200**(2): p. 125-40.
62. Pandey, S.C., et al., *Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors.* J Neurosci, 2006. **26**(32): p. 8320-31.
63. Messaoudi, E., et al., *Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo.* J Neurosci, 2007. **27**(39): p. 10445-55.
64. Schmidt, H.D. and R.C. Pierce, *Cocaine-induced neuroadaptations in glutamate transmission: potential therapeutic targets for craving and addiction.* Ann N Y Acad Sci, 2010. **1187**: p. 35-75.
65. Kauer, J.A. and R.C. Malenka, *Synaptic plasticity and addiction.* Nat Rev Neurosci, 2007. **8**(11): p. 844-58.
66. Jones, S. and A. Bonci, *Synaptic plasticity and drug addiction.* Curr Opin Pharmacol, 2005. **5**(1): p. 20-5.
67. Capriles, N., et al., *A role for the prefrontal cortex in stress- and cocaine-induced reinstatement of cocaine seeking in rats.* Psychopharmacology (Berl), 2003. **168**(1-2): p. 66-74.
68. Del Arco, A. and F. Mora, *Prefrontal cortex-nucleus accumbens interaction: in vivo modulation by dopamine and glutamate in the prefrontal cortex.* Pharmacol Biochem Behav, 2008. **90**(2): p. 226-35.
69. Sun, W. and G.V. Rebec, *The role of prefrontal cortex D1-like and D2-like receptors in cocaine-seeking behavior in rats.* Psychopharmacology (Berl), 2005. **177**(3): p. 315-23.
70. Khroyan, T.V., et al., *Dopamine D1- and D2-like receptor mechanisms in relapse to cocaine-seeking behavior: effects of selective antagonists and agonists.* J Pharmacol Exp Ther, 2000. **294**(2): p. 680-7.
71. Kleven, M.S., et al., *Effects of repeated injections of cocaine on D1 and D2 dopamine receptors in rat brain.* Brain Res, 1990. **532**(1-2): p. 265-70.
72. Schmidt, H.D., S.M. Anderson, and R.C. Pierce, *Stimulation of D1-like or D2 dopamine receptors in the shell, but not the core, of the nucleus accumbens reinstates cocaine-seeking behaviour in the rat.* Eur J Neurosci, 2006. **23**(1): p. 219-28.
73. Schmidt, H.D. and R.C. Pierce, *Cooperative activation of D1-like and D2-like dopamine receptors in the nucleus accumbens shell is required for the reinstatement of cocaine-seeking behavior in the rat.* Neuroscience, 2006. **142**(2): p. 451-61.
74. Self, D.W., et al., *Opposite modulation of cocaine-seeking behavior by D1- and D2-like dopamine receptor agonists.* Science, 1996. **271**(5255): p. 1586-9.
75. Cornish, J.L., P. Duffy, and P.W. Kalivas, *A role for nucleus accumbens glutamate transmission in the relapse to cocaine-seeking behavior.* Neuroscience, 1999. **93**(4): p. 1359-67.
76. Uys, J.D. and R.T. LaLumiere, *Glutamate: the new frontier in pharmacotherapy for cocaine addiction.* CNS Neurol Disord Drug Targets, 2008. **7**(5): p. 482-91.
77. Kozell, L.B. and C.K. Meshul, *Nerve terminal glutamate immunoreactivity in the rat nucleus accumbens and ventral tegmental area after a short withdrawal from cocaine.* Synapse, 2004. **51**(4): p. 224-32.
78. McFarland, K., C.C. Lapish, and P.W. Kalivas, *Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior.* J Neurosci, 2003. **23**(8): p. 3531-7.
79. Pierce, R.C., et al., *Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization.* J Neurosci, 1996. **16**(4): p. 1550-60.
80. Isaac, J.T., M.C. Ashby, and C.J. McBain, *The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity.* Neuron, 2007. **54**(6): p. 859-71.
81. Kerchner, G.A. and R.A. Nicoll, *Silent synapses and the emergence of a postsynaptic mechanism for LTP.* Nat Rev Neurosci, 2008. **9**(11): p. 813-25.
82. Thomas, M.J., et al., *Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine.* Nat Neurosci, 2001. **4**(12): p. 1217-23.
83. Malenka, R.C. and M.F. Bear, *LTP and LTD: an embarrassment of riches.* Neuron, 2004. **44**(1): p. 5-21.
84. Argilli, E., et al., *Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area.* J Neurosci, 2008. **28**(37): p. 9092-100.
85. Bellone, C. and C. Luscher, *mGluRs induce a long-term depression in the ventral tegmental area that involves a switch of the subunit composition of AMPA receptors.* Eur J Neurosci, 2005. **21**(5): p. 1280-8.
86. Bonci, A. and R.C. Malenka, *Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area.* J Neurosci, 1999. **19**(10): p. 3723-30.

87. Nugent, F.S., et al., *High-frequency afferent stimulation induces long-term potentiation of field potentials in the ventral tegmental area.* Neuropsychopharmacology, 2008. **33**(7): p. 1704-12.
88. Pennartz, C.M., et al., *Synaptic plasticity in an in vitro slice preparation of the rat nucleus accumbens.* Eur J Neurosci, 1993. **5**(2): p. 107-17.
89. Kombian, S.B., et al., *Dopamine and adenosine mediate substance P-induced depression of evoked IPSCs in the rat nucleus accumbens in vitro.* Eur J Neurosci, 2003. **18**(2): p. 303-11.
90. Kombian, S.B. and R.C. Malenka, *Simultaneous LTP of non-NMDA- and LTD of NMDA-receptor-mediated responses in the nucleus accumbens.* Nature, 1994. **368**(6468): p. 242-6.
91. Schotanus, S.M. and K. Chergui, *Dopamine D1 receptors and group I metabotropic glutamate receptors contribute to the induction of long-term potentiation in the nucleus accumbens.* Neuropharmacology, 2008. **54**(5): p. 837-44.
92. Goto, Y., C.R. Yang, and S. Otani, *Functional and dysfunctional synaptic plasticity in prefrontal cortex: roles in psychiatric disorders.* Biol Psychiatry, 2010. **67**(3): p. 199-207.
93. Zhong, P., et al., *Serotonin facilitates long-term depression induction in prefrontal cortex via p38 MAPK/Rab5-mediated enhancement of AMPA receptor internalization.* J Physiol, 2008. **586**(Pt 18): p. 4465-79.
94. Luu, P. and R.C. Malenka, *Spike timing-dependent long-term potentiation in ventral tegmental area dopamine cells requires PKC.* J Neurophysiol, 2008. **100**(1): p. 533-8.
95. Saal, D., et al., *Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons.* Neuron, 2003. **37**(4): p. 577-82.
96. Ungless, M.A., et al., *Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons.* Nature, 2001. **411**(6837): p. 583-7.
97. Chen, B.T., et al., *Cocaine but not natural reward self-administration nor passive cocaine infusion produces persistent LTP in the VTA.* Neuron, 2008. **59**(2): p. 288-97.
98. Nugent, F.S. and J.A. Kauer, *LTP of GABAergic synapses in the ventral tegmental area and beyond.* J Physiol, 2008. **586**(6): p. 1487-93.
99. Fourgeaud, L., et al., *A single in vivo exposure to cocaine abolishes endocannabinoid-mediated long-term depression in the nucleus accumbens.* J Neurosci, 2004. **24**(31): p. 6939-45.
100. Schramm-Sapyta, N.L., C.M. Olsen, and D.G. Winder, *Cocaine self-administration reduces excitatory responses in the mouse nucleus accumbens shell.* Neuropsychopharmacology, 2006. **31**(7): p. 1444-51.
101. Martin, M., et al., *Cocaine self-administration selectively abolishes LTD in the core of the nucleus accumbens.* Nat Neurosci, 2006. **9**(7): p. 868-9.
102. Moussawi, K., et al., *N-Acetylcysteine reverses cocaine-induced metaplasticity.* Nat Neurosci, 2009. **12**(2): p. 182-9.
103. Famous, K.R., et al., *Phosphorylation-dependent trafficking of GluR2-containing AMPA receptors in the nucleus accumbens plays a critical role in the reinstatement of cocaine seeking.* J Neurosci, 2008. **28**(43): p. 11061-70.
104. Volkow, N.D., et al., *Role of dopamine, the frontal cortex and memory circuits in drug addiction: insight from imaging studies.* Neurobiol Learn Mem, 2002. **78**(3): p. 610-24.
105. Lasseter, H.C., et al., *Prefrontal cortical regulation of drug seeking in animal models of drug relapse.* Curr Top Behav Neurosci, 2010. **3**: p. 101-17.
106. Huang, C.C., et al., *Repeated cocaine administration impairs group II metabotropic glutamate receptor-mediated long-term depression in rat medial prefrontal cortex.* J Neurosci, 2007. **27**(11): p. 2958-68.
107. Huang, C.C., H.J. Lin, and K.S. Hsu, *Repeated cocaine administration promotes long-term potentiation induction in rat medial prefrontal cortex.* Cereb Cortex, 2007. **17**(8): p. 1877-88.
108. Deroche-Gamonet, V., D. Belin, and P.V. Piazza, *Evidence for addiction-like behavior in the rat.* Science, 2004. **305**(5686): p. 1014-7.
109. Kasanetz, F., et al., *Transition to addiction is associated with a persistent impairment in synaptic plasticity.* Science, 2010. **328**(5986): p. 1709-12.
110. Kasanetz, F., et al., *Prefrontal synaptic markers of cocaine addiction-like behavior in rats.* Mol Psychiatry, 2012.
111. Robinson, T.E., et al., *Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex.* Synapse, 2001. **39**(3): p. 257-66.
112. Nestler, E.J., *Molecular basis of long-term plasticity underlying addiction.* Nat Rev Neurosci, 2001. **2**(2): p. 119-28.
113. Grahame, N.J. and C.L. Cunningham, *Intravenous self-administration of ethanol in mice.* Curr Protoc Neurosci, 2002. **Chapter 9**: p. Unit 9 11.

114. Scemes, E., D.C. Spray, and P. Meda, *Connexins, pannexins, innexins: novel roles of "hemi-channels".* Pflugers Arch, 2009. **457**(6): p. 1207-26.
115. Willecke, K., et al., *Structural and functional diversity of connexin genes in the mouse and human genome.* Biol Chem, 2002. **383**(5): p. 725-37.
116. Phelan, P., *Innexins: members of an evolutionarily conserved family of gap-junction proteins.* Biochim Biophys Acta, 2005. **1711**(2): p. 225-45.
117. Kumar, N.M. and N.B. Gilula, *The gap junction communication channel.* Cell, 1996. **84**(3): p. 381-8.
118. Yeager, M., V.M. Unger, and M.M. Falk, *Synthesis, assembly and structure of gap junction intercellular channels.* Curr Opin Struct Biol, 1998. **8**(4): p. 517-24.
119. Sohl, G. and K. Willecke, *Gap junctions and the connexin protein family.* Cardiovasc Res, 2004. **62**(2): p. 228-32.
120. Unger, V.M., et al., *Three-dimensional structure of a recombinant gap junction membrane channel.* Science, 1999. **283**(5405): p. 1176-80.
121. Boassa, D., et al., *Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane*. J Biol Chem, 2007. **282**(43): p. 31733-43.
122. Bosco, D., J.A. Haefliger, and P. Meda, *Connexins: key mediators of endocrine function.* Physiol Rev, 2011. **91**(4): p. 1393-445.
123. Elenes, S., et al., *Heterotypic docking of Cx43 and Cx45 connexons blocks fast voltage gating of Cx43.* Biophys J, 2001. **81**(3): p. 1406-18.
124. Falk, M.M., et al., *Cell-free synthesis and assembly of connexins into functional gap junction membrane channels.* EMBO J, 1997. **16**(10): p. 2703-16.
125. Harris, A.L., *Connexin channel permeability to cytoplasmic molecules.* Prog Biophys Mol Biol, 2007. **94**(1-2): p. 120-43.
126. Rackauskas, M., et al., *Gating properties of heterotypic gap junction channels formed of connexins 40, 43, and 45.* Biophys J, 2007. **92**(6): p. 1952-65.
127. Gaietta, G., et al., *Multicolor and electron microscopic imaging of connexin trafficking.* Science, 2002. **296**(5567): p. 503-7.
128. Laird, D.W., *Life cycle of connexins in health and disease.* Biochem J, 2006. **394**(Pt 3): p. 527-43.
129. Lauf, U., et al., *Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10446-51.
130. Falk, M.M., et al., *Gap junction turnover is achieved by the internalization of small endocytic double-membrane vesicles.* Mol Biol Cell, 2009. **20**(14): p. 3342-52.
131. Kjenseth, A., et al., *Regulation of gap junction intercellular communication by the ubiquitin system.* Cell Signal, 2010. **22**(9): p. 1267-73.
132. Jordan, K., et al., *The origin of annular junctions: a mechanism of gap junction internalization.* J Cell Sci, 2001. **114**(Pt 4): p. 763-73.
133. Dobrowolski, R. and K. Willecke, *Connexin-caused genetic diseases and corresponding mouse models.* Antioxid Redox Signal, 2009. **11**(2): p. 283-95.
134. Firouzi, M., et al., *Polymorphisms in human connexin40 gene promoter are associated with increased risk of hypertension in men.* J Hypertens, 2006. **24**(2): p. 325-30.
135. Gerido, D.A. and T.W. White, *Connexin disorders of the ear, skin, and lens.* Biochim Biophys Acta, 2004. **1662**(1-2): p. 159-70.
136. Simon, A.M. and D.A. Goodenough, *Diverse functions of vertebrate gap junctions.* Trends Cell Biol, 1998. **8**(12): p. 477-83.
137. White, T.W. and D.L. Paul, *Genetic diseases and gene knockouts reveal diverse connexin functions.* Annu Rev Physiol, 1999. **61**: p. 283-310.
138. Ebihara, L., *Physiology and biophysics of hemi-gap-junctional channels expressed in Xenopus oocytes.* Acta Physiol Scand, 2003. **179**(1): p. 5-8.
139. Goodenough, D.A. and D.L. Paul, *Beyond the gap: functions of unpaired connexon channels.* Nat Rev Mol Cell Biol, 2003. **4**(4): p. 285-94.
140. Kar, R., et al., *Biological role of connexin intercellular channels and hemichannels.* Arch Biochem Biophys, 2012. **524**(1): p. 2-15.
141. Goldberg, G.S., P.D. Lampe, and B.J. Nicholson, *Selective transfer of endogenous metabolites through gap junctions composed of different connexins.* Nat Cell Biol, 1999. **1**(7): p. 457-9.
142. Valiunas, V., E.C. Beyer, and P.R. Brink, *Cardiac gap junction channels show quantitative differences in selectivity.* Circ Res, 2002. **91**(2): p. 104-11.

143. Weber, P.A., et al., *The permeability of gap junction channels to probes of different size is dependent on connexin composition and permeant-pore affinities.* Biophys J, 2004. **87**(2): p. 958-73.
144. Maeda, S. and T. Tsukihara, *Structure of the gap junction channel and its implications for its biological functions.* Cell Mol Life Sci, 2011. **68**(7): p. 1115-29.
145. Gonzalez, D., J.M. Gomez-Hernandez, and L.C. Barrio, *Molecular basis of voltage dependence of connexin channels: an integrative appraisal.* Prog Biophys Mol Biol, 2007. **94**(1-2): p. 66-106.
146. Revilla, A., M.V. Bennett, and L.C. Barrio, *Molecular determinants of membrane potential dependence in vertebrate gap junction channels.* Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14760-5.
147. Bukauskas, F.F. and V.K. Verselis, *Gap junction channel gating.* Biochim Biophys Acta, 2004. **1662**(1-2): p. 42-60.
148. Verselis, V.K., C.S. Ginter, and T.A. Bargiello, *Opposite voltage gating polarities of two closely related connexins.* Nature, 1994. **368**(6469): p. 348-51.
149. Musa, H., et al., *Amino terminal glutamate residues confer spermine sensitivity and affect voltage gating and channel conductance of rat connexin40 gap junctions.* J Physiol, 2004. **557**(Pt 3): p. 863-78.
150. Neyton, J. and A. Trautmann, *Physiological modulation of gap junction permeability.* J Exp Biol, 1986. **124**: p. 993-114.
151. Peracchia, C., *Chemical gating of gap junction channels; roles of calcium, pH and calmodulin.* Biochim Biophys Acta, 2004. **1662**(1-2): p. 61-80.
152. Gonzalez-Nieto, D., et al., *Regulation of neuronal connexin-36 channels by pH.* Proc Natl Acad Sci U S A, 2008. **105**(44): p. 17169-74.
153. Harris, A.L., *Emerging issues of connexin channels: biophysics fills the gap.* Q Rev Biophys, 2001. **34**(3): p. 325-472.
154. Trexler, E.B., et al., *Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation.* J Gen Physiol, 1999. **113**(5): p. 721-42.
155. Moreno, A.P. and A.F. Lau, *Gap junction channel gating modulated through protein phosphorylation.* Prog Biophys Mol Biol, 2007. **94**(1-2): p. 107-19.
156. Saez, J.C., et al., *Regulation of gap junctions by protein phosphorylation.* Braz J Med Biol Res, 1998. **31**(5): p. 593-600.
157. Shah, M.M., A.M. Martinez, and W.H. Fletcher, *The connexin43 gap junction protein is phosphorylated by protein kinase A and protein kinase C: in vivo and in vitro studies.* Mol Cell Biochem, 2002. **238**(1-2): p. 57-68.
158. Solan, J.L. and P.D. Lampe, *Connexin phosphorylation as a regulatory event linked to gap junction channel assembly.* Biochim Biophys Acta, 2005. **1711**(2): p. 154-63.
159. Leithe, E. and E. Rivedal, *Ubiquitination of gap junction proteins.* J Membr Biol, 2007. **217**(1-3): p. 43-51.
160. Su, V. and A.F. Lau, *Ubiquitination, intracellular trafficking, and degradation of connexins.* Arch Biochem Biophys, 2012. **524**(1): p. 16-22.
161. Kjenseth, A., et al., *The gap junction channel protein connexin 43 is covalently modified and regulated by SUMOylation.* J Biol Chem, 2012. **287**(19): p. 15851-61.
162. Juszczak, G.R. and A.H. Swiergiel, *Properties of gap junction blockers and their behavioural, cognitive and electrophysiological effects: animal and human studies.* Prog Neuropsychopharmacol Biol Psychiatry, 2009. **33**(2): p. 181-98.
163. Kim, S.K., et al., *Natural killer activity and antibody-dependent cellular cytotoxicity in patients with primary lung cancer.* Yonsei Med J, 1992. **33**(1): p. 41-7.
164. Panchin, Y., et al., *A ubiquitous family of putative gap junction molecules.* Curr Biol, 2000. **10**(13): p. R473-4.
165. Baranova, A., et al., *The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins.* Genomics, 2004. **83**(4): p. 706-16.
166. Panchin, Y.V., *Evolution of gap junction proteins--the pannexin alternative.* J Exp Biol, 2005. **208**(Pt 8): p. 1415-9.
167. Bruzzone, R., et al., *Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes.* J Neurochem, 2005. **92**(5): p. 1033-43.
168. Ambrosi, C., et al., *Pannexin1 and Pannexin2 channels show quaternary similarities to connexons and different oligomerization numbers from each other.* J Biol Chem, 2010. **285**(32): p. 24420-31.
169. Locovei, S., J. Wang, and G. Dahl, *Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium.* FEBS Lett, 2006. **580**(1): p. 239-44.

170. Charles, A.C., et al., *Intercellular calcium signaling via gap junctions in glioma cells.* J Cell Biol, 1992. **118**(1): p. 195-201.
171. Boitano, S., E.R. Dirksen, and M.J. Sanderson, *Intercellular propagation of calcium waves mediated by inositol trisphosphate.* Science, 1992. **258**(5080): p. 292-5.
172. Dietrich, H.H., et al., *Red blood cell regulation of microvascular tone through adenosine triphosphate.* Am J Physiol Heart Circ Physiol, 2000. **278**(4): p. H1294-8.
173. Harrington, L.S. and J.A. Mitchell, *Novel role for P2X receptor activation in endothelium-dependent vasodilation.* Br J Pharmacol, 2004. **143**(5): p. 611-7.
174. Locovei, S., L. Bao, and G. Dahl, *Pannexin 1 in erythrocytes: function without a gap.* Proc Natl Acad Sci U S A, 2006. **103**(20): p. 7655-9.
175. Boassa, D., et al., *Trafficking dynamics of glycosylated pannexin 1 proteins.* Cell Commun Adhes, 2008. **15**(1): p. 119-32.
176. Barbe, M.T., H. Monyer, and R. Bruzzone, *Cell-cell communication beyond connexins: the pannexin channels.* Physiology (Bethesda), 2006. **21**: p. 103-14.
177. Bunse, S., et al., *The potassium channel subunit Kvbeta3 interacts with pannexin 1 and attenuates its sensitivity to changes in redox potentials.* FEBS J, 2009. **276**(21): p. 6258-70.
178. Dermietzel, R., et al., *Differential expression of three gap junction proteins in developing and mature brain tissues.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 10148-52.
179. Gibson, J.R., M. Beierlein, and B.W. Connors, *Functional properties of electrical synapses between inhibitory interneurons of neocortical layer 4.* J Neurophysiol, 2005. **93**(1): p. 467-80.
180. Kandler, K. and L.C. Katz, *Neuronal coupling and uncoupling in the developing nervous system.* Curr Opin Neurobiol, 1995. **5**(1): p. 98-105.
181. Balice-Gordon, R.J., L.J. Bone, and S.S. Scherer, *Functional gap junctions in the schwann cell myelin sheath.* J Cell Biol, 1998. **142**(4): p. 1095-104.
182. Kleopa, K.A., J. Orthmann-Murphy, and I. Sargiannidou, *Gap junction disorders of myelinating cells.* Rev Neurosci, 2010. **21**(5): p. 397-419.
183. Meier, C., et al., *Connexin32-containing gap junctions in Schwann cells at the internodal zone of partial myelin compaction and in Schmidt-Lanterman incisures.* J Neurosci, 2004. **24**(13): p. 3186-98.
184. Nagy, J.I., et al., *Connexin29 and connexin32 at oligodendrocyte and astrocyte gap junctions and in myelin of the mouse central nervous system.* J Comp Neurol, 2003. **464**(3): p. 356-70.
185. Scherer, S.S., et al., *Transgenic expression of human connexin32 in myelinating Schwann cells prevents demyelination in connexin32-null mice.* J Neurosci, 2005. **25**(6): p. 1550-9.
186. Rash, J.E., et al., *Grid-mapped freeze-fracture analysis of gap junctions in gray and white matter of adult rat central nervous system, with evidence for a "panglial syncytium" that is not coupled to neurons.* J Comp Neurol, 1997. **388**(2): p. 265-92.
187. Rash, J.E., T. Yasumura, and F.E. Dudek, *Ultrastructure, histological distribution, and freeze-fracture immunocytochemistry of gap junctions in rat brain and spinal cord.* Cell Biol Int, 1998. **22**(11-12): p. 731-49.
188. Bennett, M.V. and R.S. Zukin, *Electrical coupling and neuronal synchronization in the Mammalian brain.* Neuron, 2004. **41**(4): p. 495-511.
189. Belluardo, N., et al., *Expression of connexin36 in the adult and developing rat brain.* Brain Res, 2000. **865**(1): p. 121-38.
190. Condorelli, D.F., et al., *Expression of Cx36 in mammalian neurons.* Brain Res Brain Res Rev, 2000. **32**(1): p. 72-85.
191. Buhl, D.L., et al., *Selective impairment of hippocampal gamma oscillations in connexin-36 knock-out mouse in vivo.* J Neurosci, 2003. **23**(3): p. 1013-8.
192. Vandecasteele, M., J. Glowinski, and L. Venance, *Electrical synapses between dopaminergic neurons of the substantia nigra pars compacta.* J Neurosci, 2005. **25**(2): p. 291-8.
193. Buzsaki, G. and A. Draguhn, *Neuronal oscillations in cortical networks.* Science, 2004. **304**(5679): p. 1926-9.
194. Traub, R.D., et al., *High-frequency population oscillations are predicted to occur in hippocampal pyramidal neuronal networks interconnected by axoaxonal gap junctions.* Neuroscience, 1999. **92**(2): p. 407-26.
195. Venance, L., et al., *Connexin expression in electrically coupled postnatal rat brain neurons.* Proc Natl Acad Sci U S A, 2000. **97**(18): p. 10260-5.
196. Nagy, J.I. and J.E. Rash, *Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS.* Brain Res Brain Res Rev, 2000. **32**(1): p. 29-44.

197. Nagy, J.I., et al., *Connexin26 in adult rodent central nervous system: demonstration at astrocytic gap junctions and colocalization with connexin30 and connexin43.* J Comp Neurol, 2001. **441**(4): p. 302-23.
198. Rash, J.E., et al., *Identification of cells expressing Cx43, Cx30, Cx26, Cx32 and Cx36 in gap junctions of rat brain and spinal cord.* Cell Commun Adhes, 2001. **8**(4-6): p. 315-20.
199. Rash, J.E., et al., *Cell-specific expression of connexins and evidence of restricted gap junctional coupling between glial cells and between neurons.* J Neurosci, 2001. **21**(6): p. 1983-2000.
200. Naus, C.C., et al., *Altered gap junctional communication, intercellular signaling, and growth in cultured astrocytes deficient in connexin43.* J Neurosci Res, 1997. **49**(5): p. 528-40.
201. Rash, J.E., et al., *Immunogold evidence that neuronal gap junctions in adult rat brain and spinal cord contain connexin-36 but not connexin-32 or connexin-43.* Proc Natl Acad Sci U S A, 2000. **97**(13): p. 7573-8.
202. Dermietzel, R., et al., *Oligodendrocytes express gap junction proteins connexin32 and connexin45.* Glia, 1997. **20**(2): p. 101-14.
203. Kunzelmann, P., et al., *Coexpression of connexin45 and -32 in oligodendrocytes of rat brain.* J Neurocytol, 1997. **26**(1): p. 17-22.
204. Li, X., et al., *Connexin47, connexin29 and connexin32 co-expression in oligodendrocytes and Cx47 association with zonula occludens-1 (ZO-1) in mouse brain.* Neuroscience, 2004. **126**(3): p. 611-30.
205. Dobrenis, K., et al., *Human and mouse microglia express connexin36, and functional gap junctions are formed between rodent microglia and neurons.* J Neurosci Res, 2005. **82**(3): p. 306-15.
206. Eugenin, E.A., et al., *Microglia at brain stab wounds express connexin 43 and in vitro form functional gap junctions after treatment with interferon-gamma and tumor necrosis factor-alpha.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 4190-5.
207. Takeuchi, H., et al., *Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner.* J Biol Chem, 2006. **281**(30): p. 21362-8.
208. Orellana, J.A., et al., *Modulation of brain hemichannels and gap junction channels by pro-inflammatory agents and their possible role in neurodegeneration.* Antioxid Redox Signal, 2009. **11**(2): p. 369-99.
209. Ray, A., et al., *Site-specific and developmental expression of pannexin1 in the mouse nervous system.* Eur J Neurosci, 2005. **21**(12): p. 3277-90.
210. Vogt, A., S.G. Hormuzdi, and H. Monyer, *Pannexin1 and Pannexin2 expression in the developing and mature rat brain.* Brain Res Mol Brain Res, 2005. **141**(1): p. 113-20.
211. Bruzzone, R., et al., *Pannexins, a family of gap junction proteins expressed in brain.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13644-9.
212. Huang, Y., et al., *Pannexin1 is expressed by neurons and glia but does not form functional gap junctions.* Glia, 2007. **55**(1): p. 46-56.
213. Zappala, A., et al., *Expression of pannexin1 in the CNS of adult mouse: cellular localization and effect of 4-aminopyridine-induced seizures.* Neuroscience, 2006. **141**(1): p. 167-78.
214. Zoidl, G., et al., *Localization of the pannexin1 protein at postsynaptic sites in the cerebral cortex and hippocampus.* Neuroscience, 2007. **146**(1): p. 9-16.
215. Zappala, A., et al., *Expression of pannexin2 protein in healthy and ischemized brain of adult rats.* Neuroscience, 2007. **148**(3): p. 653-67.
216. Pakhotin, P. and A. Verkhratsky, *Electrical synapses between Bergmann glial cells and Purkinje neurones in rat cerebellar slices.* Mol Cell Neurosci, 2005. **28**(1): p. 79-84.
217. Ray, A., et al., *Pannexin expression in the cerebellum.* Cerebellum, 2006. **5**(3): p. 189-92.
218. Hormuzdi, S.G., et al., *Impaired electrical signaling disrupts gamma frequency oscillations in connexin 36-deficient mice.* Neuron, 2001. **31**(3): p. 487-95.
219. Lai, C.P., et al., *Tumor-suppressive effects of pannexin 1 in C6 glioma cells.* Cancer Res, 2007. **67**(4): p. 1545-54.
220. Zoidl, G. and R. Dermietzel, *On the search for the electrical synapse: a glimpse at the future.* Cell Tissue Res, 2002. **310**(2): p. 137-42.
221. LeBeau, F.E., et al., *Fast network oscillations induced by potassium transients in the rat hippocampus in vitro.* J Physiol, 2002. **542**(Pt 1): p. 167-79.
222. Draguhn, A., et al., *Electrical coupling underlies high-frequency oscillations in the hippocampus in vitro.* Nature, 1998. **394**(6689): p. 189-92.
223. Schmitz, D., et al., *Axo-axonal coupling. a novel mechanism for ultrafast neuronal communication.* Neuron, 2001. **31**(5): p. 831-40.

224. Traub, R.D. and A. Bibbig, *A model of high-frequency ripples in the hippocampus based on synaptic coupling plus axon-axon gap junctions between pyramidal neurons.* J Neurosci, 2000. **20**(6): p. 2086-93.
225. Maier, N., et al., *Reduction of high-frequency network oscillations (ripples) and pathological network discharges in hippocampal slices from connexin 36-deficient mice.* J Physiol, 2002. **541**(Pt 2): p. 521-8.
226. Deans, M.R., et al., *Synchronous activity of inhibitory networks in neocortex requires electrical synapses containing connexin36.* Neuron, 2001. **31**(3): p. 477-85.
227. Landisman, C.E. and B.W. Connors, *Long-term modulation of electrical synapses in the mammalian thalamus.* Science, 2005. **310**(5755): p. 1809-13.
228. Landisman, C.E., et al., *Electrical synapses in the thalamic reticular nucleus.* J Neurosci, 2002. **22**(3): p. 1002-9.
229. Mann-Metzer, P. and Y. Yarom, *Electrotonic coupling synchronizes interneuron activity in the cerebellar cortex.* Prog Brain Res, 2000. **124**: p. 115-22.
230. Venance, L., J. Glowinski, and C. Giaume, *Electrical and chemical transmission between striatal GABAergic output neurones in rat brain slices.* J Physiol, 2004. **559**(Pt 1): p. 215-30.
231. Szente, M., et al., *Involvement of electrical coupling in the in vivo ictal epileptiform activity induced by 4-aminopyridine in the neocortex.* Neuroscience, 2002. **115**(4): p. 1067-78.
232. Naus, C.C., J.F. Bechberger, and D.L. Paul, *Gap junction gene expression in human seizure disorder.* Exp Neurol, 1991. **111**(2): p. 198-203.
233. Li, J., et al., *Upregulation of gap junction connexin 32 with epileptiform activity in the isolated mouse hippocampus.* Neuroscience, 2001. **105**(3): p. 589-98.
234. Gajda, Z., et al., *Involvement of gap junctions in the manifestation and control of the duration of seizures in rats in vivo.* Epilepsia, 2003. **44**(12): p. 1596-600.
235. Gajda, Z., et al., *Quinine, a blocker of neuronal cx36 channels, suppresses seizure activity in rat neocortex in vivo.* Epilepsia, 2005. **46**(10): p. 1581-91.
236. Velazquez, J.L., D. Han, and P.L. Carlen, *Neurotransmitter modulation of gap junctional communication in the rat hippocampus.* Eur J Neurosci, 1997. **9**(12): p. 2522-31.
237. Rorig, B., G. Klausa, and B. Sutor, *Dye coupling between pyramidal neurons in developing rat prefrontal and frontal cortex is reduced by protein kinase A activation and dopamine.* J Neurosci, 1995. **15**(11): p. 7386-400.
238. O'Donnell, P. and A.A. Grace, *Different effects of subchronic clozapine and haloperidol on dye-coupling between neurons in the rat striatal complex.* Neuroscience, 1995. **66**(4): p. 763-7.
239. Onn, S.P. and A.A. Grace, *Dye coupling between rat striatal neurons recorded in vivo: compartmental organization and modulation by dopamine.* J Neurophysiol, 1994. **71**(5): p. 1917-34.
240. Hampson, E.C., D.I. Vaney, and R. Weiler, *Dopaminergic modulation of gap junction permeability between amacrine cells in mammalian retina.* J Neurosci, 1992. **12**(12): p. 4911-22.
241. Cepeda, C., et al., *Dye-coupling in the neostriatum of the rat: I. Modulation by dopamine-depleting lesions.* Synapse, 1989. **4**(3): p. 229-37.
242. Onn, S.P. and A.A. Grace, *Repeated treatment with haloperidol and clozapine exerts differential effects on dye coupling between neurons in subregions of striatum and nucleus accumbens.* J Neurosci, 1995. **15**(11): p. 7024-36.
243. O'Donnell, P. and A.A. Grace, *Dopaminergic modulation of dye coupling between neurons in the core and shell regions of the nucleus accumbens.* J Neurosci, 1993. **13**(8): p. 3456-71.
244. Allison, D.W., et al., *Mefloquine effects on ventral tegmental area dopamine and GABA neuron inhibition: a physiologic role for connexin-36 GAP junctions.* Synapse, 2011. **65**(8): p. 804-13.
245. Belousov, A.B., *The regulation and role of neuronal gap junctions during development.* Commun Integr Biol, 2011. **4**(5): p. 579-81.
246. Wang, Y., et al., *Neuronal gap junction coupling is regulated by glutamate and plays critical role in cell death during neuronal injury.* J Neurosci, 2012. **32**(2): p. 713-25.
247. Alev, C., et al., *The neuronal connexin36 interacts with and is phosphorylated by CaMKII in a way similar to CaMKII interaction with glutamate receptors.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20964-9.
248. Ye, Z.C., et al., *Functional hemichannels in astrocytes: a novel mechanism of glutamate release.* J Neurosci, 2003. **23**(9): p. 3588-96.
249. Jiang, S., et al., *Glutamate release through connexin 43 by cultured astrocytes in a stimulated hypertonicity model.* Brain Res, 2011. **1392**: p. 8-15.

250. Pennartz, C.M., H.J. Groenewegen, and F.H. Lopes da Silva, *The nucleus accumbens as a complex of functionally distinct neuronal ensembles: an integration of behavioural, electrophysiological and anatomical data.* Prog Neurobiol, 1994. **42**(6): p. 719-61.
251. O'Donnell, P., et al., *Modulation of cell firing in the nucleus accumbens.* Ann N Y Acad Sci, 1999. **877**: p. 157-75.
252. Cameron, C.M. and R.M. Carelli, *Cocaine abstinence alters nucleus accumbens firing dynamics during goal-directed behaviors for cocaine and sucrose.* Eur J Neurosci, 2012. **35**(6): p. 940-51.
253. Carelli, R.M., *The nucleus accumbens and reward: neurophysiological investigations in behaving animals.* Behav Cogn Neurosci Rev, 2002. **1**(4): p. 281-96.
254. Lee, R.S., G.F. Koob, and S.J. Henriksen, *Electrophysiological responses of nucleus accumbens neurons to novelty stimuli and exploratory behavior in the awake, unrestrained rat.* Brain Res, 1998. **799**(2): p. 317-22.
255. Schultz, W., *Multiple reward signals in the brain.* Nat Rev Neurosci, 2000. **1**(3): p. 199-207.
256. Kokarovtseva, L., et al., *Excitability and gap junction-mediated mechanisms in nucleus accumbens regulate self-stimulation reward in rats.* Neuroscience, 2009. **159**(4): p. 1257-63.
257. Bennett, S.A., et al., *Long-term changes in connexin32 gap junction protein and mRNA expression following cocaine self-administration in rats.* Eur J Neurosci, 1999. **11**(9): p. 3329-38.
258. McCracken, C.B., et al., *Extended cocaine self-administration and deprivation produces region-specific and time-dependent changes in connexin36 expression in rat brain.* Synapse, 2005. **58**(3): p. 141-50.
259. Matesic, D.F., et al., *Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells.* Mol Carcinog, 1994. **10**(4): p. 226-36.
260. Nakata, Y., et al., *Prolonged decrease in hepatic connexin32 in chronic liver injury induced by carbon tetrachloride in rats.* J Hepatol, 1996. **25**(4): p. 529-37.
261. Oguro, K., et al., *Global ischemia-induced increases in the gap junctional proteins connexin 32 (Cx32) and Cx36 in hippocampus and enhanced vulnerability of Cx32 knock-out mice.* J Neurosci, 2001. **21**(19): p. 7534-42.
262. Temme, A., O. Traub, and K. Willecke, *Downregulation of connexin32 protein and gap-junctional intercellular communication by cytokine-mediated acute-phase response in immortalized mouse hepatocytes.* Cell Tissue Res, 1998. **294**(2): p. 345-50.
263. Postma, F., et al., *Electrical synapses formed by connexin36 regulate inhibition- and experience-dependent plasticity.* Proc Natl Acad Sci U S A, 2011. **108**(33): p. 13770-5.
264. Wang, Y. and A.B. Belousov, *Deletion of neuronal gap junction protein connexin 36 impairs hippocampal LTP.* Neurosci Lett, 2011. **502**(1): p. 30-2.
265. Crawford, F.C., et al., *Cocaine induced inflammatory response in human neuronal progenitor cells.* J Neurochem, 2006. **97**(3): p. 662-74.
266. Poon, H.F., et al., *Cocaine-induced oxidative stress precedes cell death in human neuronal progenitor cells.* Neurochem Int, 2007. **50**(1): p. 69-73.
267. Orellana, J.A., et al., *ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels.* J Neurochem, 2011. **118**(5): p. 826-40.
268. Orellana, J.A., et al., *Hypoxia in high glucose followed by reoxygenation in normal glucose reduces the viability of cortical astrocytes through increased permeability of connexin 43 hemichannels.* Glia, 2010. **58**(3): p. 329-43.
269. Retamal, M.A., et al., *Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia.* J Neurosci, 2007. **27**(50): p. 13781-92.
270. Ozog, M.A., et al., *The complex of ciliary neurotrophic factor-ciliary neurotrophic factor receptor alpha up-regulates connexin43 and intercellular coupling in astrocytes via the Janus tyrosine kinase/signal transducer and activator of transcription pathway.* Mol Biol Cell, 2004. **15**(11): p. 4761-74.
271. Berhow, M.T., et al., *Influence of cocaine on the JAK-STAT pathway in the mesolimbic dopamine system.* J Neurosci, 1996. **16**(24): p. 8019-26.
272. Okamoto, T., et al., *Connexin32 protects against vascular inflammation by modulating inflammatory cytokine expression by endothelial cells.* Exp Cell Res, 2011. **317**(3): p. 348-55.
273. Haydon, P.G., et al., *Astrocytic control of synaptic transmission and plasticity: a target for drugs of abuse?* Neuropharmacology, 2009. **56 Suppl 1**: p. 83-90.
274. Panatier, A., et al., *Glia-derived D-serine controls NMDA receptor activity and synaptic memory.* Cell, 2006. **125**(4): p. 775-84.

275. Kanneganti, T.D., et al., *Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling.* Immunity, 2007. **26**(4): p. 433-43.
276. Pelegrin, P. and A. Surprenant, *Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor.* EMBO J, 2006. **25**(21): p. 5071-82.
277. Yirmiya, R. and I. Goshen, *Immune modulation of learning, memory, neural plasticity and neurogenesis.* Brain Behav Immun, 2011. **25**(2): p. 181-213.
278. Papp, L., E.S. Vizi, and B. Sperlagh, *P2X7 receptor mediated phosphorylation of p38MAP kinase in the hippocampus.* Biochem Biophys Res Commun, 2007. **355**(2): p. 568-74.
279. Chu, Y.X., et al., *Involvement of microglial P2X7 receptors and downstream signaling pathways in long-term potentiation of spinal nociceptive responses.* Brain Behav Immun, 2010. **24**(7): p. 1176-89.

8 Acknowledgements

Un grande ringraziamento va al prof. Federico Cicirata, coordinatore del dottorato e mio tutor. Egli mi ha permesso di approfondire varie tematiche scientifiche e conoscere anche varie realtà grazie agli stages che ha organizzato in questi anni.
Grazie al prof. PierVincenzo Piazza per l'opportunità datami di svolgere il mio lavoro di tesi nel suo laboratorio al Neurocentre Magendie di Bordeaux.
Un sincero ringraziamento non può che andare poi al prof. Guillaume Drutel, per avermi seguito durante tutta la parte sperimentale e nella stesura della tesi, per le interessanti conversazioni scientifiche e per la disponibilità mostratami durante la mia permanenza a Bordeaux.
Vorrei ringraziare tutti i componenti dell'equipe Piazza che sono stati fondamentali affinché potessi portare a compimento la mia ricerca.
La mia gratitudine va inoltre al prof. Massimo Alessio per avermi accolto nel suo laboratorio all'unità di Biochimica del Proteoma del San Raffaele di Milano. Oltre alla sua grande professionalità ho apprezzato molto la sua disponibilità e l'avermi fatto sentire sempre parte del gruppo. Grazie anche a Marco e Sheila per i piacevoli momenti.
Grazie alla dr.ssa Stefania Aurino e agli altri ragazzi del TIGEM di Napoli; grazie a loro ho appreso la maggior parte delle tecniche di cui sono a conoscenza.
Grazie alla dr.ssa Zappalà e ai colleghi di dottorato per i gradevoli anni passati insieme in dipartimento.
Il mio più grande ringraziamento non può che andare però alla mia famiglia e ai miei genitori in particolare. Senza di loro non sarei mai arrivato a questo punto e a essere quello che sono. Anche nei mille momenti di difficoltà ho sempre saputo di poter avere un appoggio e un aiuto, e questo è stato fondamentale per proseguire nella mia strada.
Infine, ma non per importanza, grazie a te Imma. Sei stata la prima a insegnarmi le basi delle metodiche di laboratorio; quando arrivai a Napoli, non sapevo come muovermi in un lab di biologia molecolare. Grazie per essermi stata vicina anche nei momenti più difficili e per aver resistito a questi lunghi periodi di lontananza. E' stata dura ma insieme ce l'abbiamo fatta.