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***MOLECULAR ANALYSIS OF SIGMA-1
RECEPTOR MODULATION OF
THE DOPAMINE TRANSPORTER***

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To my beloved wife Giulia

Abstract

Sigma (σ) receptors are well established as a non-opioid, non-phencyclidine, and haloperidol-sensitive receptor family with its own binding profile and a characteristic distribution in the central nervous system (CNS) as well as in endocrine, immune, and some peripheral tissues. Two σ receptors subtypes, termed σ_1 and σ_2 , have been pharmacologically characterized, but, to date, only the σ_1 has also been cloned.

Activation of σ_1 receptors alter several neurotransmitter systems and dopamine (DA) neurotransmission has been often shown to constitute an important target of σ receptors in different experimental models; however the exact role of σ_1 receptor in dopaminergic neurotransmission remains unclear. The DA transporter (DAT) modulates the spatial and temporal aspects of dopaminergic synaptic transmission and interpret the primary mechanism by which dopaminergic neurons terminate the signal transmission. For this reason present studies have been focused in understanding whether, in cell models, the human subtype of σ_1 ($h\sigma_1$) receptor is able to directly modulate the human DA transporter (hDAT).

In the first part of this thesis, HEK-293 and SH-SY5Y cells were permanently transfected with the $h\sigma_1$ receptor. Subsequently, they were transfected with another plasmid for transiently expressing the hDAT. The hDAT activity was estimated using the described [3 H]DA uptake assay and the effects of σ ligands were evaluated by measuring the uptaken [3 H]DA after treating the cells with known σ agonists and antagonists. Results illustrated in this thesis demonstrate that

activation of overexpressed h σ_1 receptors by (+)-pentazocine, the σ_1 agonist prototype, determines an increase of 40% of the extracellular [³H]DA uptake, in comparison to non-treated controls and the σ_1 antagonists BD-1047 and NE-100 prevent the positive effect of (+)-pentazocine on DA reuptake

DA is likely to be considered a neurotoxic molecule. In fact, when levels of intracellular DA abnormally increase, vesicles can't sequester the DA which is metabolized by MAO (A and B) and COMT with consequent overproduction of oxygen reactive species and toxic catabolites. Stress induced by these molecules leads cells to death. Thus, for the second part of this thesis, experiments have been performed in order to investigate functional alterations caused by the (+)-pentazocine-mediated increase of DA uptake; particularly it has been investigated if the increase of intracellular [DA] could affect cells viability. Results obtained from this study demonstrate that (+)-pentazocine alone increases DA cell toxicity in a concentration-dependent manner only in cells co-expressing h σ_1 and hDAT and σ_1 antagonists are able to revert the (+)-pentazocine-induced increase of cell susceptibility to DA toxicity.

In the last part of this thesis, the functional cross-talking between h σ_1 receptor and hDAT has been further investigated using confocal microscopy. From the acquired data it could be suggested that, following exposure to (+)-pentazocine, the h σ_1 receptors massively translocate towards the plasma membrane and colocalize with the hDATs. However, any physical interaction between the two proteins remains to be proved.

In conclusion, the presented study shows for the first time that, in cell models, h σ_1 receptors directly modulate the hDAT activity.

Facilitation of DA uptake induced by (+)-pentazocine is reflected on the increased cell susceptibility to DA toxicity; these effects are prevented by σ_1 selective antagonists. Since numerous compounds, including several drugs of abuse, bind to σ_1 receptors and activating them could facilitate the damage of dopaminergic neurons, the reported protective effect showed by σ_1 antagonists would represent the pharmacological basis to test these compounds in experimental models of dopaminergic neurodegenerative diseases (i.e. Parkinson's Disease).

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INTRODUCTION

CHAPTER 1,

THE SIGMA-1 RECEPTOR

1.1 Historical background

The concept of sigma (σ) receptors was first introduced in literature by Martin and colleagues in 1976 (Martin et al., 1976). Basing on the physiological action induced by some benzomorphans-related molecules on morphine-dependent and non-dependent chronic spinal dogs, Martin and co-workers were able to discern three different groups. They hypothesized that the observed differences were caused by the interactions with three different subtypes of opioid receptors:

- 1) the μ subtype, which mediated the action of morphine and related compounds
- 2) the κ subtype, which mediated the action of ketocyclazocine and related compounds
- 3) the σ subtype, which mediated the action of racemic mixture of N-allylnormetazocine (SKF-10,047) and related compounds

Two subsequent findings convincingly demonstrated that σ receptors are not members of the opioid receptor family. First, the opioid receptors and the σ binding site exhibited an opposite enantioselectivity; opioid receptors are stereoselective for the (-)-isomers of benzomorphans, whereas σ receptors are stereoselective for the (+) counterpart. Second, traditional opioid receptors antagonists, such as naloxone or naltrexone, were ineffective against both the *in vivo* and the *in vitro* effects of the (+)-SKF-10,047 (Brady et al., 1982; Iwamoto, 1981; Katz et al., 1985; Slifer and Balster, 1983; Vaupel, 1983). In addition, the (-)-SKF-10,047 has been found to possess high affinity for μ and κ opioid receptors (Zukin et al., 1982).

Binding experiments further determined that the (+)- isomer of SKF-10,047 is also able to displace the tritiated phenylcyclohexylpiperidine ($[^3\text{H}]$ -PCP) in rat brains homogenates (Mendelshon et al., 1985; Zukin et al., 1982). For this reason, σ receptors were often cited as σ /opioid/PCP receptors and many researchers believed that σ and PCP were the same binding site. The finding that the two potent σ ligands haloperidol and (\pm)-pentazocine aren't able to displace the $[^3\text{H}]$ -PCP from its binding site (Tam et al., 1983; Tam and Cook, 1984) convinced the scientific community that the (+)-SKF-10,047 is able to bind two different sites: the PCP binding site, subsequently identified as the N-methyl-D-aspartate (NMDA) glutamate receptor (Mendelshon et al., 1985; Wong et al., 1988) and a second different binding site, the σ receptor. Further autoradiographic studies also demonstrated that the PCP receptor and the σ receptors have a different distribution in the rat brain (Gundlach et al., 1986a and b; Manallac et al., 1987; Sircar et al., 1986). At present, the σ receptor is considered to be a unique receptor family.

1.2 Pharmacological characterization of the σ receptors: the σ_1 and the σ_2 receptors

The main characteristics of the σ receptors class include that they can bind with high to moderate affinity to a wide spectrum of known compounds of different structural class, such as benzomorphans (pentazocine, SKF-10,047), butyrophenones (haloperidol), guanidines (DTG), piperidines ((+)-3-PPP), morphinans (dextromethorphan), tricyclic antidepressants (imipramine), phenothiazines (chlorpromazine), serotonin-uptake inhibitors

(sertraline), monoamine oxidase inhibitors (clorgyline), psychostimulants (cocaine, 3,4-Methylenedioxymethamphetamine), certain steroids (progesterone) (reviewed in Hayashi and Su, 2004a).

The presence of at least two σ receptors subtypes was first suggested by binding experiments, using two highly selective radiolabeled probes for σ receptors: [^3H]-DTG and [^3H](+)-3-PPP. In fact, (+)-benzomorphans such as (+)-pentazocine and (+)-SKF-10,047 were able to significantly displace the two radiolabeled probes from guinea pig brain homogenates and they also showed a marked lower affinity when tested on PC12 membranes (Hellewell and Bowen, 1990). When binding the rat hepatic σ sites with [^3H]-DTG, the receptor profile of σ ligands was found to be similar to that observed in PC12 cells; moreover, when the σ radioligand [^3H](+)-pentazocine was used, the results showed the same σ ligand selectivity previously observed in guinea pig brain (Hellewell and Bowen, 1990; Walker et al., 1990). Though, basing on the differences in the binding pattern, it was possible to distinguish two different σ receptor subtypes, called σ_1 and σ_2 (Hellewell and Bowen 1990; Quirion et al. 1992). The σ_1 receptors exhibit a major stereoselectivity towards the dextrorotatory isomers of the benzomorphans and many other opioids whereas the σ_2 receptors display a reverse stereospecificity, since they bind with a similar affinity both the levorotatory and the dextrorotatory isomers of the benzomorphans (Table 1.1). For more than a decade the 1,3-di(2-tolyl)guanidine (DTG) remained the only slightly selective σ_2 ligand; for this reason the [^3H]-DTG was widely employed in the σ_2 binding experiments, masking the σ_1 binding with the cold (+)-pentazocine. Anyhow, today, some σ_2 preferring ligands, such as the SM-21 (Ghelardini et al., 2000), have been synthesized and characterized. The progesterone is also able to differentiate between σ_1 and σ_2 , interacting with only the σ_1 subtype (Kahoun and Ruoho, 1992); for this reason the

progesterone is believed to be a putative endogenous ligand of σ_1 (Ganapathy et al., 1999). A third σ receptors subtype was also proposed; it was called σ_3 (Quirion et al., 1992), but, to date, no convincing binding data or selective ligands are available for this putative receptor subtype.

Table 1.1. Pharmacology of σ receptor ligands; following K_i values are obtained with different experimental approaches which are described in respective references.

Drugs	Affinity [K_i ; nM]		Function on σ_1 receptors	Other activities	References
	σ_1	σ_2			
BENZOPORPHANS					
(+)-Pentazocine	16.7	6611	Agonist	-	Vilner and Bowen, 2000
(-)-Pentazocine	807	2324	Agonist	κ_1 agonist, μ_1 , μ_2 , ligand, low affinity δ and κ_3 opioid ligand	Vilner and Bowen, 2000 + Chien and Pasternak 1995b
(+)-SKF-10,047	597	39740	Agonist	NMDA receptor ligand	Vilner and Bowen, 2000 + Hayashi and Su, 2004
(-)-SKF-10,047	50399	41461	Agonist	-	Vilner and Bowen, 2000
ANTIPSYCHOTICS					
Haloperidol	6.44	221	Antagonist	Dopamine D_2 and D_3 antagonist; σ_2 agonist	Vilner and Bowen, 2000 + Maurice et al., 2001a° + Jaen et al., 1993
Chlorpromazine	453	1628	?	Dopamine D_2 antagonist	Matsumoto and Pouw, 2000 + Hayashi and Su, 2004
ANTIDEPRESSANTS					
Clorgyline	2.9	505	Agonist?	Irreversible monoamine oxidase A inhibitor	Bermack and Debonnel, 2005 + Itzhak et al., 1991
Fluoxetine	240	16100	Agonist	Selective 5-HT reuptake inhibitor	Hayashi and Su, 2008 + Narita et al., 1996
Fluvoxamine	36	8439	Agonist	Selective 5-HT reuptake inhibitor	Hayashi and Su, 2008 + Narita et al., 1996
Imipramine	343	2107	Agonist	Monoamine reuptake inhibitor	Hayashi and Su, 2008 + Narita et al., 1996
Sertraline	57	5297	Agonist	Selective 5-HT reuptake inhibitor	Hayashi and Su, 2008 + Bermack and Debonnel, 2005 + Narita et al., 1996
ANTITUSSIVES					
Carbetapentane	128	1953	Agonist	Muscarinic antagonist, σ_2 agonist	Matsuno et al., 1996 + Calderon et al., 1994 + Maurice et al., 2001a
Dextromethorphan	205	11060	Agonist	NMDA receptor allosteric antagonist	LePage et al., 2005 + Shin et al., 2005 + Maurice et al., 2001a
Dimemorfan	151	4421	Agonist	?	Shin et al., 2005 + Wang et al., 2003 + Chou et al., 1999
PARKINSON'S AND/OR ALZHEIMER'S DISEASE					
Amantadine	7440	?	Agonist?	NMDA antagonist, antiviral properties	Peeters et al., 2004 + Chen and Lipton, 2006
Donepezil	14.6	?	Agonist	Cholinesterase inhibitor	Kato et al., 1999 + Maurice et al., 2006a + Meunier et al., 2006
Memantine	2600	?	Agonist?	NMDA antagonist, antiviral properties	Peeters et al., 2004 + Chen and Lipton, 2006

DRUG OF ABUSE					
Cocaine	2000	31000	Agonist	Monoamine transporters inhibitor, amongst other actions, psychostimulant	Matsumoto et al., 2002 + Rothman and Bauerman, 2003
Metamphetamine	2160	46670	?	Preferential DAT inhibitor, amongst other actions, psychostimulant	Nguyen et al., 2005 + Fleckenstein et al., 2007
MDMA	3057	8889	?	Preferential SERT inhibitor, among other actions, psychostimulant	Brammer et al., 2006 + Green et al., 2003
PUTATIVE ENDOGENOUS REGULATORS					
Neuropeptide Y (NPY)	~10*	?	Agonist?	Anti-amnesic, anti-convulsivant	Maurice et al., 2001a + Roman et al., 1989 + Ault and Werling, 1997
DHEAS	5200	?	Agonist	GABA _A negative modulator	Hong et al., 2004 + Maurice et al., 2001a
Pregnenolone sulfate	980	?	Agonist	NMDA positive/GABA _A negative modulator	Hong et al., 2004 + Maurice et al., 2001a
Progesterone	130	?	Antagonist	NMDA negative/GABA _A positive modulator	Hong et al., 2004 + Maurice et al., 2001a
DMT	14750	21710	Agonist	agonist 5-HT _{2A} and 5-HT _{2A} receptors, psychedelic drug	Fontanilla et al., 2009 + Su et al., 2009 + Smith et al., 1998
D-erythro-sphingosine	140	13000	Agonist?	Endogenous amine involved in lipid signalling	Ramachandran et al., 2009
N,N-dimethyl-sphingosine	120	2800	Agonist?	Endogenous amine involved in lipid signalling	Ramachandran et al., 2009
L-threo-sphingosine	20	8300	Agonist?	Endogenous amine involved in lipid signalling	Ramachandran et al., 2009
Sphinganine	70	35000	Agonist?	Endogenous amine involved in lipid signalling	Ramachandran et al., 2009
ANTICONVULSANTS					
Phenytoin (DPH)	Not measurable	Not measurable	Allosteric modulator	Delayed rectifier K ⁺ channel blocker, T-type Ca ²⁺ current inhibitor, Na ⁺ current inhibitor	Walker et al., 1990 + Cobos et al., 2005 and 2006 + Nobile and Lagostena, 1998 + Todorovic and Lingle, 1998 + Rush et al., 1997
Ropizine	Not measurable	Not measurable	Allosteric modulator	?	Walker et al., 1990
OTHER σ DRUGS					
AC-927	30	138	Antagonist	σ_2 antagonist	Matsumoto et al., 2008
BD-737	8.78	68.3	Agonist	-	McCracken 1999b + Vilner and Bowen, 2000 + Hellewell et al., 1994 + Guitart et al., 2004
BD-1008	2	8	Antagonist	σ_2 agonist	McCracken 1999b + Vilner and Bowen, 2000 + Hayashi and Su, 2004
BD-1047	0.9	47	Antagonist	α adrenoceptor ligand	McCracken et al., 1999 ^o + Matsumoto et al., 1995 + Maurice et al., 2001a
BD-1063	9	449	Antagonist	-	Matsumoto et al., 1995 + McCracken 1999b + Brammer et al., 2006 + Maurice et al., 2001a
BMY-14802	66	51	Antagonist	5-HT _{1A} agonist	Matsumoto and Pouw, 2000 + Guitart et al., 2004 + Matos et al., 1996
DTG	77	43	?	σ_2 agonist	Kedjourar et al., 1999 + Maurice et al., 2001a + Matsumoto and Pouw, 2000
Dup734	10	?	Antagonist	5-HT ₂ antagonist	Tam et al., 1992
Eliprodil (SL-82.0715)	132	634	?	NMDA antagonist, α_1 adrenoceptor ligand	Hashimoto and London, 1995

E-5842	4	220	Antagonist	High affinity for α_{2B} , α_{1a} and α_{1B} adrenergic receptors, low to moderate affinity for dopamine, 5-HT and glutamate receptors	Guitart et al., 1998 + Guitart et al., 2004
JO-1784 (Igmesine)	39	?	Agonist	-	Guitart et al., 2004
MR-200	1.5	21.9	Agonist	-	Ronsisvalle et al., 2001b, Kanzaki et al., 1992
NE-100	1.5	84.6	Antagonist	-	Chaki et al., 1996
OPC-14523	47 (IC ₅₀ nM)	56 (IC ₅₀ nM)	Agonist	Agonist of pre- and post-synaptic 5-HT _{1A} receptors, SERT inhibitor	Tottori et al., 2001 + Guitart et al., 2004 + Bermack and Debonnel, 2007
(+)-3-PPP	79	120	Agonist	σ_2 agonist, NMDA receptor ligand, dopaminergic agonist	Walker et al., 1990 + Höfner and Wanner, 2000 + Hayashi and Su 2004
(-)-3-PPP	~310	~1200	?	-	Itzhak et al., 1991
PRE-084	44	?	Agonist	-	Maurice et al., 1999
Rimcazole (BW-234U)	2380	1162	Antagonist	DAT inhibitor	Matsumoto and Pouw, 2000 + Matsumoto et al., 2001c
SA-4503	17.4	1784	Agonist	-	Matsuno and Mita, 1998
SR-31742A	5.3 for the whole σ population		-	High affinity for C8-C7 sterol isomerase	Poncelet et al., 1993
SM-21	>1000	67.5	?	Agonist σ_2	Ghelardini et al., 2000

?: to date not studied or unclear

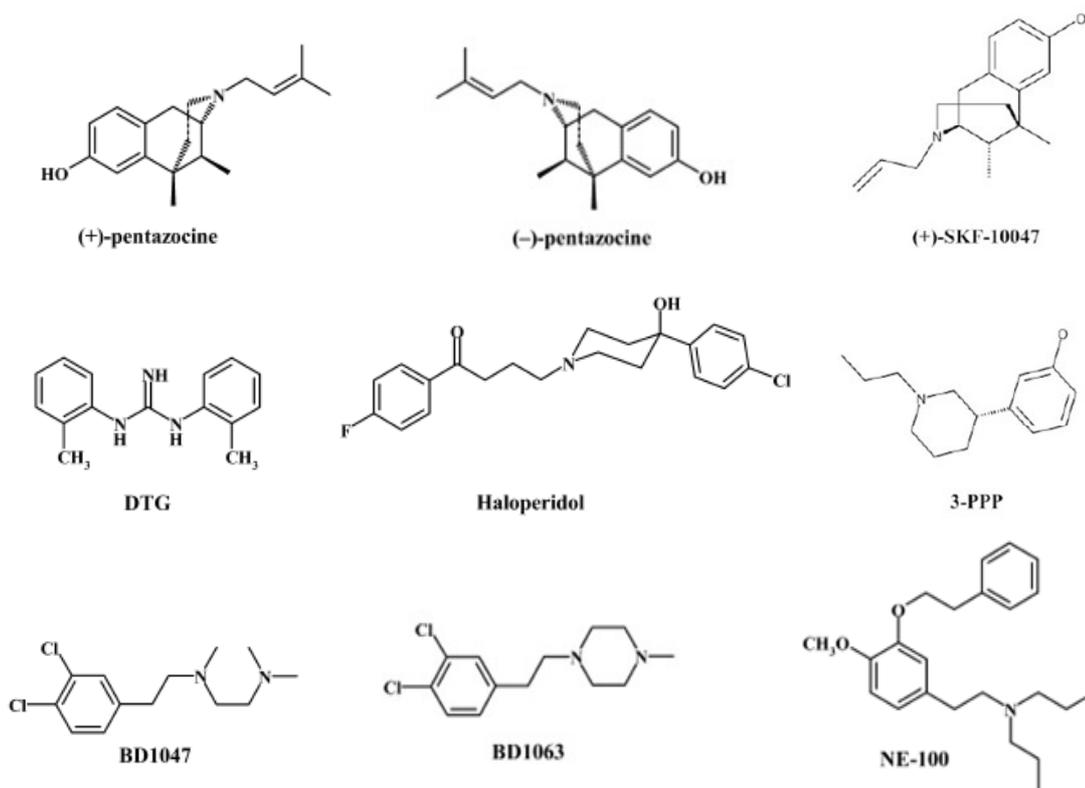
-: no other pharmacological target has been described.

* This result has been first reported by Roman et al. in the 1989; however it hasn't been confirmed later in other researches.

Another distinguish feature between the two classes of σ receptors is their interaction with ropizine and phenytoin (Musacchio et al., 1989a; DeHaven-Hudkins et al., 1993). σ_1 receptors are allosterically modulated by these compounds whereas σ_2 receptors are not affected. Furthermore, these two classes of receptors are also distinguishable on the basis of their molecular weight. In fact the σ_1 receptor was found to be a 25 kDa single protein which has been cloned (see section 1.3) in several animal species and humans (Kekuda et al., 1996; Prasad et al., 1998; Hanner et al., 1996; Pan et al., 1998; Seth et al., 1997, 1998); on the other hand, the σ_2 receptors, not yet been cloned, have an apparent molecular weight of 18-21 kDa, detected with [³H]azido-DTG photoaffinity labelling in the presence of dexrallorphan to mask σ_1 binding site (Hellewell and Bowen, 1990; Hellewell et al., 1994).

Currently, the classification of σ receptors comprising the σ_1 and the σ_2 subtypes is widely accepted by the scientific community.

Figure 1.1. Chemical structure of the most important sigma ligands



1.3 Cloning and molecular characterization of σ_1 receptors

One of the most important step in the σ receptor comprehension was the cloning of the σ_1 receptor. In 1996, Hanner et al. (Hanner et al., 1996) successfully purified and cloned the σ_1 binding site from guinea pig liver microsomes. Hanner et al. used the aminoacid (AA) sequence to degenerate nucleotide primers and they succesfully used these primers to amplify a RT-PCR product, with guinea pig liver as RNA source. From then on, the σ_1 was also cloned from other different sources: from the JAR human choriocarcinoma cell line (Kekuda et al., 1996; Prasad et al., 1998), from the rat brain and from the mouse brain (Seth et al., 1998; Mei and Pasternak, 2001; Pan et al., 1998). The σ_1 cDNA cloned from guinea pig is 1857 bp long and it codes for a 223 amino acids protein. The estimated molecular mass was 25,3 kDa, then very similar to the values coming from the photoaffinity labeling experiments. The human σ_1 cDNA is 1,653 bp long, with a coding sequence of 672 bp long that codes for a 223 AA protein (Kekuda et al., 1996). Moreover, the AA sequence of the human σ receptor bears 93% of homology with the guinea pig σ_1 (Kekuda et al., 1996). Analysis of the AA sequence showed that both the human and the guinea pig σ_1 receptors carry, at the N-terminus, the AA sequence motif MQWAVGRR, wich is considered an endoplasmic reticulum retention signal. As showed in figure (Fig.1.2), also the mouse and the rat brain σ_1 AA sequence is highly conserved, showing high homologies both with the guinea pig and the human σ_1 receptors.

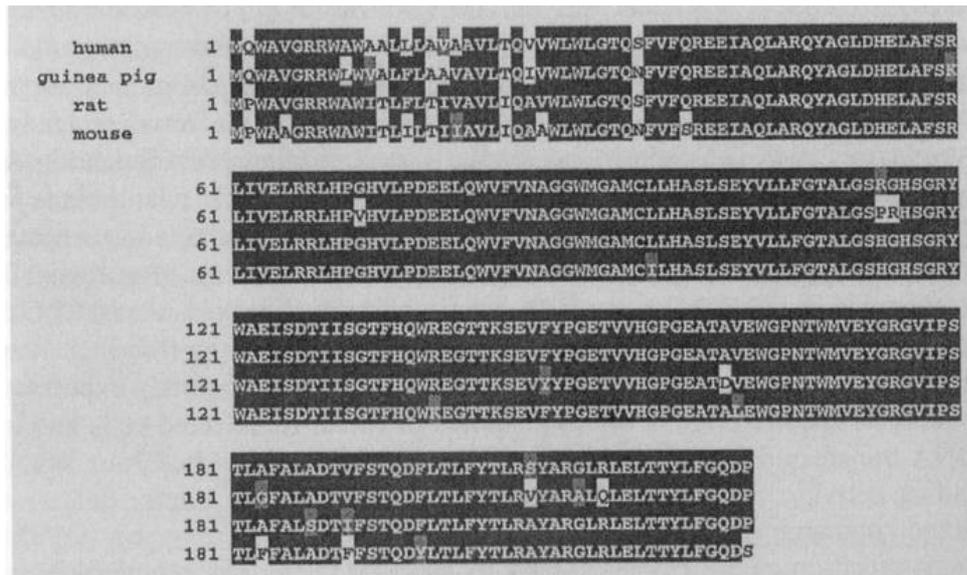


Figure 1.2. Comparison of the AA sequence of σ_1 receptors from different mammalian species. Regions with identical AA are indicated by dark shaded boxes and region with conservative substitution are indicated by lightly shadow boxes (image from *Sigma Receptors Handbook*, Matsumoto, Bowen and Su, 2007)

The AA sequence of the cloned σ_1 showed no homology to any other mammalian protein, but it was found to share around 30% of homology with ERG2, a fungal gene product that possess a sterol C8-C7 isomerase activity (Moebius et al., 1997). Interestingly, the most conserved domain in both the peroteins carries a sterol/steroid-binding domain, obligatory for a protein involved in sterol synthesis (Hanner et al., 1996a), but, probably, also a key feature for σ_1 to play its endogenous role. In fact, the existence of a lipophilic sterol/steroidbinding site also explains the surprising ability of the cloned σ_1 to bind so many drugs from distinct pharmacological classes with high affinity (Hanner et al., 1996). However, the cloned σ_1 receptor doesn't possess any sterol isomerase activity similar to ERG2 fungal enzyme (Hanner et al., 1996). Moreover, the mammalian sterol isomerase has been cloned, it is called emopamil-binding protein and it doesn't show any structural similarity to either the yeast C8-C7 sterol isomerase or mammalian σ_1 receptors (Hanner et al, 1995; Silve et al., 1996).

The cloned mammalian σ_1 receptors has been shown to be an integral membrane protein able to localize to the nuclear and the endoplasmic reticulum (Jbilo et al., 1997). The hydropathy analyses have led to the prediction of two different transmembrane topology models consisting of one (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997; Seth et al., 1998) or two (Jbilo et al., 1997; Pan et al., 1998; Mei and Pasternak, 2001) transmembranes domains (Fig. 1.3). Evidence supporting the two transmembranes model were provided later by Aydar et al. (Aydar et al., 2002); using a GFP probe and two fusion protein, the C-terminal GFP- σ_1 (GFP-C- σ_1) and the N-terminal GFP- σ_1 (GFP-N- σ_1), Aydar et al. (Aydar et al., 2002) were able to prove that, when expressed in in *Xenopus laevis* oocytes, the σ_1 receptor posseses two transmembrane segments and both the termini are located in the cytoplasmatic lumen (Fig. 1.3).

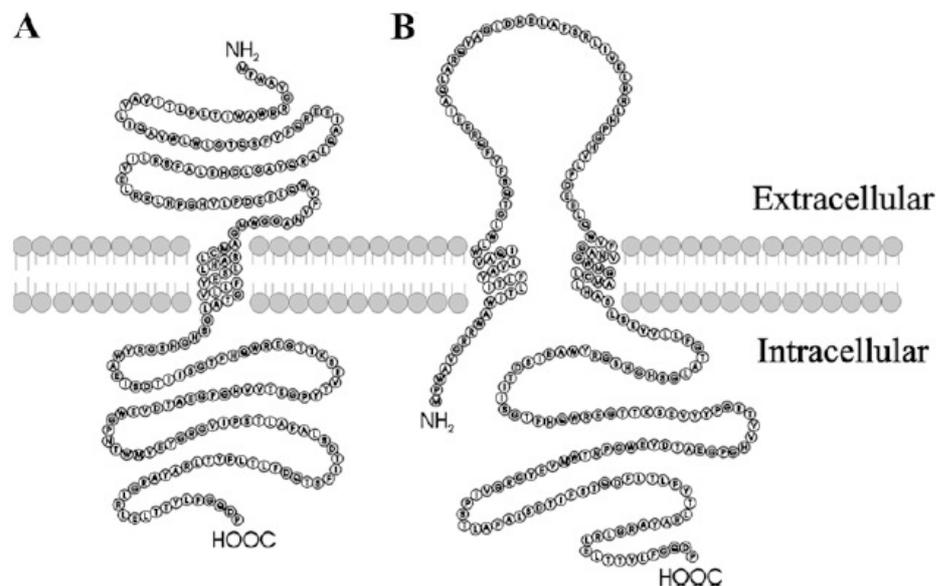


Figure 1.3. Structural models proposed for the σ_1 receptor, with a single putative transmembrane domain (A) and two putative transmembrane domains (B) (Guitart et al., 2004)

Another relevant finding in σ_1 receptor biology was the discovery of an alternatively spliced variant of the human σ_1 receptor, expressed in the human T lymphocyte cell line Jurkatt (Ganapathy et al., 1999). This splice variant, that lacks 31 AA in the position 119-149, doesn't have the ability to bind the classical σ_1 ligands. At present, it is not known whether the σ_1 splice variant is expressed in human tissues and which could be his pathophysiological implications.

1.4 Distribution of σ_1 receptors

1.4.1 Anatomical distribution of σ_1 receptors

σ receptors are widely distributed throughout the whole body. In order to understand the physiological roles of the σ receptors, many efforts were made in studying their anatomical localization.

In the central nervous system (CNS), they have been extensively studied because of their potential role in several CNS disorders (reviewed in Hayashi and Su, 2004a). The initial binding studies were performed using the two not σ_1/σ_2 selective probes [^3H](+)-3-PPP and [^3H](+)-DTG (Bouchard and Quirion, 1997; Gundlach et al., 1986a; McLean and Weber, 1998); these studies showed that the highest concentration of σ receptors in the brain are in the brainstem motor nuclei. In fact high densities of σ receptors were found in the major constituents of brainstem motor circuits, like the cerebellum, the red nucleus, the gray matter of the spinal cord and both the basal ganglia and the *pars compacta* of the substantia nigra (Bouchard and Quirion, 1997; Gundlach et al., 1986a; McLean and Weber,

1998). The functional studies confirmed that the σ receptors have a role in modulating some motor functions (Bouchard and Quirion, 1997; Gundlach et al., 1986a; McLean and Weber, 1998); moreover, the σ agonists are able to directly stimulate motor behaviors via nigrostriatal dopaminergic pathways (Bastianetto et al., 1995; Goldstein et al., 1989; Patrick et al., 1993; Walker et al., 1993) With the availability of new σ_1/σ_2 more selective compounds, it was also possible to demonstrate that both the σ_1 and σ_2 subtypes are involved in the motor function modulation (Bouchard and Quirion, 1997; Patrick et al., 1993; Matsumoto and Pouw, 2000)

The σ binding sites appear to be highly concentrated also in the spinal cord (Kovacs and Larson, 1995, 1998; Alonso et al., 2000), mainly in the dorsal horn (Alonso et al., 2000), in the hippocampus and in many other limbic and paralimbic areas, such as the cingulate cortex and the amygdala,

The studies focused on the anatomical distribution of σ_1 receptors have been extensively described in the rodent brain with autoradiographic methods employing selective σ_1 ligands (Okuyama et al., 1995; Walker et al., 1992; Ishiwata et al., 1998 and 2006; Kawamura et al., 2000a and b), with in situ hybridization (Kitaichi et al., 2000; Zamanillo et al., 2000) and with immunohistochemical techniques (Alonso et al., 2000). In these studies, σ_1 appeared to be particularly concentrated in all brain areas where dopamine (DA) is widely released, especially in the gyrus dentatus of the hippocampus suggesting that they may modulate cognitive behaviors, mood and affective states (Bouchars and Quirion, 1997), or maybe involved in psychostimulant actions (reviewed in Matsumoto et al., 2003). High concentrations of σ_1 receptors were also found in the facial nucleus, in the thalamic and hypothalamic nuclei, in the striatum, in the cerebellum, in the dorsal raphe nucleus and in the locus coeruleus (Bouchard and Quirion 1997; Inoue et al., 2000), whereas the σ_2 appeared more concentrated in the

brain areas involved in the control of posture and movement (Bouchard and Quirion 1997).

The σ_1 receptor is also widely distributed in the peripheral organs. It has been found in the digestive tract (Samovilova and Vinogradov 1992), in the kidney (Hellewell et al., 1994), in the liver (McCann and Su, 1991; Hellewell et al., 1994; DeHaven-Hudkins et al., 1994), in the heart (Ela et al., 1994; Monassier and Bousquet, 2002; DeHaven-Hudkins et al., 1994), in the lungs (Stone et al., 2006), in the vas deferens (DeHaven-Hudkins et al., 1991), in the testis (Wolfe et al., 1989; DeHaven-Hudkins et al., 1994), in the ovaries (Wolfe et al., 1989), and also in blood mononuclear cells (Wolfe et al., 1988). The σ_2 receptors have also been found in different peripheral organs; it appeared to be particularly concentrated in the liver and in the kidney (Hellewell et al. 1994).

1.4.2 Subcellular distribution of σ_1 receptors

Early studies aimed in clarifying the subcellular localization of σ receptors were difficult and not much accurate because the ligand binding assays were the only strategy available to mark the σ binding sites. However by combining rat membrane fractionation and radioligand binding experiments, some groups obtained some preliminary data (Cagnotto et al., 1994; DeHaven-Hudkins et al., 1994; Itzhak et al., 1991; Jbilo et al., 1997; Kekuda et al., 1996; McCann et al., 1989; McCann et al., 1994; Tanaka et al., 1995). Interestingly, the not σ_1/σ_2 selective radioactive ligands [^3H](+)-SKF-10,047, [^3H](+)-3-PPP and [^3H]DTG were found to bind some subcellular fraction, respectively with the following order of affinity: microsomal > mitochondrial > synaptosomal > myelin > nuclear fraction (Itzhak et al., 1991). On the other hand, when using the σ_1 selective [^3H](+)-

pentazocine as radioactive probe, the affinity for the same subcellular fractions was slightly different, respectively: microsomal > nuclear fraction > myelin > mitochondrial > synaptosomal (Cagnotto et al., 1994; McCann et al., 1989). Taken together, these results indicated for the first time that the distribution of the σ_1 receptors in the synaptic regions of plasma membrane or in the mitochondria is minimum, whereas the σ_1 are predominately enriched in microsomes, suggesting a localization on the endoplasmatic reticulum (ER).

The availability of the specific antibodies against the σ_1 receptors led to more accurate studies. Western blotting experiments confirmed the data obtained with binding results not only in the rat brain neurons, but also in oligodendrocytes, in lymphocytes and in the NG108-15 neuroblastoma x glioma cell line (Hayashi et al., 2000; Dussossoy et al., 1999; Alonso et al., 2000). In the immunocytochemical researches, performed with the promonocytic cell line THP, the σ_1 specifically localized on the ER membrane and on nuclear envelopes (Dussossoy et al., 1999; Jbilo et al., 1997). In others immunofluorescence studies, σ_1 polyclonal antibodies were able to selectively stain the ER in the cytoplasmatic area of neuronal and retinal rat cells (Alonso et al., 2000; Hayashi et al., 2001, Morin-Surun et al., 1999; Ola et al., 2001).

However, the most accurate data come from the confocal fluorescence microscopy investigations. In the NG108 cell line, the endogenous expressed σ_1 receptors were found to localize on both the ER and the nuclear envelope, but, at the ER level, they are highly clustered in globular structures enriched in cholesterol and neutral lipids, similar to the lipid rafts (Hayashi and Su, 2003a; Hayashi and Su, 2003b; Hayashi and Su, 2007). Interestingly, the fusion protein σ_1 -C-terminally enhanced yellow fluorescence protein (EYFP-C- σ_1) showed the same distribution of the

endogenous expressed σ_1 receptors, whereas the fusion protein σ_1 -N-terminally enhanced yellow fluorescence protein (EYFP-N- σ_1) didn't behave like the endogenous receptor; in fact the EYFP-N- σ_1 receptor was diffused in the ER tubules, but it couldn't target the lipid raft-like structures. Moreover, the EYFP-N- σ_1 transfected cells lines failed in the compartmentalization of neutral lipids in lipid rich globular structures, indicating a putative role of the σ_1 receptors in the intracellular lipid homeostasis (Hayashi and Su, 2003b).

To date, the σ_1 receptors are considered by the scientific community an ER protein with a unique distribution pattern.

1.5 Potential σ_1 receptor endogenous ligand(s)

The endogenous ligand of the σ_1 receptors have not yet been unequivocally identified.

First efforts to discover endogenous σ ligand(s) were based on the fractionation techniques, since the classical strategy for identifying an endogenous ligand is to extract it from the tissue where it acts. These studies provided proofs of the existence of a peptide-related substance, called "sigmaphin", isolated from the guinea pig brain (Su et al., 1986; Contreras et al., 1987) and a porcine liver extract able to bind the σ binding sites (Nagornaia et al., 1988). Even if these efforts proved the existence of endogenous extracts able to bind to the σ receptors, none of these substances were ever purified or characterized.

Early binding experiments reported that two endogenous neuropeptides, neuropeptide Y (NPY) and peptide YY (PYY), were able to strongly displace the [3 H](+)-SKF-10,047 from rat brain homogenates σ receptors (K_i

of 9.8 and 4.9 nM, respectively) (Roman et al., 1989). However, subsequent experiments couldn't confirm this hypothesis (McCann and Su, 1991; Tam and Mitchell, 1991).

The search for an endogenous ligand led to the discovery that certain neurosteroids possess affinity for σ_1 receptors. The interaction between neurosteroids and σ_1 receptors was first reported by Su and co-workers (Su et al., 1988). Among the other tested neurosteroids, the progesterone appeared to bind to the σ receptors with the highest affinity (Su et al., 1988). Furthermore, the binding experiments, using [^3H]progesterone as radioactive probe, unveiled a drug displacement pattern consistent with σ receptors (McCann and Su, 1991; Yamada et al., 1994). The possibility that neurosteroids could be the endogenous σ_1 receptors ligands is further supported by the discovery that the σ_1 receptor possess a sterol/steroid binding pocket (Mei and Pasternak, 2001). However, the affinity of progesterone for the σ_1 receptors subtype, that falls in the μM range, doesn't appear high enough for an endogenous ligand and the progesterone concentration in free serum is insufficient to occupy the σ_1 receptors in the brain (Schwarz et al., 1989). In addition, the other steroids such as dehydroepiandrosterone sulfate (DHEAS), pregnenolone sulfate, testosterone and deoxycorticosterone exhibit even lower affinities than progesterone for the σ_1 receptor subtype (Su et al., 1988 and 1990; Hayashi and Su, 2004a). Anyhow, several publications keep confirming the ability of progesterone to bind the σ receptors (Hanner et al., 1996; Ganapathy et al., 1999; Klein et al., 1994; Maurice et al., 1996; Ramamoorthy et al., 1995). The few available functional studies indicate that some neurosteroids act as agonists (e.g. pregnenolone), while others act as antagonists (e.g. progesterone) (Maurice et al., 2001a).

Recently, the endogenous *N,N*-dimethyltryptamine (DMT) was found to be able to bind and regulate the σ_1 receptors (Fontanilla et al., 2009). The DMT is historically known to be the most active psychoactive compound in some sacramental teas (such as *ayahuasca* and *yagé*) used in native shamanic rituals in South America. However, DMT can be produced by enzymes in mammalian lung (Axelrod, 1961) and in rodent brain (Savaedra and Axelrod, 1962) and it has also been found in human urine, blood and cerebrospinal fluid (Franzen and Gross, 1965; Beaton and Morris, 1984). The affinity of DMT for the σ_1 receptors falls in the μM range (Fontanilla et al., 2009) and it is similar to the affinity of progesterone for the same σ receptors subtypes. Moreover, in mouse models, μM concentrations of DMT induced the same hypermobility effects observed with the σ_1 selective ligand (+)-SKF-10,047, while both the DMT and (+)-SKF-10,047 can't cause the same pharmacological effects in the σ_1 KO mice (Fontanilla et al., 2009). These results strongly support that the behavioral actions of DMT are mediated by the σ_1 receptors.

The endogenous sphingolipids were also recently found to compete with [^3H](+)-pentazocine on the σ_1 binding site (Ramachandran et al., 2009). In particular, the p-erythro-sphingosine, or just sphingosine, was found to bind to the σ_1 receptors with the highest affinity, but not its major metabolite, the sphingosine-1-phosphate, that couldn't bind at all both the σ receptors subtypes (Ramachandran et al., 2009). This finding suggests a putative cross-talking between the enzyme sphingosine kinase and the σ_1 receptors, mediated by the sphingosine itself (Ramachandran et al., 2009). Though, also the sphingosine was proposed as endogenous σ_1 mediator.

To date, it appears clearer and clearer that σ receptors possess high affinity for many endogenous molecules and numerous research groups share the opinion that these enigmatic receptors could be regulated by a

broad spectrum of endogenous products. Moreover, since the involvement of σ receptors in the modulation of several subcellular effectors has been also reported (Section 1.6), the hypothesis that the σ receptors could affect many biological functions is widely accepted. Even the term “ σ endogenous ligand” is disappearing from literature, substituted by the less specific definition of “ σ endogenous regulator”.

1.6 Allosteric modulation of the σ_1 receptors

The anticonvulsivant drug phenytoin (DPH) is known to allosterically modulates the binding of σ_1 radioligands without affecting σ_2 radioligands (Quirion et al., 1992). However, DPH not only discriminates between σ_1 and σ_2 ligands, but it is also able to differently modulate the activity of σ_1 ligands. In fact, DPH increased the binding of [3 H]dextromethorphan, [3 H](+)-SKF-10,047, [3 H](+)-3-PPP and [3 H](+)-pentazocine (Musacchio et al., 1987, 1988, 1989a and b; Craviso and Musacchio, 1983; Karbon et al., 1991; Bailey and Karbon, 1993; McCann and Su, 1991; Culp et al., 1992; Rothman et al., 1991; Bonhaus et al., 1993; Chaki et al., 1996; DeHaven-Hudkins et al., 1993), but not the binding of σ_1 [3 H]haloperidol, [3 H]progesterone, [3 H]DTG, [3 H]DuP 734, [3 H]RS-23597-190 and [3 H]-N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy) phenyl]-ethylamine ([3 H]NE-100) (Karbon et al., 1991; Meyer et al., 1998; Culp et al., 1992; Bonhaus et al., 1994; Tanaka et al., 1995; Chaki et al., 1996).

It was initially proposed that the differential sensitivity of σ_1 compounds to the DPH allosteric modulation should be restricted to compounds that bind selectively the σ_1 receptor subtype (DeHaven-Hudkins et al., 1993). However, this hypothesis is not entirely consistent with the

more recent data on the σ ligands selectivity patterns. The newest researches demonstrated that the allosteric modulation by DPH of the affinity of σ_1 receptor ligands depends on the agonist or antagonist profile of the ligand (Cobos et al., 2006).

1.7 Modulation of cellular effectors by σ_1 receptors

1.7.1 Is the σ_1 coupled to the G-proteins?

One of the earliest issues regarding the cellular effects of the σ_1 receptors was their possible coupling to G-proteins. Even if this idea has been investigated with many different approaches, to date, the results are still contradictory (Bermack and Debonnel, 2005; Guitart et al., 2004).

With the availability of the cloned σ_1 receptor and a deeper knowledge of its molecular conformation (fig.1.2 and 1.3), it was suddenly clear that the σ_1 doesn't possess at all the typical structure of a G-protein-coupled receptor (with seven transmembrane domains). Even so, the literature is still filled with controversial data. For example, it has been reported that G-proteins mediate some electrophysiological and behavioural responses of σ_1 ligands (Soriani et al., 1998, 1999a; Bergeron et al., 1996; Ueda et al., 2001) whereas, in other experimental models, some σ_1 agonists, such as (+)-pentazocine, (+)-SKF-10,047, (+)-3-PPP and BD-737 were unable to increase GTPase activity in rat brain membranes (Hong and Werling, 2000; Odagaki et al., 2005).

The existence of both metabotropic and non-metabotropic σ_1 receptor subtypes was also proposed (Maruo et al., 2000). However, unequivocally

proofs of the existence of this hypothetic σ_1 metabotropic subtype have not yet been carried out.

1.7.2 σ_1 modulation of PLC-PKC-IP₃ Ca²⁺ release system: intracellular dynamics following the σ_1 receptors activation

It is known that the ER can work as calcium (Ca²⁺) deposit and, under certain stimuli, it can release the Ca²⁺ in the cytoplasm (Hayashi and Su, 2000), or directly to mitochondria (Hayashi and Su, 2007). The ankyrins are a member of a family of cytoskeletal adapter proteins that interconnect membrane proteins with the cell cytoskeleton (Bennett and Stenbuck, 1979), they are present on the ER, on the plasma membrane and on the Golgi complex (De Matteis and Morrow, 1998; Tuvia et al., 1999) and they are known to affect the Ca²⁺ efflux interacting with inositol 1,4,5-trisphosphate receptors (IP₃Rs) (Chien and Pasternak, 1994; De Waard et al., 1997).

In the 2000 Hayashi and Su first described the role of σ_1 receptors in regulating the Ca²⁺ efflux from the ER storages, physically interacting with the ankyrin-IP₃Rs complex.

The regulation of the Ca²⁺ signalling by σ_1 receptors is a complex one and involves the translocation of σ_1 receptors from the lipid rafts of the ER to the plasma membrane, to the nucleus and, partially, σ_1 can also dislocate around the whole cell without leaving the ER. At the resting state, in the previously described highly clustered globular structures, the σ_1 receptors form a complex with the ankyrin (specifically the ANK220 isomer) and the IP₃Rs (Hayashi and Su, 2001) The σ_1 receptors activation by agonists induces the dissociation of the σ_1 receptor-ANK220 complex from the IP₃Rs channel (Hayashi and Su, 2001), potentiating the calcium efflux induced by the 1,4,5-trisphosphate (IP₃) in a bell-shaped curve fashion (Hayashi et al.,

2000). This issue directly correlates the σ_1 receptors to the known PLC/PKC/IP₃ Ca²⁺ release mechanism. In fact, the activities of the receptors that activates the PLC system, enhancing the inositol IP₃ cytoplasmatic concentration level, such as receptors for bradykinin and brain-derived neurotrophic factor (TrkB) (Hong et al., 2004; Peeters et al., 2004; Yagasaki et al. 2006), are undoubtedly affected by the σ_1 activation.

In the NG-108 cell line, the enhancement of Ca²⁺ efflux has been reported not only when treating the cells with the known selective σ_1 agonists such as PRE 084 or (+)-pentazocine, but also when using some neurosteroids, such as pregnenolone sulfate and DHEA (Hayashi et al., 2000, Hong et al., 2004). The destiny of the released σ_1 receptor/ANK220 complex is still unknown, but, as described later on the introduction (section 1.7.3), it is clear that the σ_1 receptors could modulate many other systems by protein-protein interactions. Interestingly, once activated, σ_1 receptors are also able to reach the plasma membrane and they can even be exocytosed (Hayashi and Su, 2003a). Though, so far, it's not possible to exclude the hypothesis that the σ_1 receptor/ANK220 complex could bind other protein systems, modulating their functionality.

In the presence of a σ_1 antagonist, σ_1 receptors dissociates from the ANK220, which, in turn, remains associated to the IP₃Rs (Hayashi and Su, 2001). In this case, the ANK220 is still able to counteract the bradykinin-induced Ca²⁺ efflux and the σ_1 agonist-induced Ca²⁺ release potentiation is completely prevented. This latter effect has been also observed after the pre-treatment with progesterone and with a specific σ_1 receptor antisense oligodeoxynucleotide (Hayashi et al., 2000, Hong and Werling, 2002). Additionally, *in vivo*, the silencing of IP₃Rs receptors induces a decrease of the σ_1 receptor mRNA levels (Novakova et al., 2007), suggesting a putative

cross-talking between the two systems. The Hayashi and Su model is illustrated in figure 1.4.

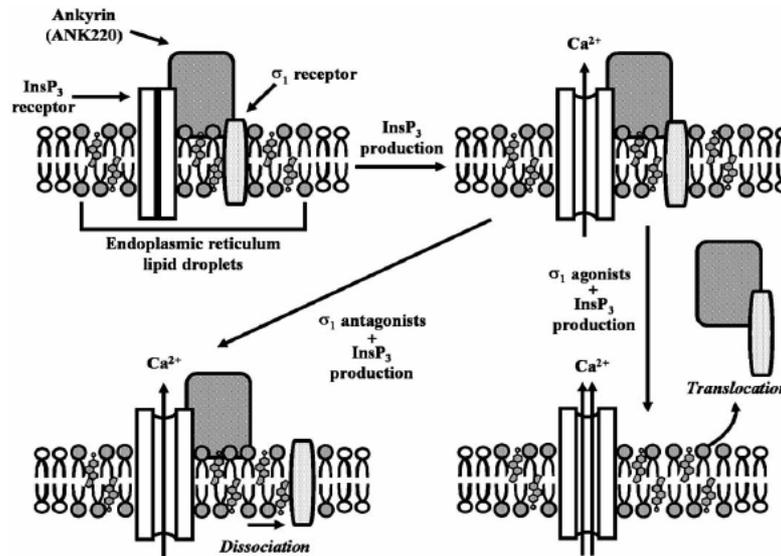


Figure 1.4. The σ_1 receptors regulation of the ankyrin-IP₃Rs complex at ER level (image taken from Cobos et al., 2008)

Since the Ca²⁺ intracellular concentration ($[Ca^{2+}]_i$) is one of the most important second messengers, it is likely to hypothesize that the σ_1 receptors are able to affect many others proteins systems through the modulation of the Ca²⁺ concentration itself.

The physical association between the ER and mitochondria, which is known as the mitochondria-associated ER membrane (MAM), has been also described in literature (reviewed in Hayashi et al., 2009) The MAM plays important roles in various cellular ‘housekeeping’ functions including the non-vesicular transport of phospholipids (reviewed in Hayashi et al., 2009). The σ_1 receptors were found also in the MAM; at this level, the σ_1 receptors play a role in the regulation of the direct Ca²⁺ exchanges between the ER and the mitochondria (Hayashi and Su, 2007). The mechanism is a very complex one and it’s well reviewed in Hayashi et al., 2009.

1.7.3 Modulation of plasmalemma ion channels

1.7.3.1 Calcium channels

In addition to the effects discussed in the section 1.7.2, σ_1 receptors are able to regulate the $[Ca^{2+}]_i$, triggering the plasmalemma voltage dependent Ca^{2+} channels.

Church and Fletcher first reported in 1995 a σ_1 interference, while studying the variation of the $[Ca^{2+}]_i$ in cultured hippocampal pyramidal neurons preparations (Church and Fletcher, 1995). In their model, several σ_1 ligands, such as haloperidol, BD-737, carbetapentane, ifenprodil, rimcazole and dextromethorphan, blocked the increase of $[Ca^{2+}]_i$ mediated by high-voltage-activated channels (Church and Fletcher, 1995). σ_1 ligands were also found to inhibit the depolarization-induced increasing in the $[Ca^{2+}]_i$ in primary cultures of neurones and forebrain synaptosomes (Klette et al., 1997; Brent et al., 1996 and 1997). However, in these experiments σ_1 agonists and antagonists produced the same effects which might be also due to the involvement of the σ_2 receptors subtype (reviewed in Monnet, 2005). In another series of experiments performed in rat forebrain synaptosomes, the (+)-pentazocine, the (-)-pentazocine, the BD-1008 and the DTG not only inhibited the raising of the Ca^{2+} levels induced by the depolarization, but they also reduced the basal $[Ca^{2+}]_i$, indicating that the σ receptors are able to directly affect the $[Ca^{2+}]_i$ (Brent et al, 1996 and 1997). These latter effects were prevented by the pretreatment with the σ_1 antagonist rimcazole.

The involvement of σ_1 in the Ca^{2+} plasmalemma channels regulation has been further confirmed. Hayashi and Su reported that, in the NG-108 cell line, the σ_1 agonist (+)-pentazocine inhibits the $[Ca^{2+}]_i$ increase induced by KCl depolarization, whereas another σ_1 agonist, PRE-084, induce the

opposite effects, potentiating the KCl $[Ca^{2+}]_i$ increase (Hayashi and Su, 2000). Since the pretreatment with the σ_1 receptor antisense oligodeoxynucleotide blocked the effects of both the molecules, it has been ruled out that the σ_1 is the subtype involved in the modulation of the plasmalemma Ca^{2+} channels (Hayashi and Su, 2000).

1.7.3.2 Potassium channels

In different research models, potassium (K^+) channels have been reported to constitute one of the σ receptors target. The σ_1 selective ligands (+)-pentazocine and igmesine (JO-1784) were firstly reported as negative modulators of several K^+ currents in frog melanotropic cells (Soriani et al., 1998, 1999a and b). The effects described in these latter experiments seem to be mediated by a G_s -protein (Soriani et al., 1998, 1999a). However, in the rat neurohypophysial terminals, the voltage-gated K^+ channels are inhibited by the σ agonists (\pm)-pentazocine and (\pm)-SKF-10,047, despite intra-terminal perfusion with GTP-free solutions, a G-protein inhibitor (GDP β S), a G-protein activator (GTP γ S) or a non-hydrolysable ATP analogue (AMPPcP) (Lupardus et al., 2000). Though, Lupardus and co-workers excluded that the G-proteins could mediate the σ signalling (Lupardus et al., 2000). The same research group also reported that the K^+ channels expressed in oocytes without the σ_1 receptors are unresponsive to the σ agonists, while, when both the channel and the σ_1 were co-expressed, the K^+ current can be inhibited while triggering the σ_1 receptors (Lupardus et al., 2000). These findings indicate that the inhibition of K^+ channels by σ receptor agonists does not arise from direct interactions of these ligands with the channel itself. The involvement of the G-proteins has been also excluded while studying the effects of σ receptor activation on voltage-activated K^+

channels and action potentials in isolated rat intracardiac neurons, using the whole-cell patch-clamp recording techniques (Zhang and Cuevas, 2005). In this research, different types of voltage-activated K^+ channels were inhibited by several σ ligands and the observed blockade was not sensitive to the application of $GDP_{\beta}S$ (Zhang and Cuevas, 2005). The observed inhibition of K^+ channels is dose-dependent, and the rank order potency of (+)-pentazocine > ibogaine > DTG suggests that the effects are mediated by the σ_1 receptor subtype activation.

Since the G-protein doesn't appear to be the signalling pathway connecting the σ_1 receptors to the K^+ currents, to date the exact mechanism of this regulation remains still unknown; however, a physical protein-protein interaction between σ_1 and the K^+ channel has been observed to occur. Aydar and co-workers (Aydar et al., 2002) studied, in *Xenopus oocytes*, the heterologous expression of the σ_1 receptors to explore the effects on K^+ channels Kv 1.4 and 1.5. While confirming that the σ_1 ligands modulate the K^+ channel functions, this study also led to the discovery that Kv 1.4 and 1.5 channel coimmunoprecipitate with the σ_1 receptor (Aydar et al., 2002). Therefore, it was proposed that the two protein could form a stable macromolecular complex with functional implications (Aydar et al., 2002).

The σ_1 modulation of K^+ currents, as well as the Ca^{2+} currents suggests that σ_1 receptors could form complexes with several other proteins. The hypothesis that these complexes could be involved in many different processes is in line with the observed wide variety of actions that the σ_1 ligands produce in the CNS and, partially, it might also explain some of the discrepancies found in the literature (reviewed in Bermack and Debonell, 2005).

1.7.3.3 Sodium channels

Cheng and co-workers (Cheng et al., 2008) found out that, in rat medial prefrontal cortex slices, the σ_1 receptor agonist DHEA sulfate inhibits persistent sodium (Na^+) currents. The observed inhibitory effect is ameliorated by G_i protein inhibitors and protein kinase C (PKC) inhibitors and it is blocked by σ_1 receptor antagonists (Chang et al., 2008). These results indicate the existence of a sodium current-controlling mechanism via the σ_1 receptor- G_i protein-PKC signaling pathway in cortical neurons (Cheng et al., 2008).

1.8 Functions of σ_1 receptors on ER lipid rafts

As previously described (section 1.4.2.), on the ER the σ_1 receptors are highly clustered in globular structures enriched in cholesterol and neutral lipids, similar to the lipid rafts (Hayashi and Su, 2003a; Hayashi and Su, 2003b; Hayashi and Su, 2007). At this level, they are proposed to regulate the lipid storage sites and the turnover of the lipid droplets associated with the ER (Hayashi and Su, 2005a). The endoplasmic reticulum lipid droplets are formed by coalescence of neutral lipids into discs inside the bilayer of the ER membranes. When they reach a critical size, they bud off to form cytosolic lipid droplets (Murphy and Vance, 1999), which are proposed to be a novel transport pathway of lipids between the endoplasmic reticulum and Golgi apparatus or plasma membrane (Ohashi et al., 2003) that can be mediated by σ_1 receptors (Hayashi and Su, 2003a and 2005b). This σ_1 feature might explain how the σ_1 receptors can interact with so many

neurotransmitter systems (see next section), whose receptors are located at the plasma membrane level.

The over-expression of the σ_1 receptors in PC12 and NG-108 cell lines also alters the functional lipid composition of the ER lipid rafts. First of all, the cholesterol contents are appreciably increased in the σ_1 transfected cells, showing that the σ_1 upregulation would potentiate the lipid rafts formation (Takebayashi et al., 2004a; Hayashi and Su, 2005a). Secondly, the overexpression of σ_1 alters the proportion of some critical lipid raft-forming gangliosides, which have been previously proposed to be involved in the re-localization of many cytoplasmatic proteins (Simons and Ikonen, 1997). Among other lipid rafts proteins, the growth factors receptors appears to be especially triggered by the σ_1 receptors activation. In fact, it was reported that, in a classical agonist/antagonist fashion, the σ_1 ligands could alter the action of the brain-derived neurotrophic factor (BDNF) on the glutamatergic neurotransmission (Yagasaki et al., 2006) and they can enhance the nerve growth factor-induced neurite sprouting in stable σ_1 transfected PC12 cells (Takebayashi et al., 2002). Moreover, while using a σ_1 dominant-negative model, the not functional σ_1 receptor attenuates the differentiation of the transfected rat primary hippocampal cultured cells (Hayashi and Su, 2004b), reinforcing the role of σ_1 receptors on cell differentiation.

1.9 Modulation of others major neurotransmitter systems

1.9.1 Modulation of the adrenergic system

Early studies showed that both in rat brain synaptosomes and in cultured bovine chromaffin cells, a mixed spectra of σ_1 agonists and antagonists (Haloperidol, (+)-3PPP, (+)-SKF-10,047 and DTG) are able to presynaptically inhibit the [3 H]norepinephrine reuptake (Rogers and Lemaire, 1991).

Also the NMDA-stimulated [3 H]norepinephrine release is affected by σ_1 receptors. In isolated rabbit iris-ciliary body, flunarizine and (+)-pentazocine inhibited [3 H]norepinephrine release and this action was sensitive to the NE-100 antagonism (Campana et al., 2002). On the other hand, in rat hippocampal slices, σ agonists, both σ_1 and non- σ_1 selective, potentiate NMDA-induced [3 H]norepinephrine release. In this same experimental paradigm, the σ_1 antagonist BD-1063 and the haloperidol prevent the effects of the agonists, without affecting the basal values of the NMDA-induced [3 H]norepinephrine release (Monnet et al., 1992a, 1995 and 1996).

Neurosteroids were again showed to be involved in the σ_1 receptors dynamics. In fact, DHEA, wich in many experiments acts as σ_1 selective agonist, has been shown to potentiate NMDA-evoked [3 H]norepinephrine release. This effect is reverted by the σ_1 antagonists BD-1063 and progesterone and also by haloperidol (Monnet et al., 1995)

1.9.2 Modulation of cholinergic system

Behavioral effects of the σ receptors, such as cognition and memory, connect these protein to the regulation of cholinergic processes.

In vivo microdialysis studies, subcutaneous applications of both σ_1 receptors agonist (+)-SKF-10,047 and the non selective σ ligand DTG were shown to display positive effects on the release of acetylcholine from rat prefrontal cortex; the observed effect is reverted by NE-100 and haloperidol (Matsuno et al., 1992, 1993, 1995a). Moreover, in line with the classical σ_1 pharmacology, (+)-SKF-10,047 induces a greater effect than its enantiomer, (-)-SKF-10,047.

Using the same experiment model, it was found that several σ ligands like (+)-3PPP, SA-4503, (+)-pentazocine, (+)-SKF-10,047, and DTG increase the spontaneous acetylcholine release in the hippocampus (Kobayashi et al., 1996a and b), in the frontal cortex (Kobayashi et al., 1996b), but not in the striatum (Kobayashi et al., 1996a). These results also give important informations about the CNS distribution of σ_1 receptors. In addition, the σ_1 agonists JO-1784, (+)-SKF-10,047 and SA-4503 positively affect the KCl-induced [3 H]acetylcholine release from rat hippocampal slices (Junien et al, 1991; Horan et al., 2002). This effect is antagonized by the presence of haloperidol and DTG alone is able to inhibit the KCl-stimulated [3 H]acetylcholine release (Junien et al., 1991).

The ability to regulate cholinergic system, exhibited by some σ ligand, confirms that these receptors are an important target since the dysregulation of the cholinergic system could have an enormous therapeutic potential in the area of Alzheimer's disease and of others forms of dementia.

1.9.3 Modulation of serotonergic neurotransmission

In the last decade, an increasing number of studies have been aimed to describe the involvement of σ receptors in the modulation of serotonergic neurotransmission.

In rat, a two days treatment with 4-(N-benzylpiperidin-4-yl)-4-iodobenzamide (4-IBP) and (+)-pentazocine produces a ~35% increase in the basal firing rate of 5-HT neurons, compared to saline-treated animals (Bermack and Debonnel, 2001). Since JO-1784 and PRE-084 failed to increase the firing activity of serotonergic neurons, this difference has been ascribed to the existence of two different subtypes of σ_1 receptors, defined metabotropic and non-metabotropic (Bermack and Debonnel, 2001). Thereafter OPC-14523, which is known to bind both the σ_1 and 5HT_{1A} receptors (Oshiro et al., 2000; Tottori et al., 2001), was shown to increase the firing activity of serotonergic neurons (Bermack et al., 2004). Since NE-100 prevents the serotonergic OPC-14523 stimulation, it has been postulated that this effect is probably mediated by σ_1 receptors (Bermack et al., 2004).

Moreover, neurosteroids were proposed to modulate the serotonergic system via σ_1 receptor activation. In fact, in female rats dorsal raphe nucleus, subchronic administration of allopregnelone and DHEA increases the firing activity of the serotonergic neurons (Robichaud and Debonnel, 2004 and 2006), whereas the putative σ_1 antagonist progesterone alone has no effect (Robichaud and Debonnel, 2004).

In summary, the activation of σ_1 receptors seems to enhance the 5-HT neurones firing activity. These findings suggest that σ_1 ligands and some neurosteroids might represent interesting adjuvants in the treatment of mood disorders.

1.9.4 Modulation of GABAergic system

To date, few studies have described the effects of σ_1 ligands on GABAergic neurotransmission. They all seem to indicate an inhibiting role of σ_1 receptors. DTG has been reported to suppress the firing rate of GABAergic interneurons in anaesthetized rats (Zhang et al., 1993a). (+)-SKF-10,047, serotonin and trazodone inhibit the KCl-evoked GABA release in rat mossy fibre cerebellar synaptosomes, but only the effects of (+)-SKF-10,047 and trazodone are prevented by pretreatment with BD-1047 and 3-PPP (Garrone et al., 2000). Nanomolar concentrations of neurosteroids have been also found to interfere with GABA neurotransmission via σ_1 receptors. In fact, in cultured hippocampal pyramidal neurons, the pregnenolone sulfate inhibits GABAergic synaptic transmission by a presynaptic effect (Mtchedlishvili and Kapur, 2003) and this effect is counteracted by BD-1063 and haloperidol.

Even if these preliminary informations were reported in literature, more studies are necessary to confirm a putative role of σ_1 in GABAergic neurotransmission modulation.

1.9.5 Modulation of glutamatergic system

The connection between σ_1 receptors and glutamatergic system has been often reported. Among all glutamate-gated ion channels (NMDA, kainate and AMPA receptors), σ_1 regulation of the NMDA receptor subtype has been deeper investigated. Moreover, adopting *in vivo* paradigms, some σ ligands have been shown to produce the same effects of the NMDA receptor agonists, such as mood, motricity and memory alterations (Maurice and Lockhart, 1997).

In electrophysiological models, the application of σ ligands enhances the responsiveness of pyramidal neurons in hippocampal CA₁ and CA₃ regions to microiontophoretic applications of NMDA (Bergeron et al., 1997). Since the NMDA potentiation effects of JO-1784 and DTG are prevented by pre-treatment with pertussis toxin, but not the effects of (+)-pentazocine, again, the two different patterns of data support the possibility of the co-existence of two different types of σ_1 receptors, metabotropic and non-metabotropic (Monnet et al., 1994).

How the NMDA receptor-mediated activity is modified by σ ligands is yet not known. While a σ binding site on the NMDA receptor/channel has been postulated, autoradiographic and radioligand binding studies suggest that a colocalization between NMDA and σ sites is likely to exclude (Largent et al., 1986, Monaghan et al., 1989).

In the 90's, several researchers believed that neurosteroids were the endogenous ligands of σ_1 receptors; for this reason neurosteroid-induced potentiation of the NMDA-evoked responses has been extensively studied. In rat CA₃ pyramidal neurons, low doses of DHEA potentiate the NMDA response selectively and in a dose-dependent manner. The effect of DHEA is reversed by the selective σ_1 antagonist NE-100 and by haloperidol (Bergeron et al, 1996;. Debonnel et al., 1996). Progesterone have no effect by itself but, at low doses, it reverses the potentiation of NMDA response induced by DHEA as well as those induced by other nonsteroidal σ ligands: (+)-pentazocine, JO-1784 and DTG (Bergeron et al, 1996). The σ potentiation of NMDA response in the CA₃ region of rat dorsal hippocampus is significantly greater in ovariectomized rats, a classical progesterone-lacking model, than in males and in non-ovariectomized females (Debonnel et al., 1996; Bergeron et al., 1996). In addition, in pregnant rats, where progesterone production is greater enhanced, the

potentiation of neuronal response to NMDA requires 10-fold higher doses of DTG, (+)-pentazocine and DHEA than in female control rats (Bergeron et al., 1999). These results indicate that endogenous progesterone, acting as a σ_1 antagonist, might induce a tonic decrease in NMDA receptor function. More recent studies have shown that the neurosteroid DHEA also enhances glutamatergic neurotransmission in hippocampus *via* σ_1 receptors. In fact, chronic administration of DHEAs facilitates the induction of long-term potentiation and this effect is reversed by σ_1 antagonists NE-100 and haloperidol (Chen et al., 2006).

The glutamate receptor-induced increase of $[Ca^{2+}]_i$ is also modulated by σ_1 ligands. In early studies, some σ ligands like JO-1784, (+)-pentazocine, (+)-SKF-10,047, carbetapentane, dextromethorphan, (+)-cyclazocine, DTG and haloperidol were reported to reduce NMDA-stimulated increase of the $[Ca^{2+}]_i$ (Hayashi et al., 1995; Klette et al., 1997). On the contrary, more recent studies have documented that σ_1 ligands are able to enhance NMDA-evoked increase of $[Ca^{2+}]_i$. For example, the potentiation of glutamate response in pyramidal neurons is increased by σ_1 agonists JO-1784, (+)-pentazocine and (+)-SKF-10,047. This σ_1 effect is reversed by both the σ_1 antagonist NE-100 and the PKC inhibitor Gö-6976, suggesting that the modulation of NMDA responses involves somehow the PLC-PKC signalling cascade (Monnet et al., 2003).

σ ligands could also regulate the glutamate release. In fact, in rat cultured cortical neurons, not only the glutamate release evoked by BDNF stimulation of the PLC γ is enhanced in an agonist/antagonist fashion by the σ agonists imipramine and fluvoxamine, but the over-expression of σ_1 receptors itself is sufficient to enhance the induction of the PLC γ pathway by BDNF and, hence, the glutamate release (Yagasaki et al., 2006).

Up to now, it is commonly accepted that σ_1 receptors enhance NMDA-mediated effects. Since the glutamatergic pathways are widely distributed in the CNS, the interactions of σ ligands with the glutamate systems might be crucial in several mental pathologies, including depression and psychosis.

1.9.6 Modulation of the dopaminergic neurotransmission

DA neurotransmission has been often shown to constitute an important target of σ receptors in different experimental models. Since the reported results are frequently contradictory, the exact role of σ_1 receptor in dopaminergic neurotransmission remains unclear.

Early electrophysiological studies have reported that intravenous (i.v.) administration of σ agonists (+)-3-PPP, (+)-pentazocine and DTG decreases the firing rate of rats DA neurons (Steinfels et al., 1989). Interestingly, the σ antagonist BMY-14802 alone increases the firing rate in the same brain areas (Steinfels et al., 1989). In a similar model, it has been shown that i.v. administration of (+)-pentazocine is more effective in increasing the mesolimbic DA cell firing rate than the nigrostriatal one (Zhang et al., 1992); however, DTG and JO-1784 did not alter DA neuronal activity at nontoxic doses (Zhang et al., 1992). In this same study, the σ antagonist BMY-14802 was able to cause a dose-dependent increase of DA cell firing rate in both the brain regions (Zhang et al., 1992). (+)-3-PPP is able to inhibit both nigrostriatal and mesolimbic DA cell firing rates, but these effects are reversed by (-)-eticlopride and (+)-butaclamol, suggesting that 3-PPP-induced inhibition could be ascribed at the DA agonist properties of the drug (Zhang et al., 1992). More recently, Minabe and co-workers (Minabe et al., 1999) reported that acute administration of the σ_1 agonist SA-4503 produces a significant decrease in the number of spontaneously active

neurons of the substantia nigra pars compacta (SNPC), whereas, in the ventral tegmental area (VTA), the same compound induces the opposite effect, enhancing the firing rate of the DA neurons. Further data have confirmed the involvement of σ_1 receptors, since the σ_1 selective antagonist NE-100 is able to reverse both these effects (Minabe et al., 1999). In contrast, other studies reported that one-hour pretreatment with the σ agonists (+)-pentazocine, DTG or JO 1784 (Zhang et al., 1993b) and with the σ_1 antagonist E-5842 (Sánchez-Arroyos and Guitart, 1999) do not alter the number of spontaneously active DA neurons, both in SNPC and in VTA (Zhang et al., 1993b; Sánchez-Arroyos and Guitart, 1999).

Interestingly, a model of chronic administration led to a different pattern of data. Repeated treatments with DTG and with σ_1 agonists (+)-pentazocine (Zhang et al., 1993b) and SA-4503 (Minabe et al., 1999) produces a significant increase in the number of spontaneously active VTA DA neurons (Zhang et al., 1993b; Minabe et al., 1999), whereas, in the same area, the σ_1 antagonist E-5842 induces the opposite effect, reducing the firing rate of DA neurons (Sánchez-Arroyos and Guitart, 1999). In addition, in the SNPC, repeated administration of JO-1784 (Zhang et al., 1993b) and SA-4503 (Minabe et al., 1999) decrease the number of the spontaneously active DA neurons and the E-5842 is devoid of activity (Sánchez-Arroyos and Guitart, 1999).

In summary, the *in vivo* electrophysiological studies have shown that DA neurones responses to σ ligands might be dissimilar among different brain areas and they can also be affected by the extent of the treatment.

Since the observed fluctuations in the firing rate of the DA neurons should be mediated by the enhancement of extracellular [DA], several investigations have been carried out to determine if σ receptors could directly regulate the intersynaptic concentrations of the major dopaminergic

neurotransmitter. From these studies, it has been brought to the light that the σ ligands deeply modify the extracellular [DA].

First solid evidences were reported by Gudelsky in 1995 (Gudelsky, 1995). In fact, he observed that, following systemic administration of (+)-pentazocine and (+)-SKF-10,047 (but not its (-)-enantiomer), striatum and medial prefrontal cortex extracellular [DA] increase of about 50% of the basal values in the first sixty minutes, followed by a rapid decrease (Fig.1.5 A) (Gudelsky, 1995).

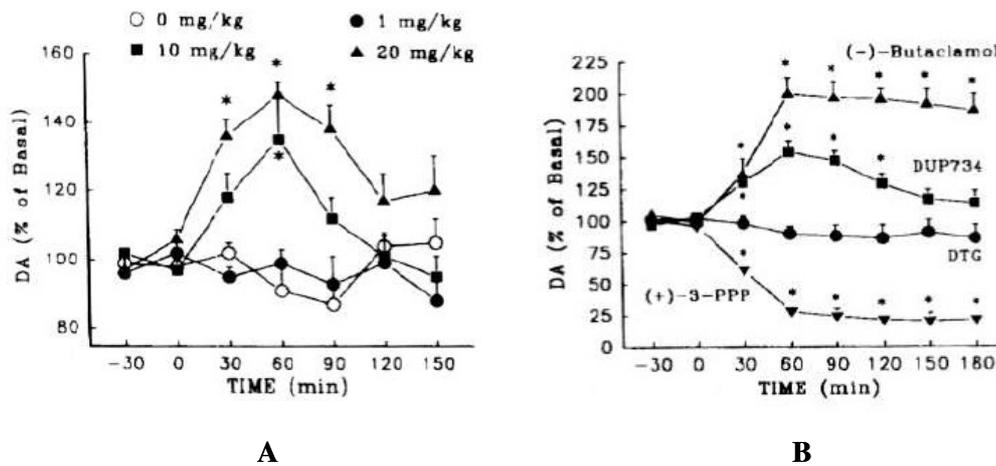


Figure 1.5. Effect on the extracellular [DA]. **A)** (+)-pentazocine concentration dependent increase of the extracellular [DA] (Gudelsky, 1995) **B)** Different effects of other ligands: dopamine receptors antagonist (-)-butaclamol, σ_1 antagonist Dup734, σ agonist DTG and σ_1 agonist (+)-3-PPP (Gudelsky, 1995).

These effects are shared also by the σ_1 antagonist Dup734 and by the DA receptor antagonist (-)-butaclamol (Fig.1.5 B), but (-)-butaclamol pharmacological profile appears quite different from the one of the σ ligands, as the DA receptors antagonist produces a major extracellular [DA] increase, followed by a much slower decrease. This could indicate that the σ ligands and the (-)-butaclamol act at a different level in the extracellular [DA] regulation; in fact, since the (-)-butaclamol directly antagonizes the DA receptors, the observed increase in the DA release could be

physiologically induced for counteracting the blockade of the dopaminergic transmission. However, in the same experiment, the extracellular [DA] was unaffected by the systemic administration of DTG and it was suppressed by the σ_1 agonist (+)-3-PPP (Gudelsky, 1995; Kanzaki et al., 1992); the (+)-3-PPP effect was reversed by the preadministration of the σ_1 antagonist BMY-14802, whereas this last ligand alone did not affect the extracellular levels of DA (Kanzaki et al., 1992). Further studies clarified that the extent of the administered dose could greatly affect the profile of action of the σ ligands. In fact, in rats, the intra-striatal administration of lower doses of (+)-pentazocine, DTG and a σ selective ligand MR200 induce a decrease of the extracellular [DA] (Fig.1.6 A and B) (Gudelsky 1999; Moison et al., 2003), but, when administered at higher doses, these ligands induce a biphasic effect, deeper discussed in the next section (see section 1.9.7) and consisting of a rapid increase of the extracellular [DA], followed by a prolonged and marked decrease (Fig.1.6 A and B) (Gudelsky 1999; Moison et al., 2003). Because the excitatory responses among the pharmacological effects induced by σ ligands occur only when these drugs are administered at higher concentrations, it has been speculated that the observed biphasic effect is due to the aspecific involvement of other receptors that regulate the DA release (Gudelsky 1999; Moison et al., 2003). Thus, the negative regulation of extracellular [DA] appears to be a more specific effect of the σ ligands than the increase of the DA levels (Gudelsky 1999; Moison et al., 2003).

Other studies have confirmed that the σ_1 receptors are able to inhibit DA release. The σ_1 agonists (+)-pentazocine and BD-737 inhibited the KCl-stimulated [3 H]DA release of about the 40% of the basal values both in slices of rat nucleus accumbens and in SH-SY5Y (Ault and Werling, 1999 and 2000). The σ_1 antagonists Dup734 and BIMU-8 were able to block these effects (Ault and Werling, 1999 and 2000).

In line with all the above described results indicating that σ_1 receptors may have an inhibitory role in regulation of extracellular [DA], more recent studies also indicate that repeated administration of the σ_1 selective agonist PRE-084 causes important adaptive changes in the striatal dopaminergic transmission (Peeters et al., 2004). In fact, the chronic administration of PRE084 results in an increase of DA-induced GTP γ S binding and this effect is reversed where both the PRE-084 and the σ_1 antagonist BD-1047 are chronically co-administrated (Peeters et al., 2004). Thus, probably, the observed increase of DA receptors could be a neuronal physiological adaptation induced by the lack of free DA in the synaptic cleft (Peeters et al., 2004).

It is well documented that σ_1 receptors are able to modulate NMDA receptor subtype responses (see section 1.9.5). Among the major NMDA-mediated functions, σ_1 receptors have been shown to be effective in regulating NMDA-stimulated DA release.

Using *in vivo* microdialysis techniques, it has been shown that systemic administration of MS-377, a σ_1 antagonist, has no effect alone, but it reduces the increase of extracellular [DA] induced by PCP (Takahashi et al., 2001). The effects of MS-377 on NMDA-induced current were also determined in acutely dissociated DA neurons of VTA area, using patch clamp whole cell recording (Yamazaki et al., 2002). In this model, the σ_1 antagonists MS-377, haloperidol, NE-100 and BD-1063 were able to attenuate the NMDA-evoked currents (Yamazaki et al., 2002).

The NMDA-stimulated [3 H]DA release has been also investigated. In experiments on rat striatum slices, the σ agonists (+)-pentazocine, (+)-SKF-10,047, BD-737 and pregnenolone sulfate inhibited NMDA-stimulated [3 H]DA release. Interestingly, in the same studies, the putative σ_1 antagonist progesterone behaved like the agonists (Gonzalez-Alvear and Werling,

1994, 1997; Nuwayhid and Werling, 2003a and b). Moreover, in these studies the inhibition of NMDA-stimulated [³H]DA release, observed with the σ_1 ligands, including the progesterone, was reversed by the σ_1 antagonists DuP 734 and BD-1008 (Gonzalez-Alvear and Werling, 1994, 1995, 1997; Nuwayhid and Werling, 2003a and b). Experimental evidence indicate the PLC-PKC signalling system as preferring mechanism by which the σ_1 receptors mediate their modulating effects on the glutamatergic NMDA receptors; in fact, pre-treatments with the PKC $_{\beta}$ selective inhibitor LY379196 and the PLC inhibitor U-73,122 (Nuwayhid and Werling, 2003a and b), completely abolish the inhibition of NMDA-evoked [³H]DA release induced by σ receptor ligands.

The modulation of NMDA-stimulated [³H]DA release has been also studied in nucleus accumbens and in cortical slices. In both preparations, σ_1 agonists (+)-pentazocine and BD-737 inhibit [³H]DA release (Ault et al., 1998; Ault and Werling 1998). The observed inhibitory effect of σ_1 agonists was reversed by the non selective antagonist BD-1008, DTG and also by the σ_1 antagonist DuP 734 (Ault et al., 1998; Ault and Werling 1998), but not by the σ_2 antagonist BIMU-8 (Ault et al., 1998; Ault and Werling 1998), suggesting that, in these experiments, σ_1 receptors may modulate NMDA receptors.

As it concerns σ_2 receptors, also this receptor subtype has been demonstrated to modulate the extracellular levels of DA in amphetamine-stimulated [³H]DA release experiments. In fact, in this model, the enhancement of amphetamine-mediated responses was reversed by the non-selective σ antagonist BD-1008 and by the selective σ_2 antagonists BIMU-8 and Lu28-179 (Izenwasser et al., 1998; Weatherspoon and Werling, 1999) but not by the selective σ_1 antagonist DuP 734 (Izenwasser et al., 1998; Weatherspoon and Werling, 1999).

In summary, although in the studies described above, contradictory results have been often reported, σ_1 receptors seem to exert an inhibitory role in the regulation of extracellular [DA].

The effect of σ_1 ligands on the levels of DA metabolites in the cortex and striatum have been also investigated.

Studies with *in vivo* brain microdialysis techniques found that some σ_1 agonists, such as (\pm)-pentazocine and (+)-SKF-10,047, increase extracellular dihydroxy phenylacetic acid (DOPAC) levels in the striatum but not in the frontal cortex (Matsuno et al., 1995b) and panamesine, a putative σ_1 antagonist, increases extracellular DOPAC levels in the prefrontal cortex (Skuzza et al., 1998); σ_1 antagonists MR-200 and BMY-14802 do not modify extracellular DOPAC levels in the striatum (Moison et al., 2003; Kanzaki et al., 1992). Using the same experiment paradigm, also DTG was shown to increase extracellular DOPAC levels after intra-nigral administration (Bastianetto et al., 1995), however, in following researches, this result hasn't been further confirmed (Moison et al., 2003).

The σ_1 ligands modulation of the levels of extracellular homovanillic acid (HVA), another DA metabolite, has also been evaluated. σ_1 antagonist BMY-14802 doesn't affect HVA levels in the striatum (Kanzaki et al., 1992), but other σ ligands, such as DTG and panamesine, increased extracellular HVA levels in the striatum and medial prefrontal cortex, respectively (Skuzza et al., 1998; Bastianetto et al., 1995). Thus, it appears that some σ_1 ligands may modulate the DA metabolism.

In post-mortem experiments, both the two σ_1 agonist prototypes (+)-pentazocine and (+)-SKF-10,047 definitely increased tissue levels of DOPAC and HVA in the striatum (Iyengar et al., 1990); however, in the same experimental conditions, the putative σ_1 agonist SA-4503 increases DOPAC levels only in the frontal cortex, but not in the striatum (Kobayashi

et al., 1997) and the putative σ_1 antagonist panamesine increased DOPAC and HVA levels in both the brain cortex and striatum (Skuzza et al., 1998). These results show that σ_1 ligands are able to increase the DOPAC and HVA levels in brain. However, both σ_1 agonist and antagonists have been shown to produce this effect.

1.9.7 Biphasic effects of σ_1 agonists in regulating of extracellular DA concentrations

In several experimental paradigms, such as behavioural, biochemical and electrophysiological models, σ_1 ligands induce a biphasic bell-shaped effect.

As previously described, in rat, intrastriatal infusion of the σ_1 selective agonists (+)-pentazocine (Fig.1.6 A) and MR-200 (Fig.1.6 B) by a microdialysis probe results in a biphasic effect on extracellular DA concentration, consisting in a rapid increase, during the first thirty minutes, followed by a more prolonged and marked decrease (Fig.1.6 A and B) (Gudelsky 1999; Moison et al., 2003).

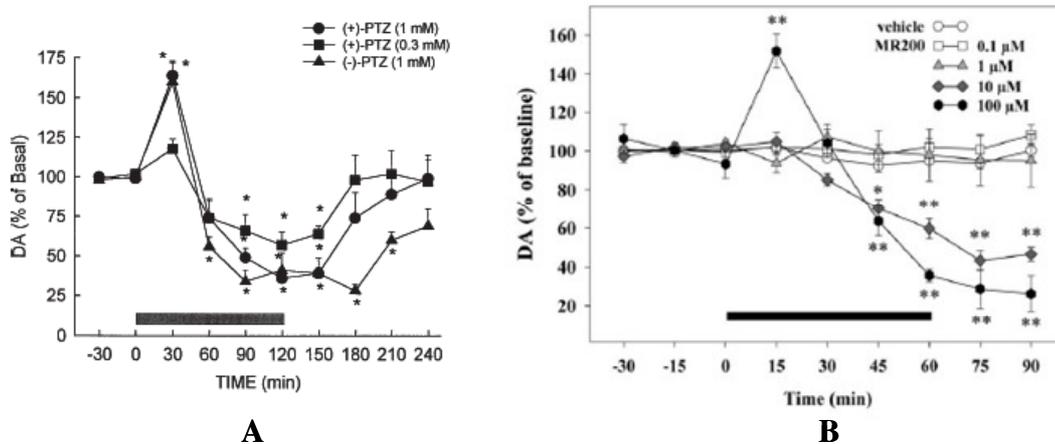


Figure 1.6. Effect on the extracellular [DA]. A) The bell-shaped response is dependent from the administered (+)-pentazocine concentration (Gudelsky, 1995); in this case, also

the less effective isomer (-)-pentazocine induces the bell-shaped response and this is probably due to the very high concentration used in the experiment (Gudelsky, 1999). **B)** The bell-shaped response is dependent from the MR-200 concentrations (Moison et al., 2003): the pharmacological effects begin at 10 μ M, but the biphasic trend only begins at 100 μ M (Moison et al., 2003).

The biphasic effect is evident only when high concentrations of σ_1 ligand are administered. As shown in figure 1.6, (+)-pentazocine induces the bell-shaped response only when administered at concentrations over 1mM (Fig.1.6 A) and MR-200 induces the same response only at concentrations over 100 μ M (Fig.1.6 B). At lower doses, both the σ_1 agonists definitely appear to induce mainly extracellular [DA] decrease (Fig.1.6 A and B) (Gudelsky 1999; Moison et al., 2003). In addition, the infusion of the NMDA antagonist 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) (100 μ M) significantly attenuates the initial increase but not the subsequent decrease of DA release, produced by the intrastriatal infusion of (+)-pentazocine (Fig.1.7) (Gudelsky 1999). These findings indicate that the increasing effect of the extracellular [DA] could be mediated by the NMDA receptor itself, directly or following the activation by other systems. However, since the subsequent decrease of the extracellular [DA] isn't affected by CPP, this latter effect should be ascribed only to σ_1 receptors (Gudelsky 1999).

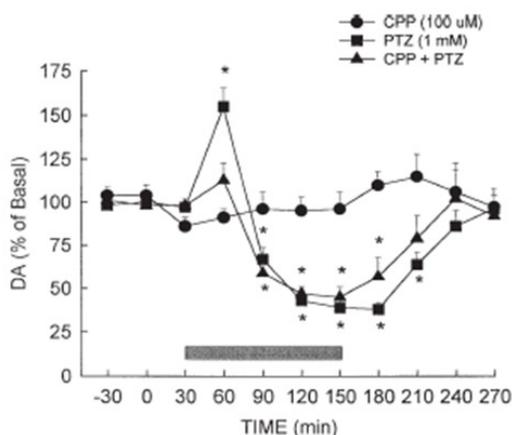


Figure 1.7. The NMDA antagonist CPP alone doesn't induce any physiological change and it prevents the rapid (+)-pentazocine-stimulated increase of the extracellular [DA]. However, the CPP isn't able to reverse the following prolonged decrease of the extracellular [DA] (Gudelsky 1999).

In the NG-108 cell line transfected with σ_1 mouse receptor, the σ_1 agonists (+)-pentazocine, PRE-084 and and pregnenolone sulfate, without effect by themselves, potentiate the bradykinin-induced increase of cytosolic free Ca^{2+} concentration in a bell-shaped manner. This effect of σ_1 ligands is also blocked by an antisense oligodeoxynucleotide against the cloned σ_1 receptors (Hayashi et al., 2000). In isolated cardiac myocytes from adult rats, the exposure to 10 nM of haloperidol or (+)-pentazocine induces an increase in the amplitude of contraction, reaching 188% and 138% (respectively) of control level. A lower concentration of haloperidol or (+)-pentazocine (1 nM) does not induce the same increase in contraction amplitude observed with higher concentration, but rather reduces the amplitude to 70-80% of control (Novakova et al., 1995). Another dual effect of σ ligands has been reported *in vivo* electrophysiological studies investigating the σ -selective modulation of NMDA-induced neuronal activation in rat CA₃ dorsal hippocampus region. At lower doses, σ ligands DTG, JO-1784 and (+)-pentazocine dose-dependently enhance NMDA-induced activation of CA₃ pyramidal neurons (Monnet et al, 1992b). However, at higher doses, the effects of selective σ_1 agonists, such as JO-1784 and (+)-pentazocine, progressively decrease until vanishing and they are even able to act as antagonists, preventing the potentiation induced by low doses of other σ agonists (Bergeron et al., 1995; Bergeron and Debonnel, 1997).

To explain the dual effect of the σ_1 ligands, many hypotheses have been proposed, such as the existence of two σ_1 receptors subtypes (Bergeron and Debonnel, 1997; Novakova et al., 1995; Bermack and Debonnel, 2005) or conformations (Novakova et al., 1995). In my opinion σ ligands simply lose their σ selectivity when administered at higher concentrations and they bind also other receptors classes, activating several disparate responses.

However, the biphasic trend of σ_1 ligands effects is an important factor to take into account while attempting to explain the controversial data about the σ_1 receptors (Bermack and Debonnel, 2005).

1.10 Therapeutical potential of σ_1 receptors

Given the widespread distribution of σ_1 receptors in the CNS and their different modulatory roles at cellular, biochemical and neurotransmission levels, the σ_1 ligands appear to be useful tools in different therapeutic fields, wich are briefly described in this chapter.

1.10.1 Role of σ_1 receptors in learning and memory

Based on studies demonstrating that σ_1 receptor agonists potentiate NMDA-induced neuronal firing in the CA₃ region of the hippocampus (Monnet et al., 1990) and that σ_1 agonists increase extracellular acetylcholine levels in the rat hippocampus and cortex, measured *in vivo* by intracerebral microdialysis (Matsuno et al., 1993b), σ_1 receptors have been extensively studied within the context of learning and memory.

It is known that deficits in cortical cholinergic activity have been observed in some cognitive diseases, such as Alzheimer's disease and pathological ageing. The learning impairment induced by the muscarinic antagonist scopolamine is reversed by several σ_1 agonists, such as (+)-pentazocine, JO-1874, SA-4503 and two neurosteroids, pregnenolone sulfate and DHEA (Maurice et al., 1997a; Maurice et al., 2001a; Monnet and Maurice, 2006) In addition, attenuation of the scopolamine-induced

amnesia, observed with σ_1 agonists, is selectively reversed by known σ_1 antagonists, including haloperidol, NE-100, the neurosteroid progesterone and also by the downregulation of σ_1 receptor expression by specific antisense oligodeoxynucleotides (Maurice et al., 1997; Maurice et al., 2001c; Monnet and Maurice, 2006). The cholinesterase inhibitor donepezil, that is used in treating Alzheimer's disease, is also a potent σ_1 receptor ligand with an affinity of 14.6 nM (Kato et al., 1999). Precise pharmacological examinations of the interaction of donepezil with σ_1 receptors indicate not only that, on these receptors, donepezil could act as agonist, but also that the activation of the σ_1 receptors themselves could be involved in the donepezil anti-amnesic therapeutic properties (Maurice et al., 2006a; Meunier et al., 2006a and b). In fact, in rats, donepezil is able to protect against dizocilpine-, amyloid β_{25-35} related peptide-, or carbon monoxide-induced mnemonic impairment, but these effects are abolished by pretreatment with the σ_1 selective antagonist BD-1047 or antisense oligodeoxynucleotides (Maurice et al., 2006a). Other cholinesterase inhibitors, such as rivastigmine and tacrine, also attenuate dizocilpine-induced learning impairments (Maurice et al., 2006a); however pharmacological effects of these latter compounds aren't antagonized by BD-1047 (Maurice et al., 2006a).

Dizocilpine is a non-competitive NMDA receptor antagonist. Since activation of NMDA receptors is a crucial step in the establishment of long-term potentiation, chronic administration of dizocilpine represent a useful tool to study the memory and learning impairments both in rats and in mice (Murray et al., 1995). In this model, learning deficits are attenuated by the σ ligands (+)-SKF-10,047, (+)-pentazocine, JO-1784, DTG, PRE 084 and SA4503 and also the putative endogenous σ_1 agonists DHEAs and pregnenolone sulfate (Maurice et al., 1999, 2001a, 2001b, 2006a, 2006b;

Monnet and Maurice, 2006). The anti-amnesic properties of these σ ligands are reverted by the known σ_1 antagonists haloperidol, BMY-14802, NE-100 and BD-1047, by the putative endogenous σ_1 antagonists progesterone and by the administration of antisense oligodeoxynucleotides against σ_1 receptor (Maurice et al., 1999b, 2001a, 2001b, 2006a, 2006b; Monnet and Maurice, 2006). The ANAVEX-41, a new potent σ_1 -muscarinic mixed agonist, has been shown to be an effective anti-amnesic agent against dizocilpine-induced learning impairments with its effect being blocked by the σ_1 receptor antagonist BD-1047 (Espallergues et al., 2007).

σ_1 agonists were also reported to soften the learning impairment induced by the nicotinic antagonist mecamylamine, or by cortical cholinergic dysfunction caused by ibotenic acid injection in the basal forebrain (Maurice et al., 1997a, 2001a; Monnet and Maurice, 2006).

Repeated exposures to carbon monoxide (CO) gas induce a long-lasting but delayed amnesia which is measurable about one week after exposure. In this model, the hippocampal cholinergic system appears markedly affected by the hypoxic toxicity caused by CO (Maurice et al., 1997a). σ_1 ligands have neuroprotective properties also in the CO-induced amnesia models, since, the σ ligands (+)-SKF-10,047, PRE-084, JO-1784 and DTG reversed CO-induced amnesia, and their effects were prevented by the σ_1 antagonists NE-100 and BD-1047 (Maurice et al., 1999; Meunier et al., 2006a).

Therefore, in conclusion, while selective σ_1 agonists failed to improve the mnemonic processes in healthy control animals, they seem to markedly improve the learning and memory impairments in animal models of amnesia and significantly attenuate the mnemonic deficits.

1.10.2 Alzheimer's disease and σ_1 receptors

Among the memory and learning disorders, Alzheimer's disease (AD) is the most common form of late-life dementia. Also in AD models of amnesia, σ_1 activation seems to ameliorate the mnemonic deficit symptoms. Nontransgenic models of AD have been characterized in rats infused with the amyloid β_{1-40} protein or in mice injected centrally with amyloid β_{25-35} peptide (Yamada and Nabeshima, 2000). In experiments performed using the amyloid β_{25-35} -induced AD model, the σ_1 receptor agonists (+)-pentazocine, PRE 084, SA4503, (+)-SKF-10,047, the antitussive drug dextromethorphan and the putative σ_1 agonists DHEAs and pregnenolone sulfate attenuated, in a dose-dependent and bell-shaped manner, the memory deficits observed in mice seven days after β_{25-35} peptide injection. These effects of σ_1 agonists were reverted by haloperidol, BD 1047 and the putative σ_1 antagonist progesterone (Maurice et al., 1998, Meunier et al., 2006c; Wang et al., 2003). In the same experimental model, the cholinesterase inhibitor donepezil (Meunier et al., 2006c) and the σ_1 -muscarinic mixed ligand ANAVEX-41 (Villard et al., 2009) were shown to be able to attenuate learning and memory deficits and hippocampal neuronal death caused by β_{25-35} peptide administrations. These findings are consistent with recent results indicating that, in cultured rat cortical neurons, the amyloid β_{25-35} -induced neuronal death is blocked by PRE-084 and the neuroprotective effects of this compound is, in turn, blocked by the σ_1 receptor antagonist, NE-100 (Marrazzo et al., 2005).

1.10.3 Schizophrenia and σ_1 receptors

Schizophrenia is a psychiatric pathology that describes a neuropsychiatric and mental disorder characterized by abnormalities in the perception or expression of reality.

The DA hypothesis of schizophrenia was first proposed in 1960s and it implicates the enhancing of mesolimbic DA functions. In particular it proposes that an excessive activation of D₂ receptors could be the cause of the positive symptoms of schizophrenia (Depatie and Lal, 2001). This hypothesis, largely resulted from the finding drugs which blocks DA function, like the phenothiazines, could reduce psychotic symptoms (Depatie and Lal, 2001). It is also supported by the observation that amphetamine, cocaine, methylphenidate and related psychostimulants, all of which enhance DA neurotransmission by presynaptic mechanisms, and other dopaminergic agonists, including apomorphine, can mimic acute positive symptoms of schizophrenia in normal subjects (Depatie and Lal, 2001).

This explanation is now believed to be overly simplistic, even because newer antipsychotic medications (called atypical antipsychotic) can be equally effective as older medications (called typical antipsychotic), but they also affect serotonin functions and may have slightly less of a DA blocking effect (Jones et al., 2002). In addition, lower levels of glutamate receptors found in postmortem brains of people previously diagnosed with schizophrenia suggest that the neurotransmitter glutamate and the reduced function of the NMDA receptor could be involved in schizophrenia pathogenesis (Konradi and Heckers, 2003) The glutamatergic hypothesis is further supported by the finding that PCP not only emulates positive symptoms but also mimics the long-lasting psychological and social

dysfunctions similarly to the negative symptoms of the disease, and can also induce schizophrenia-like psychosis in humans.

Since σ , DA and glutamate receptors share intricate relationships (see section 1.9), σ receptors are thought to be involved in the schizophrenia pathophysiology; consistent evidences arose from the finding that several σ agonists, such as (+)-SKF-10,047 and (+)-pentazocine exhibit psychotomimetic effects (Tam and Cook, 1984; Walker et al., 1990; Matsumoto and Pouw, 2000) and that many antipsychotic drugs, such as chlorpromazine, haloperidol and nemonapride and the psychotomimetics drugs used in schizophrenia models, such as amphetamine, methamphetamine, PCP and cocaine bind also to the σ receptors (reviewed in Hayashi and Su, 2004)

In behavioral models of schizophrenia in which the dopaminergic function is affected, σ_1 antagonists have been shown to ameliorate some symptoms. In the behavioral sensitization induced by repeated administrations of psychostimulants, a broad spectra of σ_1 antagonists inhibit the sensitization to methamphetamine (Akiyama et al., 1994; Takahashi et al., 2000, Ujike et al., 1992a) and cocaine (Ujike et al., 1996; witkin et al., 1993). Nonselective σ_1 antagonists, such as rimcazole, E-5842 and panamesine inhibit the amphetamine-induced locomotor activity (Guitart et al., 1998; Poncelet et al., 1993; Rückert and Schmidt., 1993; Skuza and Rogoz, 2006), but the symptoms of apomorphine-induced climbing are antagonized only by E-5842 and panamesine, but not by rimcazole (Guitart et al., 1998; Skuza and Rogoz, 2006; Takahashi et al., 1999; Taylor et al., 1993). Moreover, the σ_1 antagonist BD-1047 failed to antagonize the acute symptoms in the two mentioned models (Skuza et al., 2006). Again, the reported pharmacological actions of σ ligands show several contradictions and these results are also in contrast with the studies

showing that the σ agonists, nor the antagonists, depress the dopaminergic neurotransmission (see section 1.9.6). Since many other targets are known to be involved in schizophrenia pathophysiology, the reported differences in the effects elicited by σ ligands might be ascribed to the involvement of other neurotransmitter systems.

In addition to dopaminergic dysfunctions, alterations of glutamatergic neurotransmission are also involved in schizophrenia. *In vivo* model in which the glutamatergic system is affected, σ_1 ligands modify some animal behaviors. PCP-induced head weaving is insensitive to selective D_2 antagonists but it's attenuated by the antagonists NE-100, haloperidol, BMY 14802, Dup 734 and MS-377 (Hayashi and Su 2005; Takahashi et al., 1999). The BD 1047, rimcazole and panamesine also attenuate PCP-induced head twitching (Skuzza et al., 2006). In monoamine-depleted mice, σ_1 receptor agonists such as (+)-pentazocine, (+)-SKF-10,047 and 3-(+)-PPP enhance the dizocilpine-induced psychotomimetic effects and this enhancement is blocked by σ_1 antagonist NE-100 (Okuyama et al., 1996). For these reasons, σ_1 blockade has been proposed for treating the negative symptoms of schizophrenia. However, among negative symptoms of schizophrenia, cognitive deficits are considered the most severe features of the illness and it has been extensively reported that σ_1 agonists are able to improve PCP-induced cognitive deficits, with this effect being reversed by σ antagonists (reviewed in Hayashi and Su, 2004, 2005). In summary, these results suggest that σ_1 agonists could be useful for treating cognitive deficits of schizophrenia but, at the same time, σ_1 antagonists would counteract negative symptoms. Therefore, a purported schizophrenia therapy based exclusively on σ_1 appears to be not useful.

1.10.4 Role of σ_1 receptors in depression

Depression, also known as major depressive disorder is a mental disorder characterized by low mood accompanied by low self-esteem, and loss of interest or pleasure in normally enjoyable activities. The major depressive disorder is characterized by disfunctions in brain areas where the monoaminergic neurotransmitters are widely released, especially in the frontal cortex and the hippocampus (Delgado and Moreno, 2000).

Preclinical studies have shown that targeting the σ_1 receptors alone is sufficient (but it is not requisite) to produce antidepressant-like effects. The antidepressant effects of σ ligands have been tested in behavioral models. In the tail suspension test, the selective σ_1 agonists SA-4503 and (+)-pentazocine decrease the immobility time and this effect is antagonized by NE-100 (Ukai et al., 1998). Several σ_1 agonists, such as SA-4503, (+)-pentazocine, JO-1784, UMB23 and donepezil have been shown to decrease immobility in the forced swimming test and this effect is blocked by known σ_1 antagonists (Maurice et al., 1999, and 2006a; Skuza et al., 2002a; Urani et al., 2001 and 2002; Wang et al., 2007). Recently, also the selective σ_1 receptor agonist PRE-084 has been reported to exhibit an antidepressant-like effect in the forced swim test (Skuza and Rogoz, 2009). This effect is counteracted by BD 1047 but not by SM-21, σ_1 and σ_2 receptor antagonists, respectively (Skuza and Rogoz, 2009). Furthermore, the σ_1 -stimulated decrease of immobility is more pronounced in C57BL/6J mice than in Albino Swiss mice and this result is consistent with the finding that the brain level of the endogenous σ_1 antagonist progesterone in C57BL/6J mice is two-fold lower than that of Albino Swiss mice (Phan et al., 2002). According to this latter finding, it has been also shown that in the forced swim test the antidepressant-like effects of σ_1 agonists JO-1784 and PRE-084, in adrenalectomized mice (a model of progesterone-lacking mice) are

enhanced compared to the non-adrenalectomized control mice and these effects are blocked by BD-1047 (Urani et al., 2001). Moreover, the neurosteroids that are known to act as σ_1 receptors agonists, such as DHEAS and pregnenolone sulfate, are able decrease immobility in the forced swimming test (Maurice et al., 2001a and 2006a; Skuza and Rogoz, 2002; Urani et al., 2001 and 2002; Wang et al., 2007). Thus, again, neurosteroids are shown to be related to σ_1 functions.

Interesting is the finding that an extract of *Hypericum Perforatum*, which is used as antidepressant, seems to exert its pharmacological actions also through σ_1 receptors (Mennini and Gobbi, 2004).

σ_1 agonists are also able to potentiate the firing of serotonergic neurons of dorsal raphe nucleus after just two days of treatment, whereas the effects of serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors take several weeks to emerge. Thus, it seems that the antidepressant activity promoted by σ_1 agonists could emerge faster compared to the existing medications (Bermack and Debonnel, 2001). The OPC-14523 is a compound that shows a mixed pharmacological profile, since it has been reported to act as a SSRI and σ_1 agonist at the same time (Tottori et al., 2001; Hayashi and Su, 2004a). In the forced swimming test, this compound produces a marked antidepressant-like activity after a single oral administration and this effect is blocked by both the σ_1 and 5-HT_{1A} antagonists (Tottori et al., 2001; Hayashi and Su, 2004a). In line with this finding, coadministration of the σ_1 agonist (+)-pentazocine and the SSRI venlafaxine results in a synergistic antidepressant-like effect, also when administered at subeffective doses (Dhir and Kulkarni, 2007). Interestingly, this activity is suppressed by several σ_1 antagonists, including progesterone (Dhir and Kulkarni, 2007). Thus triggering both σ_1 and the serotonin

transporter (SERT) at the same time may represent a valid strategy to treat the major depressive disorders.

Several evidences suggest that σ_1 receptors play a role in the active mechanisms of selective SSRIs. In addition, it has been reported that some SSRIs possess moderate to high affinities for σ_1 receptors in mice brain (Hashimoto, 2009). Among them, the order of affinity for σ_1 receptors is as follows: fluvoxamine ~ imipramine > sertraline > fluoxetine > paroxetine (Takebayashi et al., 2002; Hashimoto, 2009). In a cell culture system, it has been demonstrated that the σ_1 agonist (+)-pentazocine and the antidepressants fluvoxamine and imipramine, but not sertraline or paroxetine, significantly potentiate nerve-growth factor (NGF)-induced neurite outgrowth in PC12 cells and that the effect of fluvoxamine on NGF-induced neurite outgrowth is significantly antagonized by pretreatment with the selective σ_1 antagonist NE-100 (Takebayashi et al., 2002; Hashimoto, 2009). Much interestingly, the enhancement of NGF neurite sprouting by these drugs is induced also by the overexpression of σ_1 receptors (Takebayashi et al., 2004a).

In humans, fluvoxamine binds to the σ_1 receptors at therapeutic concentrations (Ishikawa et al., 2007) and the σ_1 agonist JO-1784 exhibits a stronger antidepressant activity than the known fluoxetine, when administered at the dose of 20 mg/day (Bermack and Debonel, 2005). However, when used at 100 mg/day, the antidepressant effect of JO-1784 is the same of the placebo (Bermack and Debonel, 2005) and this data is in line with the bell-shaped dose-response curve observed following the σ_1 receptors activation in several behavioral, electrophysiological and biochemical models (see section 1.9.7).

In conclusion, σ_1 agonists exhibit a good antidepressant activity in several behavioral models. The contemporary blockade of SERT and

activation of σ_1 receptor is a promising strategy and, for this reason, the design of the mixed affinity ligands represent a good prospect in terms of efficacy.

1.10.5 Role of σ_1 receptors in anxiety

Anxiety is a psychological and physiological state characterized by cognitive, somatic, emotional, and behavioral components. This disorder is characterized by excessive anxiety and worry about everyday life events with no obvious reasons for worry. People with symptoms of generalized anxiety disorder tend to expect disaster and can't stop worrying about health, money, family, work, or school.

Recently, the role of σ receptors, particularly the σ_1 receptor subtype has been identified as a target for the pathophysiology of anxiety. The effects of σ_1 ligands have been assayed in several behavioral tests, such as in the conditioned fear stress model, in the sexual dysfunction induced by stress, in the marble-burying behavior and in the colonic motor disturbances induced by fear.

In the conditioned fear stress model, mice exhibit a marked suppression of motility when placed in an environment in which they had previously received an electric footshock. In this model, (+)-SKF-10,047, JO-1784, the neurosteroids pregnenolone sulfate and DHEAS and also the antitussive dextromethorphan attenuate, in a bell-shaped dose-response manner, the motor suppression induced by electric footshock (Kamei et al., 1996 and 1997; Noda et al., 2000; Urani et al., 2004). The involvement of σ_1 receptors has been confirmed by the known σ_1 antagonist NE-100 and progesterone which block the effects elicited by the agonists (Noda et al., 2000; Urani et al., 2004). Interestingly, in this same model, (+)-pentazocine is devoid of

any effect (Kamei et al., 1996 and 1997). It has been shown that in plasma and brain of stressed mice the concentration of endogenous σ_1 active steroids is altered (Noda et al., 2000; Urani et al., 2004). For this reason, neurosteroids are believed to modulate the fear stress responses *via* σ_1 receptors (Noda et al., 2000; Urani et al., 2004). Supporting this hypothesis is the finding that the attenuation of the motor suppression induced by σ_1 agonists JO-1784, (+)-SKF-10,047 and DHEAS is higher in a model of progesterone-lacking mice, such as rats after chronic intracerebroventricular infusion of amyloid β_{1-40} peptide (Urani et al., 2004).

In the model of sexual dysfunction induced by stress, the DHEA is able to attenuate the stress-induced effect and this activity is counteracted by NE-100 (Mizuno et al., 2006).

In mice the marble burying behavior, that is used as a model for both anxiety and obsessive compulsive disorder, is reduced by fluvoxamine. The effect of this antidepressant is prevented by the σ_1 selective antagonists BD-1063 and BD-1047, but not by the σ_2 antagonist SM-21, suggesting the involvement of the subtype 1 alone in the effect elicited by this antidepressant (Egashira et al., 2007).

The σ_1 agonist JO-1784 is able to reduce the colonic motor disturbances induced by fear stress in rats, a model that mimicks the gastrointestinal tract disorders frequently present in anxiety. This effect is reversed by the σ_1 antagonist BMY-14802 (Gue et al., 1992).

Although σ_1 receptor modulators appear as suitable therapeutic options for treating anxiety, this topic still needs further preclinical and clinical exploration (Kulkarni and Dhir, 2009).

1.10.6 σ_1 receptors and analgesia

In the CNS, the σ_1 receptors are widely distributed in the areas known to be involved in pain control, such as the superficial layers of the spinal cord dorsal horn, the periaqueductal gray matter, the locus ceruleus and rostroventral medulla (Alonso et al., 2000; Kitaichi et al., 2000). Modulation of analgesia by σ_1 receptors has been studied both in opioids-induced models and in absence of opioid agonists.

1.10.6.1 Modulation of opioid analgesia

The involvement of σ_1 receptors in the opioid-induced analgesia has been first reported on rat tail-flick experiments (Chien and Pasternak, 1993). In this model, the systemic administration of σ_1 ligands such as (+)-pentazocine and DTG results in a considerable inhibition of the antinociception induced by morphine (Chien and Pasternak, 1993, 1994 and 1995a; Mei and Pasternak, 2002). In line with this finding, the σ_1 antagonists, such as haloperidol and (+)-MR-200, not only reversed the effect of the agonists, but also increase opioid-induced analgesia; this may indicate that σ_1 receptors could also work as a tonically active anti-opioid system (Chien and Pasternak, 1993, 1994 and 1995a; Marrazzo et al., 2006; Ronsisvalle et al., 2001a). However, the σ_1 ligands (+)-SKF-10,047 and NE-100 and specific σ_1 antisense oligodeoxynucleotides weren't able to modulate the κ_1 stimulated analgesia in the acetic acid-induced writhing test (Hiramatsu et al., 2002 and 2004), suggesting that modulation of analgesia by σ_1 receptors does not affect the κ opioid receptors antinociception activity.

The activity of σ_1 receptors was found to differ, depending on the considered nervous system area. In the tail-flick model, intrathecal

administrations of the σ_1 receptor agonist (+)-pentazocine doesn't attenuate the intrathecal analgesic effect of morphine, indicating that the modulation of opioid analgesia by σ_1 receptors should not occur at spinal level (Mei and Pasternak, 2002). On the other hand, when given supraspinally (intracerebroventricular administration), (+)-pentazocine diminishes the analgesic effect of κ and μ opioid receptors agonists nalorphine and nalbuphine (Mei and Pasternak, 2002). Moreover, the finding that the intracerebroventricular administration of σ_1 specific antisense oligodeoxynucleotides enhances the opioids-induced anti-noception, further confirms the supraspinal location of the σ_1 receptor-mediated modulation of analgesia (King et al., 1997; Mei and Pasternak, 2002; Pan et al., 1998). More detailed results have been reported after microinjecting morphine, in conjunction with (+)-pentazocine, haloperidol, or both, in three different brainstem nuclei, the periaqueductal gray, the rostroventral medulla and the locus coeruleus (Mei and Pasternak, 2007). In these experiments, both the locus coeruleus and rostroventral medulla were sensitive to (+)-pentazocine, but not the periaqueductal gray. The involvement of the rostroventral medulla is particularly interesting as, in this study, it was the only region showing a tonic σ_1 activity and it could also modulate the morphine analgesia administered in periaqueductal gray (Mei and Pasternak, 2007).

1.10.6.2 Analgesic effect of the σ_1 ligands

The modulation of analgesia by σ_1 receptors has been also investigated in absence of opioids.

Even if, in both the tail-flick model and the acetic acid-induced writhing test, several σ_1 ligands and antisense treatments were shown to be ineffective in producing any analgesic response when administered alone

(Cendan et al., 2005a; Chien et al., 1993, 1994, 1995a; Marrazzo et al., 2006; Mei and Pasternak, 2002; Pan et al., 1998; Hiramatsu et al., 2002 and 2004), other investigations have reported that σ_1 receptors are able to modulate nociception in different behavioral tests, in the absence of opioids. In the nociceptive flexor response test, the σ_1 agonists (+)pentazocine, SA-4503, DHEAs and pregnolone sulfate were able to elicit dose-dependent flexor responses, blocked by σ_1 receptor antagonists NE-100, BD1063 and progesterone. In the formalin test, the formalin-induced nociception is attenuated not only by systemic administration of the σ antagonist haloperidol, with an order of potency which is correlated with affinity for σ_1 receptors (Cendan et al., 2005a), but the same result was observed also in σ_1 receptor knockout mice (Cendan et al., 2005b). In line with these latter findings, recent studies showed that intrathecal treatment with the σ_1 receptor antagonist, BD-1047, in mice reduces formalin-induced pain and concomitantly attenuates the phosphorylation of N-methyl-D-aspartate (NMDA) receptor subunit 1 induced by formalin (Kim et al., 2006). In addition, intrathecal injections of the σ_1 receptor agonists, PRE-084 and carbetapentane, increase in mice the PKC- and PKA-dependent phosphorylation of the NR1 subunit of the NMDA receptor. This increase is blocked by the σ_1 receptor antagonist BD-1047 (Kim et al., 2008; Roh et al., 2008). In sciatic-neuropathic rats, a model of neuropathic pain, the σ_1 agonist DHEA induces a rapid pronociceptive action and the σ_1 antagonist BD-1047 is able to block the transient pronociceptive effect provoked by DHEA (Kibaly et al., 2008). In addition, it has been shown that the σ_1 antagonist BD-1047 also possesses an antinociceptive activity in the capsaicin-induced headache model (Kwon et al., 2009).

In summary, it appears that σ_1 receptors antagonists alone might induce analgesia and could be suitable for treating the acute and the neuropathic

pain. In addition, while counteracting the σ_1 tonic inhibition of the opioid system, σ_1 receptor antagonists may potentiate the opioid response allowing to reduce the pharmacological active dose of opioid drugs. However, the exact molecular mechanisms of the action of σ_1 receptors in the modulation of pain still have to be established and needs further investigations.

1.11 σ_1 receptors and drug abuse

Since σ_1 receptors are able to bind several drug of abuse, it has been proposed that they may directly modulate the effects of these drug. Moreover, since it has been demonstrated that the σ_1 receptors are directly involved neuronal plasticity, it is likely that these receptors are somehow involved in drug dependence (Maurice et al., 2002; Matsumoto et al., 2003; Guitart et al., 2004; Maurice & Romieu, 2004). The role of σ_1 receptors in the action of cocaine has been extensively investigated. In addition, σ_1 receptors appear to be involved also in the methamphetamine, 3,4-methylenedioxymethamphetamine, (MDMA) and ethanol effects.

1.11.1 Cocaine and σ_1 receptors

As described in 2.6.2, cocaine acts mainly as DA uptake inhibitor; however, in order to produce its psychostimulant effects, other mechanisms may also be involved (Mateo et al., 2004).

Cocaine binds to both its main target, the dopamine transporter (DAT), and the σ_1 receptors with K_i which falls in the μM range (Rothman et al., 2003). It binds, preferentially, to the σ_1 subtype rather than to the σ_2

(Matsumoto et al., 2002). Thus, following the administration of a pharmacological active dose of cocaine, the physiological concentrations reached by this compound are higher enough to bind both DAT and σ_1 receptors. For this reason, the role of σ_1 receptors and ligands in the modulation of the effect of cocaine has been extensively studied both in acute and chronic models.

1.11.1.1 σ_1 receptors modulation of the acute effects of cocaine

Among the acute side effects elicited by cocaine, hyperlocomotion represents one of the earliest and most evident symptoms. For this reason, locomotor effects of cocaine are often used as tool for studying the antipsychostimulant properties of novel compounds. Several σ_1 antagonist, such as haloperidol, BD-1008, BD-1047, BD-1063, BMY-14802, panamesine and rimcazole as the administration of σ_1 receptor antisense oligodeoxynucleotides have been reported to attenuate the acute hyperlocomotion activity induced by cocaine in rodents (Matsumoto et al., 2001a and 2003).

Convulsions and lethality are the typical side effects of cocaine overdose. The cocaine-induced convulsions, resulting from exposure to higher doses of cocaine, represent a good model to measure the cocaine-induced toxicity and the protective effects of the antipsychostimulants. The σ_1 antagonists BMY-14802, BD-1008, BD-1063, BD-1047 and rimcazole, as well as antisense oligodeoxynucleotides silencing σ_1 receptors significantly prevent cocaine-induced convulsions (Ushijima et al., 1998; Matsumoto et al., 2001a, 2001b, 2001c, 2002, 2003 and 2004; Maurice et al., 2002; Katz et al., 2003; Daniels et al., 2006). Interestingly, the ability of these compounds to prevent cocaine-induced convulsions is more related

with their affinities for σ receptors rather than for affinity towards DAT (Matsumoto et al., 2001c). In addition, it has been shown that some σ_2 receptors preferential ligands are able to prevent the cocaine-induced convulsions; this finding suggests that both the σ receptors subtypes may be able to modulate the acute effects of the cocaine (reviewed in Matsumoto et al., 2003). As it concerns the role of σ_1 receptors on lethality induced by cocaine, σ_1 receptors antagonists revealed a protective activity, preventing death in animals not only when they are administered before cocaine, but also after (Matsumoto et al., 2001a, 2001b, 2002 and 2004; McCracken et al., 1999a; Daniels et al., 2006). These findings indicate that the σ_1 antagonists could have important clinical applications since they may even used as lifesaving drugs.

Further data confirming the involvement of σ receptors in the action of cocaine are related to the administration of DTG and of selective σ_1 agonists BD-1031, BD-1052 and SA-4503 which aggravate, in mice, the locomotor stimulatory actions and the toxic effects (measured as convulsions and lethality rate) of cocaine (Matsumoto et al., 2001a and b, 2002; McCracken et al., 1999a; Skuza, 1999).

1.11.1.2 σ_1 receptors modulate the chronic effects of cocaine

Repeated administration of cocaine to laboratory animals results in behavioural sensitization as the animals develop an enhanced response to a given dose of cocaine. The establishment of the cocaine-induced locomotor sensitization, which is considered a measurable index of nervous system plasticity resulting from repeated exposure to cocaine (Matsumoto et al., 2003) is attenuated by the coadministration of several σ_1 antagonists, such as

BMY-14802, NPC-16377, rimcazole and SR-31742 (Ujike et al., 1996; Witkin et al., 1993).

The conditioned place preference (CPP) is a useful paradigm employed to evaluate the rewarding properties of drugs of abuse after their repeated administration. σ_1 receptors seem to play a role in establishment and the expression of the CPP induced by subchronic administration of cocaine. In fact, the cocaine-induced CPP acquisition is potentiated by the σ_1 receptor agonists DHEAs, pregnenolone sulfate JO-1784 or PRE-084 (Romieu et al., 2002, Romieu et al., 2004) and the effects of the agonists are blocked by the σ_1 antagonists progesterone and BD-1047 (Romieu et al., 2002; Romieu et al., 2004). In addition, repeated administrations of selective σ_1 receptor antagonists BD 1047 and NE-100 as well as the treatment of animals with a specific σ_1 antisense oligodeoxynucleotide are effective in reducing not only the establishment, but also the expression of cocaine-induced CPP (Romieu et al., 2000).

The self-administration paradigm, as well as the CPP represent useful tools to evaluate craving and relapse related to cocaine addiction. In fact, an extinct cocaine addictive behavior can be reactivated by a discriminative stimulus associated with cocaine administration or by a priming injection of cocaine. The σ_1 antagonist BD-1047 and a σ_1 antisense oligodeoxynucleotide are able to attenuate the relapse behavior in both the described models (Martin-Fardon et al., 2007, Romieu et al., 2004). Moreover, the σ_1 agonists DHEA (Romieu et al., 2004), PRE 084, JO-1784 (Romieu et al., 2002) are capable to reactivate the CPP after extinction in rats previously conditioned to cocaine, in a BD-1047 sensitive manner (Romieu et al., 2004). These results suggest that σ_1 antagonists could be employed as therapy to prevent craving and relapse of cocaine addiction.

In summary, these studies show that cocaine might produce its behavioral and biochemical effects, at least in part, through its interaction with σ_1 receptors. Thus, σ_1 antagonists should be considered for developing potential therapies to treat different aspects of cocaine abuse, such as acute effects, long term effects and relapses.

1.11.2 Methamphetamine, MDMA and σ_1 receptors

Methamphetamine, like cocaine, binds the σ_1 receptors in the micromolar range; it binds also the σ_2 receptors with a 20-fold lower affinity than the σ_1 subtype (Nguyen et al., 2005). As previously shown in section 1.10.3, several σ_1 antagonists, such as BMY-14802, E-5842, DTG, SR-31742A, MS-377, BD-1063 or BD-1047, and σ_1 antisense oligodeoxynucleotides, attenuate *in vivo* several effects of methamphetamine, such as the enhancement of locomotor activity or the development of methamphetamine-induced behavioural sensitisation (Ujike et al. 1992b; Taylor et al. 1993; Ruckert and Schmidt, 1993; Poncelet et al., 1993; Guitart et al., 1998; Takahashi et al., 2000; Nguyen et al., 2005).

It has been reported that σ_1 receptors could modulate also the effects of MDMA, a methamphetamine-related psychostimulant. In fact, the σ_1 antagonist BD-1063 attenuates the MDMA-induced locomotor activity (Brammer et al., 2006); moreover, BMY-14802 and MS-377, other two known σ_1 antagonists, inhibit also the behavioral sensitization induced by the repeated administration of MDMA (Ujike et al., 1992a; Akiyama et al., 1994; Takahashi et al., 2000).

1.11.3 Ethanol and σ_1 receptors

The involvement of σ_1 receptors in the effect of ethanol has been examined *in vivo* behavioral paradigms. In rodents, the σ_1 receptor antagonist BD-1047 has been shown to counteract the ethanol-induced locomotor stimulation, CPP and taste aversion and also to attenuate some symptoms of the abstinence syndrome after chronic ethanol consumption (Maurice et al., 2003; Meunier et al., 2006b). In line with these findings, the σ_1 receptor agonist PRE-084 enhances the ethanol-induced CPP in a dose-dependent manner (Maurice et al., 2003). However, the σ_1 agonist JO-1784 behaves as the antagonist BD-1047 and attenuates some symptoms of the ethanol abstinence syndrome (Meunier et al., 2006b).

These observations suggest that σ_1 receptor could represent a novel target for alleviating ethanol addiction and abstinence syndrome after withdrawal.

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CHAPTER 2,

THE DOPAMINE TRANSPORTER

2.1 Historical background

Neurons rely upon chemical neurotransmitters for rapid (order of milliseconds) and precise communication with each other and with target organs. Neurotransmitters released into the synaptic cleft activate specific receptors on the postsynaptic neuron, which in turn causes opening of ion channels and activation of signal transduction cascades in the post-synaptic cell resulting in transmission of the signal.

The dopamine (DA) neurotransmitter was originally assumed to be a mere intermediate in the biosynthesis of norepinephrine (NE), until Carlsson and coworkers performed experiments on reserpinized animals and revealed a previously unrecognized role for DA in the central nervous system (CNS) (Carlsson et al., 1957). The neurotoxin reserpine alters the pH gradient required for monoamine uptake into the synaptic vesicle and the animals treated with reserpine become immobile, contributing to what is known as reserpine syndrome. Loss of NE was assumed to be the cause of this syndrome because treatment of reserpinized animals with the NE precursor 3,4-Dihydroxyphenylalanine (DOPA) reversed the reserpine syndrome (Carlsson et al., 1957). However, analysis of tissue from reserpinized and DOPA treated rabbits revealed that while animals regained mobility, there was only modest recovery of NE levels in the brain (Carlsson et al., 1957). This intriguing discovery soon led to the finding that the DA, not the NE is correlated with recovery from reserpine syndrome (Carlsson and Waldeck, 1958). For the first time, the role of DA as a potential neuromodulator of movement defects was recognized and described.

The lifetime of the neurotransmitter released into the synaptic cleft is predominantly regulated by specific proteins that mediate their reuptake into the presynaptic cell, the neurotransmitter transporters. Julius Axelrod was the first to introduce the concept of reuptake by demonstrating that synaptically released noradrenaline is taken up by specific transporters in sympathetic nerve terminals (Hertting and Axelrod, 1961). Subsequently, reuptake mechanisms have been discovered for serotonin, DA, γ -amino butyric acid (GABA), glycine and a number of other neuromodulators. To date, neurotransmitter transporters have been described to play a very important role in regulating temporal and spatial aspects of neurotransmission.

2.2 Functions of DAT

2.2.1 *Physiological activity of DAT*

The DA transporter (DAT) is a membrane protein whose physiological function is to clear released DA from the synaptic cleft, effectively reducing the concentration of DA at both pre- and post-synaptic DA receptors (Nelson, 1998). Therefore, DAT modulates the spatial and temporal aspects of dopaminergic synaptic transmission and, while it regulates the key step for terminating DA action it is considered an integral part of the DA system (Mortensen and Amara, 2003; Torres and Amara, 2007).

The DA is uptaken against its concentration gradient. As discussed in section 2.3.2, the driving force for this process is granted by the Na^+ and Cl^- ions symport. Electrophysiological studies have revealed that DAT also

exhibits channel-like properties (Kahlig et al., 2005; Carvelli et al., 2004). These studies demonstrate that in addition to a carrier, DAT also acts as a channel and directly modulates membrane potential and neuronal function (Blakely and DeFelice, 2007).

The dopaminergic system is involved in learning, reward-seeking behavior, movement, and emotion (Sotnikova et al., 2006). Due to the crucial role of DAT in terminating the dopaminergic neurotransmission, dysfunctions of DAT contribute to the development of several neurological and psychiatric disorders (Torres, 2006), such as attention-deficit hyperactivity disorder (ADHD) and Parkinson's disease (PD).

2.2.2 DAT mediated DA efflux

In addition to the physiological uptake activity, DAT has been shown to be capable of mediating DA efflux (Khoshbouei et al., 2005). Efflux of DA through DAT has been shown primarily using AMPH stimulation (Khoshbouei et al., 2003). Initial studies examining reverse transport for DAT revealed that PKC activation increased DA release from striatal slices with this effect being blocked by DAT inhibitors (Kantor and Gnegy, 1998; Cowell et al., 2000). A link to PKC and potential AMPH induced efflux came from studies using the mutagenesis techniques. In these researches, the five *N*-terminal serine residues in hDAT, which are considered to be sites of PKC phosphorylation, have been substituted with alanines. While alanine substitution of these residues did not effect DA uptake or surface expression, AMPH mediated efflux was completely abolished (Khoshbouei et al., 2004). Substitution of these alanine residues with aspartic acid residues, which mimics phosphorylation at these sites, restored AMPH mediated efflux (Khoshbouei et al., 2004). This work suggests that NH₂-

terminal phosphorylation directly by PKC or via another kinase, not only affects transporter surface expression, but also enables the transporter to efflux DA.

2.3 Structural basis of physiological functions

2.3.1 *Molecular characterization*

DAT is a member of the family of Na⁺, Cl⁻-dependent substrate-specific neuronal membrane transporters. This neurotransmitter sodium symporter family (NSSf) includes also transporters for NE, serotonin, GABA, AA such as glycine, taurine, proline, betaine, creatine and some bacterial homologues (Amara and Khuar, 1993; Giros and Caron, 1993; Uhl and Kitayama, 1993; Blakely et al., 1994; Reith et al., 1997).

The cloning of the DA transporter came in 1991 when several groups using information from other recently cloned biogenic amine transporters, expressed cDNA capable of mediating DA uptake into non-DA cells (Giros et al., 1991; Usdin et al., 1991). To date it is known that DAT gene belongs to the solute carrier 6 genes (SLC6A3) family, the same family of other monoamine transporter, such as SERT and NET. The initial determination of the membrane topology of DAT was based upon hydrophobic sequence analysis and sequence similarities with the GABA transporter. These methods predicted twelve transmembrane domains (TMD) with a large extracellular loop between the third and fourth TMDs (Kilty et al., 1991). Further characterization of this protein used proteases, which digest proteins into smaller fragments, and glycosylation, which occurs only on extracellular loops, largely verified the initial predictions of membrane

topology (Vaughan and Kuhar, 1996). Thus, to date, it's accepted that the topological arrangement of DAT consists of 12 membrane-spanning α -helices with intracellular amino and carboxyl-terminal domains (Fig.2.1). There are three putative N-linked glycosylation sites on the large extracellular loop between TM3 and TM4. DAT also contains a number of potential phosphorylation sites on the intracellular domains, which suggests that kinases may mediate regulation of the transport process.

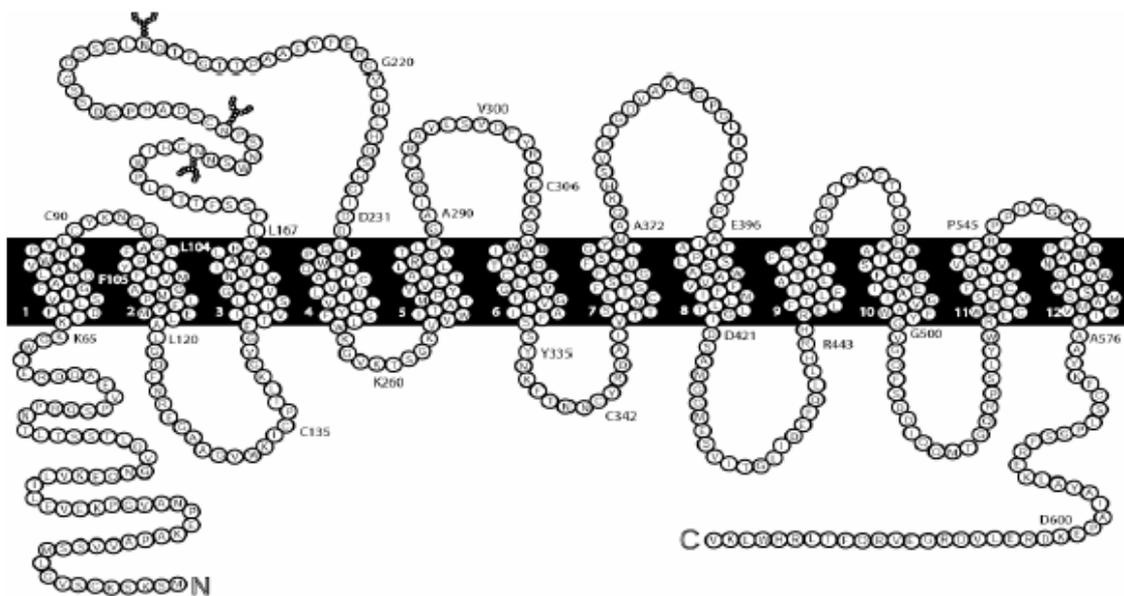


Figure 2.1. Topology of the Human Dopamine Transporter (hDAT). Illustration of the two-dimensional topology of the hDAT showing intracellular N and C termini and the amino acid sequence. There are 12 transmembrane domains with alternating intra- and extra-cellular loops. N-linked glycosyl groups are shown at the consensus asparagine glycosylation sites, Asn 181, Asn 188, and Asn 205, in the second extracellular loop. In addition, cysteines 180 and 189 in the second extracellular loop are disulfide bonded (adapted from Giros & Caron, 1993).

2.3.2 Structural basis of DA uptake

As other members of NSSf, the DAT enables the transport of substrate together with Na^+ and Cl^- as cosubstrates (Gu et al., 1994; McElvain and Schenk, 1992). When DA binds to the transporter in combination with Na^+ and Cl^- , the transporter becomes active and can transport DA inside the cell. During a transport cycle, two Na^+ , one Cl^- and one charged molecule of DA are translocated resulting in the net movement of two positive charges per molecule of DA (Gu et al., 1994; McElvain and Schenk, 1992). Another plasma membrane protein, an Na^+ -, K^+ -ATPase cotransporter, maintains an inwardly directed transmembrane Na^+ gradient by pumping Na^+ ions out of, and K^+ into, the cell. This transmembrane gradient is important, because it represents the driving force of the uptake process (Rudnick, 2002; Chen and Reith, 2003), however the exact mechanism of the transporter involved in this coupled transport process remain unclear. A great advance in understanding the NSSf has been the crystalization of a member of this family, the bacterial leucine transporter (LeuT_{Aa}), from *Aquifex aeolicus* (Yamashita et al., 2005). Despite a low similarity between the eukaryotic and prokaryotic members of the NSSf, the LeuT_{Aa} crystal structure provides the most relevant informations available for this family because few key regions are highly conserved throughout all of these symporters (Beuming et al., 2006; Zhou et al., 2007; Huang and Zhan, 2007; Rudnick, 2007). The LeuT_{Aa} consists of 12 transmembrane domains (TM#), where domains TM1 to TM5 superimpose to TM6 to TM10 by 176.5° (Fig.2.2). TM1 and TM6 are in direct contact with the binding site of leucine (the substrate) and the sodium ions (Fig.2.2).

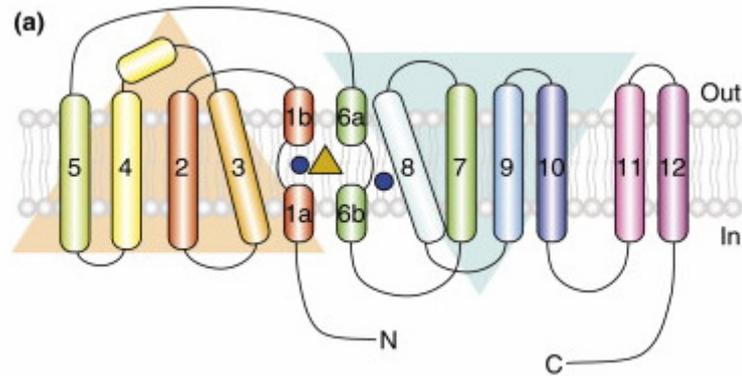


Figure 2.2. Topology of the *LeuT_{Aa}*

If the molecular structure of *LeuT_{Aa}* and other Na^+/Cl^- -dependent transporters correlate, TM1 and TM6 would play a vital role in the substrate binding of DAT, SERT, and NET.

2.3.3 Glycosylation of DAT

The glycosylation is a post-translational enzymatic process that links glycans to proteins only in specific glycosylation sites. The *N*-linked glycosylation occurs when glycans are attached to a nitrogen of asparagine or arginine side chains and it represents a crucial step for the folding of some eukaryotic proteins.

Human DAT (hDAT) is a heavily glycosylated (glycosylation accounts for ~ 30% of the molecular weight of DAT) protein *in vivo*. The large extracellular loop between TMD3 and TMD4 carries three asparagine residues at position 181, 188, and 205 which are considered three putative *N*-linked glycosylation sites (Giros et al., 1994). In order to understand whether or how glycosylation affects DAT function, several mutated transporters have been generated. In a study, Torres et al (Torres et al., 2003) describe four glycosylation mutants of human DAT: N181Q, N188Q, N205Q, and the triple mutant. The single aminoacid mutants

display normal DA uptake and surface expression, indicating that single glycosylation sites are not essential for these processes. The triple mutant has a normal K_m for DA uptake but suffers from a 2-fold reduction in V_{max} along with a distribution more toward the cell interior as compared with wild type. Similar results were obtained by Li et al. (Li et al., 2004) since they reported that the non-glycosylated DAT does not transport DA as efficiently as wild-type DAT and prevention of *N*-glycosylation also enhances the potency of cocaine-like drugs in inhibiting DA uptake into intact cells, without changing their affinity for DAT when measured in membrane preparations prepared from these cells. Thus, non-glycosylated hDAT mutants both possess an appreciably reduced catalytic activity and an altered inhibitor sensitivity compared with wild type.

2.3.4 Phosphorylation of DAT

Phosphorylation is the addition of a phosphate (PO_4) group to a protein. The addition of a PO_4 molecule to a series of AA residues can turn a hydrophobic portion of a protein into a polar and extremely hydrophilic portion of molecule, introducing deep conformational changes in the structure of the protein. This process is mainly regulated by two enzymes, the kinases that introduce and the phosphatases that cleave the PO_4 group and it represent a very important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms.

The elucidation of the primary amino acid sequence of DAT reveals the presence of several potential phosphorylation sites for protein kinases (Giros et al. 1991; Shimada et al. 1991; Usdin et al. 1991). The predicted intracellular domains of the human DAT contain 15 serine, 9 threonine, and 5 tyrosine residues, many of which are found in consensus sequences for

protein kinase C (PKC), cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and calcium calmodulin-dependent protein kinase (CaMK). Some of these sites are highly conserved throughout the neurotransmitter transporter family, including a PKC/PKG consensus sequence in intracellular loop 2 and several sites in the N- and C-terminal tails close to TMs 1 and 12 (Vaughan 2004).

Metabolic phosphorylation of DAT with $^{32}\text{PO}_4$ has been demonstrated in the human, rat, and mouse isoforms of DAT (reviewed in Vaughan 2006); moreover, it has been shown that, also in absence of exogenous treatment, both overexpressed and native DATs display a constitutive phosphorylation and dephosphorylation. Thus, DAT can be considered a phosphoprotein.

As discussed in 2.5, PMA and OA treatments stimulate DAT phosphorylation and also lead to reductions in DA transport activity (Zahniser and Doolen 2001; Vaughan 2004). For this reason, it was early hypothesized that phosphorylation could be a signal for transporter internalization (Gainetdinov et al., 2004). However, the deletion of the hDAT and rDAT N-terminal serine clusters, that abolishes transporter phosphorylation, does not affect PKC-induced downregulation or internalization, demonstrating that transporter downregulation can occur in the absence of stimulated phosphorylation (Granas et al. 2003; Cervinski et al. 2005). These findings clearly demonstrate that DAT phosphorylation is not an internalization signal and that induction of PKC-dependent downregulation might therefore occur via phosphorylation of endocytic components or other DAT-associated proteins.

As better illustrated in 2.2.2, to date the only process identified that requires DAT phosphorylation is substrate-induced efflux of DA (Granas et al. 2003; Khoshbouei et al. 2004).

2.4 Localization of DAT

2.4.1 Anatomical distribution of DAT

The anatomical and subcellular distribution of DAT can be detected using various techniques, such as *in situ* hybridization, [³H]WIN-35,065-2 binding and uptake of the tritiated DA ([³H]DA). Among these three techniques, the use of antibodies anti-DAT led to the most accurate analysis of its distribution.

The DAT is mainly present at the SNC level, but it has been also found in the periphery. At SNC level, DAT localizes exclusively on DA neurons, such as the neurons of the two major midbrain dopaminergic projection pathways:

- 4) the mesocorticolimbic DA neurons with cell bodies (somatodendritic regions/compartments) in the ventral tegmental area (VTA) and projecting to the medial prefrontal cortex and nucleus accumbens (NAc, or ventral striatum)
- 5) the nigrostriatal DA neurons with cell bodies in the substantia nigra pars compacta (SNc) and projecting to the dorsal striatum (dSTR)

DAT levels were found to vary in these different cortical area, confirming for the first time that native DAT levels differ across brain regions (Ciliax et al., 1999).

High-resolution electron microscopy has been used to visualize gold labeled antibodies directed against the rat DA transporter (rDAT); these studies demonstrate that rDAT is mainly located at the plasma membrane in various compartments of DA neurons. In the cell body, DAT is found at

the plasma membrane in tubulovesicular structures. DAT was also found associated with the plasma membranes in intermediate and distal dendrites in the VTA (Hersch et al., 1997; Nirenberg et al., 1997). DAT immunoreactivity was also noted in dendritic compartments in the VTA, with the majority of immunogold signal emanating from plasma membrane inserted DAT (Nirenberg et al., 1997).

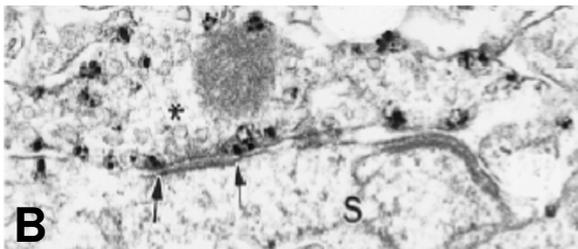
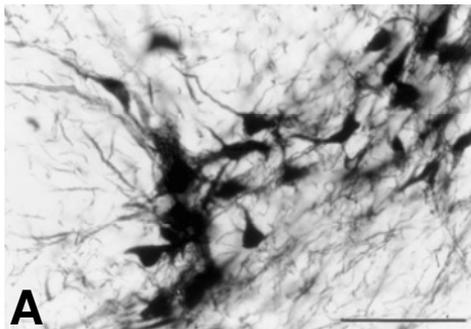


Figure 2.3. A) DAT immunostaining in the pars compacta of the substantia nigra (Hersch et al., 1997); B) High resolution imaging of a DA synapse displaying the “peri-synaptic” localization of rDAT (arrows) (Hersch et al., 1997).

Using both and NH₂-terminal and second extracellular loop antibodies, it has been also confirmed the predicted topology of the transporter, verifying that the NH₂-terminus and the second extracellular loop were found on opposing faces of the plasma membrane (Hersch et al., 1997). Also in these studies was noted a colocalization of DAT with presynaptic DA D₂ receptor (Hersch et al., 1997). This colocalization might be interesting since, at this level, the D₂ receptors work as DA autoreceptors bearing a negative feedback regulation of the extracellular DA. In fact it is known that, once activated, the presynaptic D₂ receptors inhibit the DA synthesis and release and increase the reuptake of the intersynaptic DA.

DAT is not expressed only in the SNC; in rats it has been found in lymphocytes and in spleen, kidney and thyme cells (Mignini et al., 2006).

2.4.2 *Subcellular localization of DAT*

DAT is a membrane protein and, like others membrane proteins, it is synthesized in the ER. Since DAT is proposed to be *N*-glycosylated in the extracellular loops (see section 2.3.1), the transporter must pass through the Golgi to acquire this post-translational modification. From the Golgi apparatus, DAT then traffics to the plasma membrane (reviewed in Zahniser and Sorkin, 2009).

Mutagenesis studies have shown that several regions of DAT are somehow involved in the veiculation of the newly-synthesized transporters (reviewed in Zahniser and Sorkin, 2009). Site-point mutagenesis of the carboxyl-terminal tail or truncation of this region results in inefficient plasma membrane expression of DAT (Torres et al., 2001; Bjerggaard et al., 2004). In addition, an almost complete retention of the transporter in the ER also results from a single mutation of the conserved Gly585 to alanine (Miranda et al., 2004). This dysregulation is probably due to deep changes in the intramolecular interactions necessary for the proper folding of DAT.

Anyway, the exact mechanisms that control movement of newly-synthesized DAT from Golgi to specific cell surface locations within the axons and distal dendrites are not known (reviewed Zahniser and Sorkin, 2009).

It is possible that DAT is inserted into the plasma membrane in the soma and then moves laterally along the plane of the membrane to its functional sites where it is retained by specific interactions with the resident proteins.

Alternatively, DAT could be moved from the soma to distal locations by anterograde vesicles in microtubule-dependent manner and then be inserted into the plasma membrane locally at the axonal varicosities and

distal dendrites. It has been demonstrated that a dimerization/oligomerization of DAT takes place in the ER (Sorkina et al., 2003). This oligomerization has been proposed to be necessary for the correct anterograde transport of DAT (Sorkina et al., 2003; Torres et al., 2003).

2.5 DAT regulation

Neurotransmitter transporter often interpret the primary mechanism by which neurons terminate the signal transmission. For this reason, fine and functional regulation pathways are capital step for modulating the neurotransmission.

DAT, like other neurotransmitter transporters, is subjected to constitutive and regulated endocytosis. Following the endocytotic process, the internalized DAT can be recycled back to the plasma membrane. This dynamic regulation of the transporters expression on cell surface is known as trafficking.

Trafficking of DAT is regulated by several different effectors:

- PKC
- inhibition of phosphatases
- mitogen-activated protein kinase phosphatase (MKP3)
- endogenous and exogenous ligands
- other proteins interacting with DAT

It is well established and described that rapid loss of the surface DAT occurs within minutes after the activation of the PKC by phorbol esters,

such as PMA. This issue was first reported in *xenopus* oocytes and in Sf9 insect cells expressing the cloned hDAT (Zhu et al., 1997; Pristupa et al., 1998). In addition, [³H]DA uptake experiments on dissociated embryonic mesencephalic neurons also showed that, following pretreatment with PMA the V_{\max} of DA transport is strongly inhibited.

The PKC family comprises different classes of isoforms:

- classic isoforms, Ca^{2+} - and diacylglycerol (DAG)-dependent (α , β_1 , β_2 , γ);
- non-classic isoforms, Ca^{2+} -independent, but DAG-dependent (δ , ϵ , θ , η);
- atypical isoforms Ca^{2+} and DAG-independent (λ , ζ).

Among all PKC isoforms, both β subtypes mainly appear to be responsible for regulation of DAT trafficking (reviewed in Vaughan 2006). However, the exact molecular mechanisms by which PKC down-regulates DAT remains unclear.

As previously described (see section 2.3.4), deletion of DAT phosphorylation sites doesn't affect the PKC-induced clathrin-dynamin mediated internalization of the transporter, indicating that other mechanisms are likely to be involved.

Even if it is known that a pool of plasma membrane DAT is also associated with cholesterol-rich rafts, regulating its transport activity (Foster et al., 2008), cholesterol-disrupting drugs do not affect PKC-dependent DAT endocytosis, suggesting that DAT internalization is not mediated by lipid rafts (Sorkina et al., 2005).

Recent mutagenesis studies suggest that the ubiquitination of DAT could be strictly connected to the observed PKC-stimulated endocytosis. In fact, specific site mutation of three lysine clustered in the DAT

aminoterminal, Lys19, Lys27, and Lys35 result in diminished internalization of DAT in response to PKC activation (Miranda et al., 2007). Screenings for identifying the proteins involved in PKC-dependent DAT trafficking toward the cytoplasm were made using siRNA techniques. These studies identified the NEDD4-2, a protein containing an E3 ubiquitin ligase domain, as an essential component of PKC-dependent DAT endocytosis (Sorkina et al., 2006). siRNA-induced knock-down of NEDD4-2 also results in inhibition of DAT ubiquitination, thus implicating NEDD4-2 as an E3 ligase for DAT (Sorkina et al., 2006). These data, together with DAT mutagenesis, indicate that ubiquitination of the DAT amino-terminus is crucial for PKC-dependent DAT endocytosis and that the NEDD4-2 itself may be one of the major PKC molecular effectors.

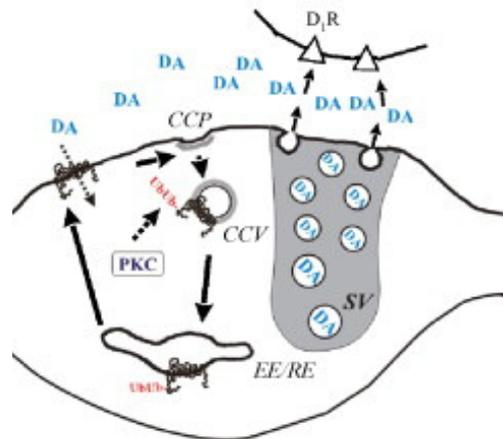


Figure 2.4. PKC dependent mechanism related to DAT internalization is the ubiquitination of the transporter. Ubiquitinated DAT is recruited into clathrin-coated pits (CCP) and then internalized via clathrin coated vesicles (CCV) to early recycling endosomes (EE/RE) which prepare the DAT for being recycled back to the surface (adapted from Zhaniser and Sorkin, 2009).

The role of phosphatases in regulation of DAT phosphorylation levels has been established using the okadaic acid (OA) a broad-spectrum protein phosphatase inhibitor. Treatment of rat striatal tissue or heterologously expressing cells with OA produces robust increases in the DAT phosphorylation and it also decreases DAT cell surface expression and activity within 5–10min (Huff et al. 1997; Vaughan et al. 1997; Cowell et al. 2000; Granas et al. 2003; Lin et al. 2003; Cervinski et al. 2005). The phosphorylation increase, observed in absence of exogenous kinase activators, reflects the rate of basal phosphorylation indicating that, in resting neurons, significant tonic phosphatase activity maintains DAT in a state of relative dephosphorylation. This robust dephosphorylation pressure may lead to the identification of endogenous signals regulating DA uptake activity.

A recent genetic complementation screening, performed in *Xenopus* oocytes, identified a mitogen-activated protein (MAP) kinase phosphatase, MKP3, as a molecule that inhibits PKC internalization of DAT (Mortensen et al., 2008). In this study, transient expression of MPK3 in MDCK cells stably expressing DAT inhibited PKC-stimulated endocytosis of DAT and enhanced DAT activity. MPK3 does not interfere with PKC-induced ubiquitylation of DAT; the molecular mechanism underlying the MPK3 effects acts at a more downstream step, to stabilize DAT on cell surface by blocking dynamin-dependent internalization and slowing the DAT degradation processes (Mortensen et al., 2008).

Exogenous and endogenous substrates also strongly modulate DAT functions. Ethanol, arachidonic acid and nitric oxide has been shown to interfere with DAT activity (Cass et al., 1991; L'hirondel et al., 1995; Mayfield et al., 2001b; Kiss et al., 1999).

Ethanol potentiates DA uptake in *Xenopus* oocytes, SK-N-SH and HEK-293 cells (Riherd et al., 2008). Since ethanol's action on DAT function and regulation is consistent across both neuronal and non-neuronal models, a direct action of ethanol on transporter trafficking in mammalian systems has been proposed (Riherd et al., 2008). In C6 rat glioma cells, arachidonic acid induces a double effect; while a brief incubation leads to an increase of the DA uptake, a longer exposition completely abolishes the DAT activity (Zhang and Reith, 1996). In the same cell line, NO reduces the number of the DAT on the plasma membrane, but, simultaneously, it increases the SERT activity (Miller and Hoffman, 1994). This finding suggests that NO may regulate these two neuronal transporters with different molecular mechanisms.

Protein-protein interactions were also shown to regulate DAT trafficking. In cells, once internalized within vesicles, DAT can alternatively be degraded by lysosomes or anchors to the microtubular net before recycle back to the plasma membrane. A neuronal protein called α -synuclein (α -Syn) is able to directly interact with recycled DAT and anchors it to the microtubules surface (reviewed in Sidhu et al., 2004). α -Syn is a small neuronal protein (140AA) implicated in many cellular processes; at presynaptic sites α -Syn is implicated in synaptic vesicle formation, axonal transport, and DA synthesis and metabolism. In a normal situation, α -Syn exists as a soluble or lipid-bound structure. From co-immunoprecipitation studies, α -Syn was found to interact directly with the DAT, forming a protein-protein heteromeric complex in transfected cells, primary cultures of mesencephalic neurons and rat SNC (Wersinger and Sidhu, 2003; Wersinger et al., 2003; Lee et al., 2001). These interactions occurs between the non-amyloid component domain (residues 58–107) of α -Syn and the last 22 amino acids of the carboxy-terminal tail of DAT

(Wersinger et al., 2003). α -Syn attenuates DAT function, trafficks DAT away from the plasma membrane and, participating in the formation of a protein-protein complex with DAT, anchors the monoamine transporter to microtubules membrane. Thus a probable α -Syn function is the maintenance of DA homeostasis through a modulation of DAT activity, by regulating its level at the cell surface (Wersinger and Sidhu, 2005).

To better understand the role of the α -Syn in the regulation of the DAT trafficking, the microtubular network has been destabilized treating the cells with colchicine, vinblastine, or nocodazole (Wersinger and Sidhu, 2005). Destabilization of the microtubular network disrupts the ability of α -Syn to modulate DAT function, resulting in an increased level of trafficking to the plasma membrane, which is accompanied by an increased rate of DA uptake, DA-induced oxidative stress, and accelerated cell death. Since DAT is the only means by which dopaminergic neurons can re-uptake synaptically released DA (Gainetdinov and Caron, 2003). The increased presence of DAT at the plasma membrane clearly results in an increased rate of re-uptake, causing excessive accumulation of high intracellular levels of ROS and reactive nitrogen species; this latter effect contributes to cytotoxic events which culminate in neuronal death (Wersinger and Sidhu, 2005) and it can partially explain why mutations of α -Syn (see section 2.7) have been implicated in the genesis of Parkinson's disease (PD).

2.6 DAT pharmacology

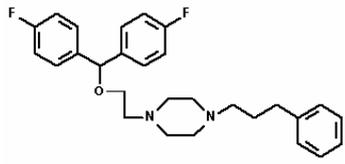
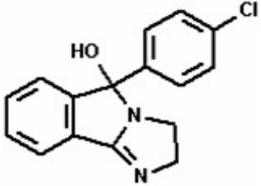
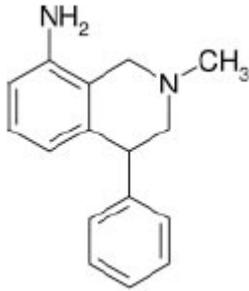
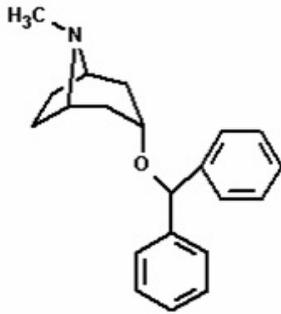
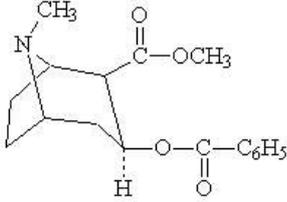
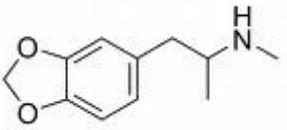
2.6.1 Dopamine reuptake inhibitor

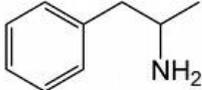
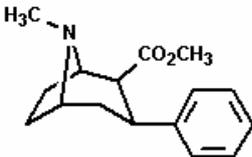
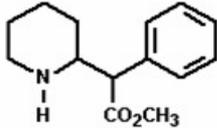
A dopamine reuptake inhibitor is a drug which is able to negatively affect the reuptake of DA by blocking DAT's action. In turn, this inhibition leads to increase extracellular concentrations of DA and therefore to potentiate the dopaminergic neurotransmission.

Inhibitors of DAT may be used in the clinical treatment of several pathologies, such as attention-deficit hyperactivity disorder (Solanto, 1998), narcolepsy (Fry, 1998), major depressive disorder (if resistant to typical treatments) (Nutt, 2008) and PD (see section 2.7). In addition, DAT inhibitors may be employed as anorectics for treating severe obesity (reviewed in Wang et al., 2002), they are studied as anticraving agents against drug addiction (reviewed in Haile et al., 2009) and they are often used as tool to indagine the site of action of cocaine, amphetamine and MDMA on DAT (Meiergerd and Schenk, 1994; reviewed in Carroll et al., 1992).

Among the DAT inhibitors, nomifensine, mazindole, GBR 12909, metilphenidate, benztropine and Win 35,065-2 have been shown to inhibit the DA reuptake at very low concentration (Table 2.1).

Table 2.1. Pharmacology data of DAT receptor ligands; K_i were obtained by competitive binding experiments using [^3H]Win-35,428 as tritiated probe

Ligand	Chemical structure	K_i (nM)	DAT source	References
GBR-12909		30 ± 2.3	Striatal rat synaptosomes	Zhu et al., 2009
Mazindole		12 ± 2	CHO cells transfected with wild type hDAT	Uakiro et al., 2007
Nomifensine		Not measurable*	-	-
Benztropine		85 ± 9	CHO cells transfected with wild type hDAT	Uakiro et al., 2007
Cocaine		197 ± 22	CHO cells transfected with wild type hDAT	Uakiro et al., 2007
MDMA		>10000	CHO cells transfected with wild type hDAT	Fantegrossi et al., 2009

AMPH		478 ± 52	CHO cells transfected with wild type hDAT	Uakiro et al., 2007
Win-35,428		2.81 ± 0.14	Sinaptosomi striatali di ratto	Zhu et al., 2009
Metilphenidate		72 ± 5	CHO cells transfected with wild type hDAT	Uakiro et al., 2007

* This value is impossible to evaluate with standards binding techniques, because nomifensine binds to the DAT on a different site than the ligand [^3H]Win-35 (Meiergerd and Schenk, 1994).

2.6.2 DAT and psychostimulants

Addiction to psychostimulants drugs is a significant medical, social and economic problem facing society. Traditionally, addiction to these drugs was treated as a psychological disorder; however, after the elucidation of the dangerous physiological effects of these drugs, their abuse has been classified as a physiological disorder. Therefore, today drug addiction is defined as a brain disorder characterized by compulsive drug-seeking behavior and uncontrollable drug intake (Koob, 2000). Although progress has been made in developing new psychosocial treatments for psychostimulants dependence, currently no medications approved for the treatment of cocaine dependence are available.

It has been shown that psychostimulant drugs interact with regions of the brain where dopaminergic terminals are abundant, specifically the mesolimbic dopaminergic system (Kreek, 2001). The two most widely abused psychostimulant drugs are cocaine and amphetamines (AMPHs).

Cocaine is the most powerful stimulant of natural origin (Fisher et al., 1987). It is extracted from the leaves of *Erythroxylon Coca* (Kreek et al.,

2005). It is still used by South Americans to relieve fatigue (Schultes, 1987). Pure cocaine (as hydrochloride) was used as a local anesthetic for surgeries in the 1880s and the main stimulant drug used in tonics and elixirs for various illnesses in the early 1900s (Koller-Becker, 1962). To date cocaine doesn't have any approved therapeutical indication, but it is widespread abused all around the planet as recreational drug. In last decade, the number of cocaine users has dramatically increased and to date cocaine abuse represent a serious health problem in many areas of the world. Only in the US, in 2001, there were an estimated 27.8 million who had used cocaine at least once in their lifetime (Gorelick et al., 2004; ONDCP, 2003) and in 2007 the estimated number of current cocaine users was 2.1 million (NIDA, research report series. Cocaine: abuse and addiction. 2009).

Amphetamine is a synthetic drug related to the plant derivative ephedrine and it was first synthesized in in 1887 (Edeleanu, 1887). Amphetamine is the first member of a group of compounds that have similar structures and biological properties and are collectively called "amphetamines". The group also includes methamphetamine, synthesized six years later and MDMA, patented in 1914 (Berman et al., 2008). AMPHs are known to produce insomnia and euphoria, enhance focus and decrease fatigue and appetite.

During time, amphetamine and methamphetamine were used for the treatment of narcolepsy, mild depression, postencephalitic parkinsonism, chronic alcoholism, cerebral arteriosclerosis and hay fever and they were also sold as decongestionant. During World War II amphetamine and methamphetamine were extensively used to combat fatigue and increase alertness in soldiers. To date, the only approved marketing indications for these two drugs are treatment of ADHD, obesity and narcolepsy (Berman

et al., 2008), but their use is tightly controlled, mainly because of their potential for abuse that can lead to severe psychological and physiological dependence (Berman et al., 2008). On the other hand, MDMA possess a too high abuse potential and it's not accepted for medical uses (Berman et al., 2008). According to the 2005 National Survey on Drug Use and Health (NSDUH), in US an estimated 10.4 million people age 12 or older (4.3 percent of the population) have been tried with AMPHs at some time of their lives and, in the same year, the estimated number of current AMPHs users was 512,000 (Research Report Series. Methamphetamine: abuse and addiction).

Both cocaine and AMPHs act increasing the extracellular DA concentrations, which is thought to mediate the rewarding and reinforcing properties of these drugs (reviewed in Torres et al., 2003); however the molecular mechanism of action of these two drugs is quite different.

Cocaine binds to the 5-HT transporter (SERT) (Woolverton and Johnson, 1992), norepinephrine transporter (NET) (Blakely et al., 1994) and DAT (Church et al., 1987; Di Chiara & Imperato, 1988; Hurd & Ungerstedt, 1989; Maisonneuve et al., 1990; Nielsen et al., 1983; Pani et al., 1990); thus cocaine inhibits presynaptic reuptake of 5-HT, norepinephrine and DA, elevating the concentration of these three neurotransmitters in the synaptic cleft. Although cocaine affects all the three monoamine transporters, the reinforcing and stimulating effects of cocaine seem to depend primarily on its interaction with DAT (Ritz et al., 1987; Koob and Bloom, 1988; Kuhar et al., 1991).

AMPHs have three effects at the monoaminergic synapse

- 1) blockade of DA uptake;
- 2) promotion of release of DA into the synaptic cleft;
- 3) inhibition of monoamine oxidase (MAO);

Concerning the first issue, it is well described that AMPHs are able to directly inhibit the DAT uptake activity (Schmitz et al., 2001).

For what it concerns the promotion of the DA release, AMPHs induce this effect through two mechanisms. On the one hand they activate the reversal DAT activity, which is described in section 2.2.2. On the other hand, AMPHs can increase the DA release through the depletion of DA vesicles. It has been demonstrated the existence of two classes of DA vesicles that are differentially depleted by AMPH (Anderson et al., 1998): at low concentrations, AMPH preferentially depletes the large vesicles, while at higher concentrations, AMPH depletes small vesicles more than large vesicles. It is known that, in the SNC, dopaminergic neurotransmission is regulated by DAT, but also by another membrane-bound transporter: the vesicular monoamine transporter-2 (VMAT2). Once the DA is transported inside the cell, VMAT2 permits DA uptake into synaptic vesicles or other intracellular organelles (Wang et al., 1997). Numerous evidences support the hypothesis that AMPH could be taken up into vesicles by VMAT2 and displace the vesicular DA (reviewed in Sora et al., 2009). However, it has also been well established that AMPHs compete with reserpine binding to VMAT2 (Peter et al., 1994); since reserpine acts by blocking the vesicular monoamine transporter VMAT2, it is still not straightforward to prove that AMPHs are actually VMAT2 transported substrates (reviewed in Sora et al., 2009).

As third and last point, it has been proved that AMPH inhibits the oxidation of aliphatic amines *ex vivo*, in tissues obtained from several sources including heart, liver and brain (Blaschko et al., 1940; Mann et al., 1937); specifically, AMPH acts as a competitive inhibitor for type A MAO (Mantle et al., 1976; Miller and Hoffman., 1980). In conclusion, the role of

MAO inhibition in the expression of the pharmacological effects of AMPH does not seem as robust as the effect of AMPH on amine transporters.

2.7 Molecular pathogenesis of Parkinson's disease

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder clinically characterized by motor symptoms such as tremor at rest, rigidity, slowness of movement (bradykinesia) and postural instability (Jankovic, 2008).

In 1817 James Parkinson first described the motor symptoms of the clinical syndrome (called "shaking palsy" by the author) that was later to bear his name (Parkinson, reprinted 2002). More than 100 years passed (1919) after the original description by Parkinson before it was recognised that patients with PD progressively lose dopaminergic neurons in the substantia nigra pars compacta (SNpc). These neurons reside in the midbrain and project axons to the forebrain where they release DA into the striatum. Striatal DA release is critical for the coordination and initiation of movement. The loss of this DA input is primarily responsible for the manifestation of motor symptoms in PD. Another pathological hallmark of PD is the presence of intracytoplasmic abnormal aggregates of protein in dopaminergic neurons, called Lewy bodies (LB); first described in 1912 by Friedrich Lewy following post-mortem analysis of brains, LB are found primarily in the SNpc and another midbrain nucleus, the locus coeruleus (LC) in PD patients and are still considered one of the standard criterion for PD diagnosis (Gibb and Lees, 1988). In 1960 it was discovered that DA concentrations are markedly decreased in the striatum of patients with PD;

this finding soon led to the first trials of levodopa in PD patients (Birkmayer and Hornykiewicz, 1961).

To date, increased oxidative stress, mitochondrial dysfunction, genetic mutations, abnormal handling of misfolded proteins by the ubiquitin–proteasome and the autophagy–lysosomal systems, inflammation and other pathogenic mechanisms have been identified as contributing factors in the death of dopaminergic and non-dopaminergic cells in the brains of patients with PD. Some of them are briefly described in following sections.

2.7.1 Dopamine metabolism and oxidative stress

DA is synthesized in the cytoplasm from the amino acid tyrosine. Tyrosine is converted to L-DOPA by the cytoplasmic enzyme tyrosine hydroxylase (TH); this step represents the rate-limiting step of catecholamine synthesis (Fig.2.5) (Kumer and Vrana, 1996). L-DOPA is decarboxylated to DA by L-amino-acid decarboxylase (AADC) (Fig.2.5) (Kumer and Vrana, 1996). Newly synthesized DA is taken up into synaptic vesicles by the vesicular VMAT2 in a ATP-dependent process. Norepinephrine and epinephrine are subsequently synthesized from DA; thus biogen amines share the same biosynthesis pathway and primary precursor. The multiple and complex mechanisms regulating TH activity and its expression are not the subjects of this PhD dissertation; however it worth remarking that TH activity is regulated through feedback inhibition by catecholamines (Zigmond et al., 1989) and that the activation of the DA D₂ autoreceptor further inhibits DA synthesis.

Vesicular DA is released into the synapse from the neuronal terminal by exocytosis following stimulation and recycled back into the terminal by DAT. Once reuptaken from the synaptic cleft, DA can either be stored into

synaptic vesicles to be reused, or metabolized by mitochondrial MAO_B (Fig.2.5) (Berry et al., 1994) to form hydrogen peroxide (H₂O₂) and 3,4-dihydroxyphenylaldehyde, which is rapidly oxidized by aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid (DOPAC). Approximately 40% of DOPAC is eliminated without further metabolism and 60% is converted to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT) (Fig.2.5) (reviewed in Eisenhofer et al., 2004)

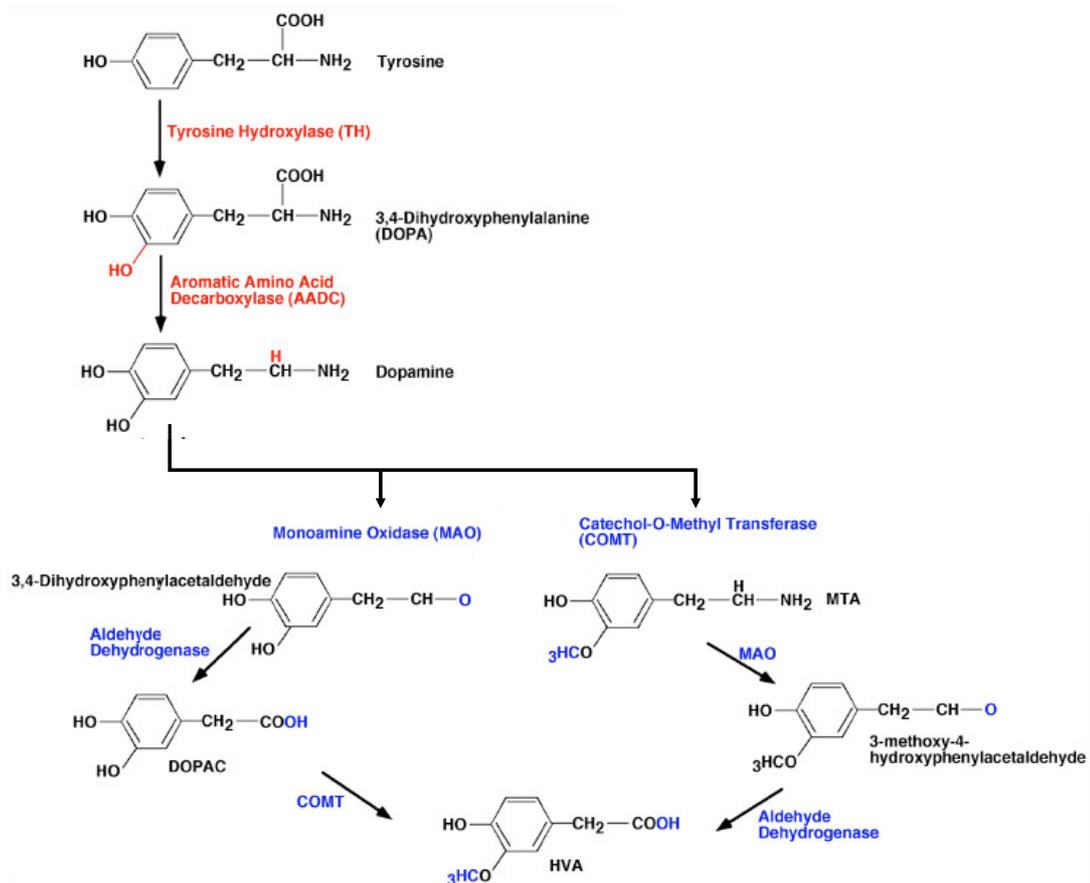


Figure 2.5. Metabolism of DA.

Cytosolic DA has the propensity to generate cytotoxic reactive oxygen species (ROS) and neurotoxic quinones (DAQs), spontaneously or by enzymatic metabolism (Graham, 1978; Hastings et al., 1996; Montine et al., 1997).

Oxidation of DA generates superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which can form an hydroxyl radical (OH^\bullet) in the iron-catalyzed Fenton reaction (Halliwell and Gutteridge, 1984). If these ROS are not inactivated by natural antioxidants (glutathione peroxidases, superoxide dismutases, catalase, reduced glutathione (GSH), ubiquinol, uric acid, essential minerals and vitamins), their high chemical reactivity can damage all types of cellular macromolecules susceptible to oxidation (German, 1999). Interestingly, GSH levels within dopaminergic neurons are depleted in PD patients, suggesting that, in these cells, the ability to manage ROS is compromised or overwhelmed by an abnormal ROS production (Jenner, 2003).

Inside cells, several enzymes can catalyze DAQs synthesis. These species react with cysteinyl residues of proteins to form 5-cysteyl-catechols adducts (LaVoie and Hastings., 1999). Since sulfhydryl groups of cysteine are often located in the active site of proteins, the normal cellular functions of the affected proteins can be inhibited by the covalent interaction with DAQs, with toxic consequences (LaVoie and Hastings., 1999).

In addition, also the DA metabolite DOPAC reacts with cystein residues of proteins to form covalent adducts (Fornstedt et al., 1986).

As reported, DA is likely to be considered a neurotoxic molecule. In normal conditions, VMAT2 sequesters about 90% of the catecholamines, leaving only a 10% to escape and being metabolized (reviewed in Eisenhofer et al., 2004). This rapid sequestration activity is thought to protect the cells from an excessive intracellular accumulation of DA and subsequent degradation to neurotoxic species. When a dysfunction causes the level of intracellular DA to abnormally increase, vesicles can't sequester the DA which is metabolized by MAO (A and B) and COMT with consequent overproduction of oxygen reactive species and toxic

catabolites (Hastings, 1995, Hastings et al., 1996). Stress induced by these molecules leads cells to death.

Thus, DA concentrations in the cytoplasm appear to be strictly connected to DA neurotoxicity and, even if it is well established that PD is a multifactorial disease, an increasing number of evidences suggests that DA metabolites-induced oxidative stress is a common mechanism by which dysfunctions in synthesis, storage, release, reuptake and metabolism of DA lead to the dopaminergic neurons degeneration observed in PD (Jenner, 2003).

2.7.2 Genetic factors contributing to Parkinson's disease

It is known that a variety of genetic and environmental factors contribute to the degeneration of nigral DA neurons observed in PD. For what it concerns the genetic component, so far mutations of five genes have been linked clearly to familial forms of PD: leucine-rich repeat kinase 2 (LRRK2), serine/threonine-protein kinase (PINK1), DJ-1 (PARK7), α -synuclein (α -Syn) and parkin (PARK2).

LRRK2, or dardarin, is a protein member of the leucine-rich repeat kinase family. The cellular and molecular mechanisms of LRRK2 toxicity remain unclear, but it has been described that mutations in LRRK2 gene are associated with an autosomal-dominant Parkinsonism, with clinical and molecular features of PD, including deposits of aggregated protein (Smith et al., 2005), known as Parkinson's disease type 8.

PINK1 is a mitochondrial enzyme which is thought to protect cells from stress-induced mitochondrial dysfunction. In fact, *in vitro* overexpression of wild type PINK1 reduces the basal neuronal pro-apoptotic activity

preventing mitochondrial cytochrome c release and this function is abrogated in familial PD-linked PINK1 mutants (Petit et al., 2005).

DJ-1 is an highly conserved 189 amino acid protein which is ubiquitously and widely expressed in most mammalian tissues, including brain and belongs to the DJ-1/ThiJ/PfpI superfamily. Deletion and point (L166P) mutations of DJ-1 have DJ-1 locus have been associated with a rare form of autosomal recessive early-onset parkinsonism, known as type 7 (Bonifati et al., 2003; Taira et al., 2004). DJ-1 eliminates hydrogen peroxide *in vitro* by oxidizing itself exhibiting intrinsic ROS scavenger properties and its expression is induced by oxidative stress (Taira et al., 2004). The protective role of DJ-1 is confirmed by the finding that mutations of DJ-1 lead to cell death *in vitro* (Taira et al., 2004).

α -Syn is a protein underlying multiple cellular functions being involved in modulation of DAT recycling (see section 2.5) and regulation of neuronal Golgi apparatus and vesicle trafficking (Cooper et al., 2006) and it is also able to interact with microtubules (Alim et al., 2004) and membrane lipids (Uversky, 2007). Three missense mutations in α -Syn gene (A53T, A30P and E46K) (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004) as well as α -Syn gene locus triplication (Singleton et al., 2003) are associated with autosomal dominant PD. The involvement of α -Syn in PD pathogenesis is further confirmed by the finding that fibrillar aggregations of α -Syn are the major structural component of Lewy bodies (Spillantini et al., 1998).

Mutated α -Syn and abnormal α -Syn expression alter multiple molecular functions of the cell to produce the neurotoxic effects. Both A30P and A53T mutants increase vulnerability of cells towards various toxic insults and enhance DAT-mediated DA toxicity (Lehmensiek et al., 2006). Mice expressing human A53T α -Syn develop mitochondrial

pathologies and dysfunctions (Stichel et al., 2007). In addition, overexpression of α -Syn leads to accumulation of the protein which cause a cytotoxic dysregulation of the vesicular trafficking from endoplasmic reticulum to Golgi apparatus (Cooper et al., 2006).

Due to the crucial roles of α -Syn in PD pathogenesis, to date the term “synucleinopathy” is often used in literature as PD synonymous (Mosharov et al., 2009).

Parkin is a component of a multiprotein E3 ubiquitin ligase complex which in turn is part of the ubiquitin-proteasome system that mediates the targeting of substrate proteins for proteasomal degradation (Shimura et al., 2000). Mutation of Parkin gene sequence have been identified in patients with a familial form of PD, known as autosomal recessive juvenile Parkinson disease (Polymeropoulos et al., 1997; Shimura et al., 2000).

A first obvious predicted mechanism for recessive Parkin mutations in PD is the accumulation of toxic proteins that would normally be degraded by Parkin. Although many substrates of E3 ubiquitin ligase have been identified (CDCrel-1, synphilin 1, α -Sp22, and α -Syn), none of them were found to accumulate in the brains of Parkin-deficient mice (Goldberg et al., 2003; Palacino et al., 2004). However, it is possible that another unidentified Parkin substrate can accumulate to toxic levels upon Parkin inactivation.

It is known that Parkin impairs the α -Syn/DAT coupling by interacting with the carboxyl-terminus of the DAT and blocks the α -Syn modulation of DAT trafficking (Moszczyńska et al., 2007). In certain conditions, i.e. overexpression, α -Syn can even enhance both DAT cell surface expression and DAT-mediated DA uptake leading to an increase of the DA induced oxidative toxicity. This effect is counteracted by wild type Parkin that acts as a neuroprotective molecular mechanism, but it is still unclear if Parkin

mediates these effects by blocking the DAT internalization or the DAT exocytosis pathways (Moszczynska et al., 2007). In addition, overexpression of Parkin alone also directly protects human DA neuroblastoma cell lines SH-SY5Y and SK-N-SH from apoptosis induced by DA or 6-OHDA and also decreases the level of ROS and protein carbonyls in the cell. (Moszczynska et al., 2007; Jiang et al., 2004). Consistent with this, PD-linked mutations of Parkin significantly abrogated the observed protective effect of wild-type Parkin, as well as its ability to suppress ROS and protein carbonylation (Jiang et al., 2004). For these reasons, to date, Parkin is considered a neuroprotective protein that is crucial for neuronal survival (Feany and Pallanck, 2003).

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AIM OF THE STUDY, METHODS AND RESULTS

CHAPTER 3,

MOLECULAR REGULATION OF DAT UPTAKE ACTIVITY BY σ_1 RECEPTORS

3.1 Aim of the study

Stimulation of σ_1 receptors has a biphasic effect on intersynaptic [dopamine] (DA) *In vivo*, σ_1 agonists rapidly produce a significant (40-50% of basal levels) increase in extracellular [DA] observed within 30 minutes of administration. This increase is followed by a more prolonged (150 minutes) and marked decrease (-70% of basal levels) of the extracellular DA levels (see section 1.9.7).

It has been shown that DA release is inhibited by σ_1 agonists in many experimental paradigms (see section 1.9.7). For this reason, inhibition of DA release has been proposed to contribute σ_1 -mediated fluctuations of extracellular [DA]. However, the hypothesis that σ_1 receptors could act through the modulation of DA uptake has never been investigated.

In dopaminergic neurons, uptake of intersynaptic DA is mediated by DA transporter (DAT) and this uptake represents the most relevant molecular mechanism extinguishing dopaminergic signal.

Due to the key role of DAT in regulating extracellular DA levels, the first part of this research has been performed to understand if σ_1 receptors may modulate DAT uptake mechanisms in cells models and, to investigate it, eight different clones have been set up.

- A) HEK-293 and SH-SY5Y native cells;
- B) HEK-293 and SH-SY5Y cells stably transfected with human σ_1 ($h\sigma_1$) receptors;
- C) HEK-293 and SH-SY5Y native cells, transiently transfected with human DAT (hDAT);
- D) HEK-293 and SH-SY5Y cells stably transfected with human $h\sigma_1$ (A), transiently transfected with human DAT (hDAT).

Cells A) were adopted to estimate basal values. B) clones have been used to check the stable transfection of h σ_1 and to produce the double transfected D) clones. Ultimately, C) and D) were employed in uptake assays as model of hDAT in presence or absence of h σ_1 overexpression. 48h after transfection with hDAT, uptake activity has been evaluated adopting the [3 H]DA uptake assay, a technique that is well characterized in literature (Cervinski et al., 2005). Once obtained basal uptake values, a molecular screening was performed to elucidate relationship between σ_1 agonists and antagonists and the amount of uptaken DA.

3.2 Methods

3.2.1 Cell cultures

For this work two different cell lines have been employed:

- 1) HEK-293 is a cell line derived from human embryonic kidney cells. This cell line has been obtained from healthy aborted fetus and immortalized using sheared adenovirus 5 DNA (Graham et al., 1977). HEK-293 are very easy to grow and transfected and are widely employed in cell biology.
- 2) SH-SY5Y is a thrice-cloned neuroblastoma sub-line of a bone marrow biopsy-derived line SK-N-SH (Biedler et al., 1978). SH-SY5Y neuroblast-like subclone preserves norepinephrine (NE) neurons characteristics since it possess endogenous NE uptake activity and dopamine- β -hydroxylase activity. However, this line loses its neuronal characteristics with increasing passage

numbers and it is not recommended to continue culture beyond 20 passages. SH-SY5Y cell line is difficult to transfect and it is very sensitive to growth conditions, but it is considered a good non-primary model of neurones.

HEK-293 were grown in 75 ml sterile tissue-treated cell culture flasks (Sarsted), in a CO₂ water-jacketed incubator at 37°C with 5% CO₂ content. The medium used for HEK cells was the Minimum Essential Eagle (MEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 µg/ml penicillin-streptomycin (Gibco), 2 mM L-glutamine (Lonza) and 1% Non Essential Amino Acids (NEAA, Gibco). Cells were splitted to 1:8 rate once they reached about 80% of confluence.

SH-SY5Y were grown in 75 ml sterile tissue-treated cell culture flasks (Sarsted), in a CO₂ water-jacketed incubator at 37°C with 5% CO₂ content. The medium used for SH-SY5Y cells was 41,5% Nutrient Mixture F-12 (Ham's F-12 Lonza) and 41,5% MEM (Gibco), supplemented with 15% fetal bovine serum (FBS, Lonza), 100 µg/ml penicillin-streptomycin (Gibco), 2 mM L-glutamine (Lonza) and 1% Non Essential Amino Acids (NEAA, Gibco). Cells were splitted to 1:6 rate once they reached about 80% of confluence.

3.2.2 *Plasmids characterization*

Three plasmids have been employed in this study.

3.2.2.1 pCMV6 Entry-hSigma-1

This plasmid was bought from OriGene Technologies Inc. and encodes for the complete h σ_1 open reading frame (ORF) (672 bp, reference sequence: NM_005866.2) fused with the expression Myc/Flag tag peptides at the C-terminal extremity.

After bacteria transformation and plasmid purification (see section 3.2.3), the integrity of plasmid DNA was checked running the product of a double restriction enzyme digestion on agarose gel (1% p/v).

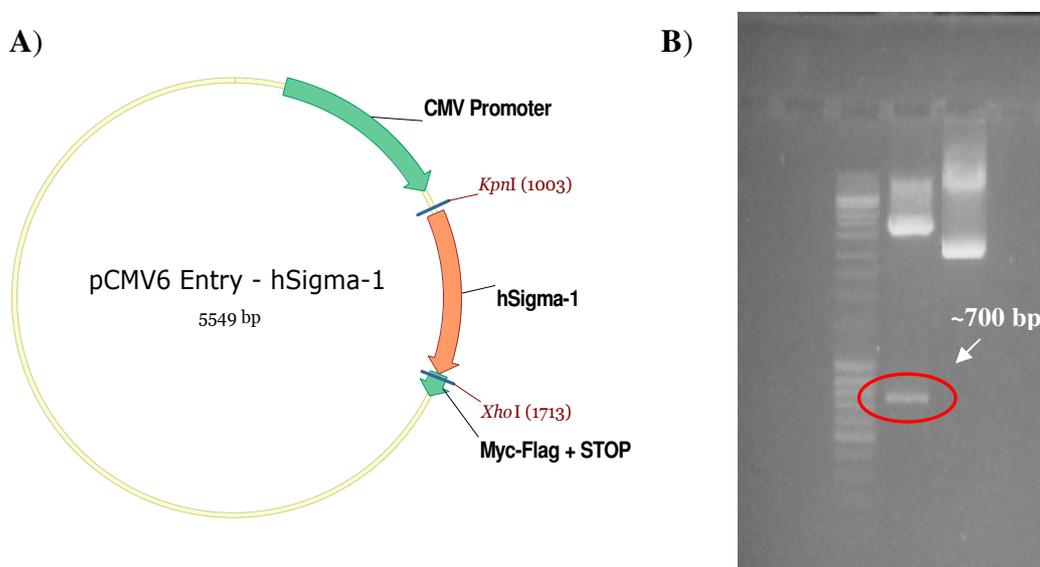


Figure 3.1. (A) schematic map of pCMV6Entry- h σ_1 ; (B) digestion of the purified plasmid with restriction enzymes KpnI and XhoI produces a predicted fragment of about 700bp.

3.2.2.2 pEGFP-C1-hDAT

This plasmid was a kind gift of Professor Susan Amara, Thomas Detre Professor and Chair of Neurobiology at University of Pittsburgh School of Medicine. pEGFP-C1-hDAT encodes for the complete hDAT ORF (1863 bp,

reference sequence: NG_015885.1) fused with the enhanced green fluorescence protein (EGFP) at N-terminus.

After bacteria transformation and plasmid purification (see section 3.2.3), the integrity of plasmid DNA was checked running the product of a double restriction enzyme digestion on agarose gel (1% p/v).

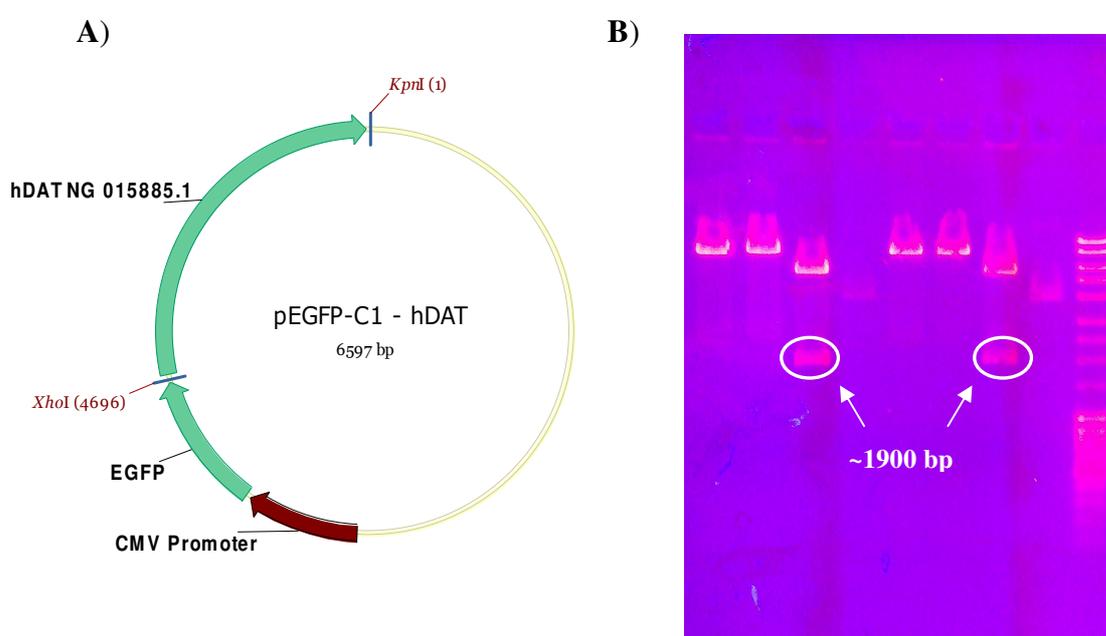


Figure 3.2. (A) schematic map of pEGFP-C1-hDAT; (B) digestion of the purified plasmid with restriction enzymes KpnI and XhoI produces a predicted fragment of about 1900bp.

3.2.2.3 pcDNA3.1(-)-hDAT

This plasmid was subcloned from pEGFP-C1-hDAT. Briefly, the tract of DNA including hDAT sequence was excised through digestion with KpnI and XhoI restriction enzymes. Simultaneously, the same restriction enzymes were used to cut the destination vector (pcDNA3.1(-)). Digestion products were then separated on agarose gel (1% p/v) and purified with a commercial kit

(Promega Wizard[®] SV Gel and PCR Clean-Up System). Once extracted, purified and quantified, a 3:1 ratio of insert and destination vector were mixed together with T4 DNA ligase (Invitrogen) and the product of ligase reaction was directly transformed into a E.Coli strain (see section 3.2.3 for transformation procedure).

After bacteria transformation and plasmid purification (see section 3.2.3), the integrity of plasmid DNA was checked running the product of a double restriction enzyme digestion on agarose gel (1% p/v).

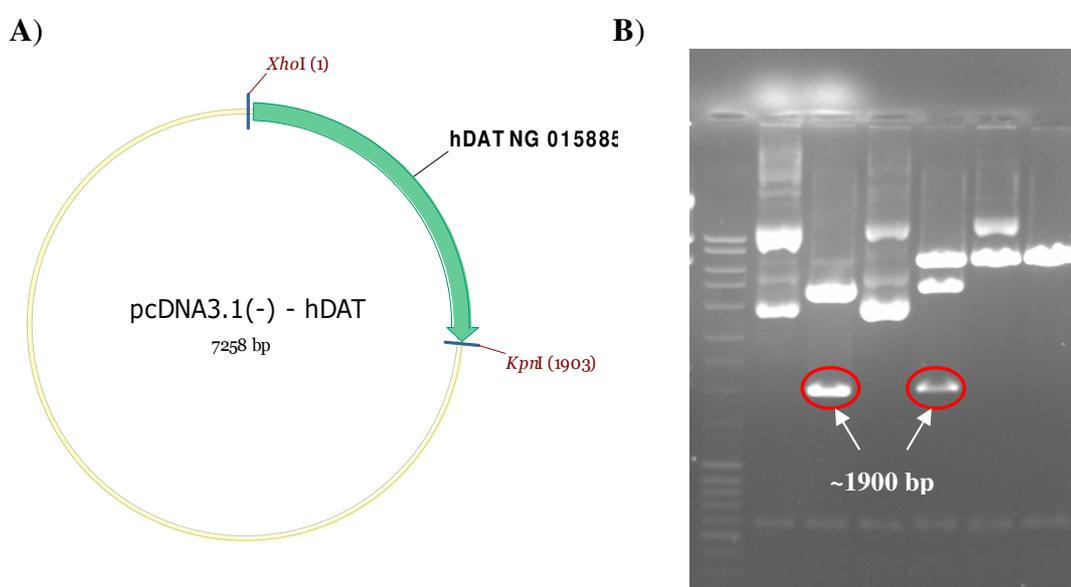


Figure 3.3. (A) schematic map of pcDNA3.1(-)-hDAT; (B) digestion of the purified plasmid with restriction enzymes KpnI and XhoI produces a predicted fragment of about 1900bp.

3.2.3 *Bacteria transformation, growth and plasmid purification*

Invitrogen One Shot TOP10[®] library efficiency chemically competent *E.Coli* were transformed according to manufacturer protocols. Briefly, 1µg of plasmid DNA was pipetted directly into the vial of competent cells and the mixture was incubated 30 minutes on ice. After this phase, the vial was incubated for 30 sec in a prewarmed water bath at 42°C and placed in ice for 3 minutes. 250 µl of pre-warmed S.O.C were then added to the bacteria and the mixture was left 1 hour at 225 rpm in a shaking incubator at 37°C. 20 µl to 100 µl of the transformation vial content were then spread on Luria-Bertani (LB) agar plates containing 50µg/ml of the required selection antibiotic and dishes were inverted and incubated at 37°C overnight. The day after, newborn monoclonal colonies were selected to grow 16 hours in 5 ml of liquid LB media; plasmids were then extracted and analyzed by enzymatic restriction digestion and sequencing.

Selected colonies were grown in 5 ml and 100 ml, respectively for mini and midi preparations. Plasmids were purified using anion-exchange tips provided in a commercial kit (QIAGEN Plasmid Midi Kit) and the extracted DNA was quantified by reading the absorbance at 260, 280 e 320 nm.

3.2.4 *Transfection and selection of the engineered eukaryotic clones*

Both HEK-293 and SH-SY5Y were transfected using the ExGen500 cationic polymer gene delivery reagent (Fermentas). Specifically, 3.3µl of ExGen500 were used to complex 1 µg of plasmid DNA for transfecting 80000 cells.

Transiently transfected cells were used 48h after transfection.

Stable transfected cells were selected by supplementing the complete medium with G418 at the final concentration of 500 µg/ml. Transfected cells were then maintained in this conditions and they were used at least 3 weeks after selection with G418.

3.2.5 *β-galactosidase enzyme assay system*

β-galactosidase enzyme assay system was used to evaluate β-galactosidase activity in lysates prepared from cells transfected with pSV-β-Gal vector. To estimate β-galactosidase activity, a commercial kit has been employed (Beta-Glo[®] Assay System–Promega). 48 h after transfection with pSV-β-Gal, about 150000 cells were lysed by freeze thawing in 200 µl of lysis buffer and 50 µl of this lysate were mixed with 50 µl of Beta-Glo[®] substrate. Samples were then incubated for 2 h, allowing the substrate (D-luciferin-o-β-galactopyranoside) to be cleaved to release luciferin. Luciferin serves as a substrate for luciferase that is also present in the reagent. As result of the luciferase activity, oxyluciferin is formed with subsequent light emission, wich is detected and quantified in a PerkinElmer Victor² luminometer/spectrophotometr. The level of pSV-β-Gal plasmid expression is directly related to luminescence, expressed in arbitrary luminescence units.

3.2.6 *Protein extraction and quantitation*

Proteins were extracted using a commercial kit (NE-PER[™] Extraction reagent; Pierce). Briefly cells were pelleted and resuspended in 100 µL of CER I buffer After 10 min incubation on ice, 5.5 µL of CER II buffer was added and the mixture was vortexed, incubated on ice for 1 min and then resuspended. The cytoplasmic fraction was separated by centrifugation at

16000 g for 5 min. Proteins were then quantified using the method of Lowry (Lowry et al., 1951).

3.2.7 Western blot experiments

Proteins of the cytoplasmic extract (50 µg) were denatured at 95°C for 3 min before being loaded and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

We used a MagicMark™ XP Western Standard as molecular weight standard. Proteins were then transferred to Protran™ nitrocellulose membranes which were blocked with 5% nonfat milk in TBS (10 mM Tris-HCl, pH 8, containing 150 mM NaCl) plus 0.1% Tween 20 for 2 h at room temperature (RT). After washing three times the membrane with TBS, blots were probed for 1 h at RT in TBS containing monoclonal ANTI-FLAG™ M5 antibody (Sigma-Aldrich) at final concentration of 10 µg/ml. The membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1:10000. Blots were developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce™). Chemiluminescence was acquired using a luminescent image analyzer LAS-3000 (Fuji-film™). The membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1:2000. Blots were finally developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce™). Chemiluminescence was acquired using a luminescent image analyzer LAS-3000 (Fuji-Film™).

3.2.8 *DA uptake experiments*

[³H]DA assays were performed in 24-wells plates (Corning) precoated with poly-L-lysine. Four days before the experiments, 70000 cells were seeded in each well of the plate. 24h later, cells were transfected with pcDNA3.1(-)-hDAT and then left 48 h in the incubator at 37 °C. The day of the experiment, cells were washed once with 1 ml of PBS and incubated for 2 hours in serum free media. Cells were then washed twice with PBS and triplicate wells were pretreated at 37 °C with 300µl of filtered Krebs-Ringer HEPES (KRH) buffer (25 mM HEPES, 125mM NaCl, 4.8mM KCl, 1.2mM KH₂PO₄, 1.3mM CaCl₂, 1.2 mM MgSO₄, 5.6 mM glucose, pH 7.4) containing vehicle, σ ligands, or phosphorylation modulators. σ ligands and PKC modulators were added respectively. 1 h and 30 min prior to start the assay for DA transport. Compounds were dissolved at high concentration (10⁻²M) in DMSO followed by dilution in KRH buffer to a final DMSO concentration range varying from 1% to 0.0000001% v/v. At the end of the pretreatment interval, cells were placed on ice and uptake was initiated by adding 3.03 µl of a 100X DA stock solution to bring the final concentration of [³H]DA (PerkinElmer, 35Ci/mmol) to 10 nM and that of total DA to 3 µM. Control wells didn't receive any treatment and nonspecific uptake was determined in the presence of nomifensine 10 µM. Uptake assays were carried out at 37 °C for 10 min and terminated by rapidly washing the wells three times with 1 ml of ice-cold KRH. Cells were solubilized with 500 µl of a 2% SDS solution and lysates were then measured for incorporated radioactivity by liquid scintillation after 2 h of incubation in scintillation cocktail.

3.2.9 *Analysis of data*

Data were analyzed with GraphPad Prism[®] software (version 4.0; GraphPad Software Inc., San Diego, CA, USA).

[³H]DA uptake data were normalized by setting DPM values of controls as 100% and of background as 0%. Controls were transfected with pcDNA3.1(-)-hDAT and didn't receive any treatment before being assayed for [³H]DA uptake activity. Background data were obtained by measuring [³H]DA uptake in cells not transfected cells with the hDAT plasmid and treating transfected cells with nomifensine (DAT selective inhibitor) for 30 minutes at final concentration of 10 μM (see section 3.3.3).

All data are presented as mean ± standard error of the mean (SEM) for the indicated number of experiments. Statistical significance was determined by Newman-Keuls test after analysis of variance (ANOVA) using GraphPad Prism Prism[®] software (version 4.0; GraphPad Software Inc., San Diego, CA, USA). Newman-Keuls test post ANOVA was used to compare all data versus controls and among multiple groups. P-values < 0.05 were considered to be significant.

3.2.10 *Immunofluorescence and confocal microscopy*

70000 cells/well were seeded in a 6-wells plate containing a sterilized poly-L-lysine precoated microscope glass coverslip (Carlo Erba). After 48 h cells were transfected with pEGFP-C1-hDAT. 48 h after transfection cells were incubated for 1 h in serum free medium in presence, or in absence of (+)-pentazocine at final concentration of 1 μM and then fixed with 0.5 ml of a 3% solution paraformaldehyde (PFA) in PBS at 25 °C for 15 minutes. Fixed cells were then washed three times with a PBS-BSA 3%

solution and permeabilized in 70% ice-cold ethanol for 3 minutes. After washing three times with PBS-BSA 3%, samples were incubated 2h at RT in PBS-BSA 3% containing monoclonal mouse ANTI-FLAG™ M2 antibody (Sigma-Aldrich) at final concentration of 1 µg/ml. Cells were washed three times with PBS-BSA 3% and incubated for 1h at RT with PBS-BSA 3% containing 4',6-diamidino-2-phenylindole (DAPI) at final concentration of 300 nM to stain nuclei and CF™555 goat anti-mouse anti-mouse secondary antibody (Biotium) at final concentration of 7 µg/ml. Cells were washed three times with with PBS-BSA 3% and coverslip were mounted onto a preclean slide (Carlo Erba) using ProLong® Gold Antifade Reagent (Invitrogen) as mounting media. Mounted slides were left overnight at RT in the dark and then analyzed by confocal microscopy using a Nikon C1si equipped with Nikon eclipse TE300 confocal laser-scanning microscope.

DAPI, EGFP and CF™555 secondary antibody were excited respectively at 405 nm, 488 nm and 543 nm and multiple images were detected respectively at 450 nm, 515 nm and 605 nm.

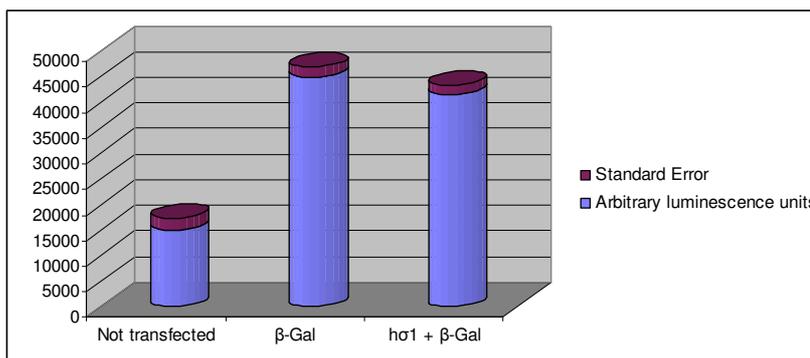
3.3 Controls employed to validate experimental groups

Several controls were performed to validate the experimental procedure adopted to investigate cell [^3H]DA uptake and cell viability.

3.3.1 Validation of transfection techniques using β -galactosidase enzyme assay system

Efficiency of transfection conditions was checked by the β -galactosidase enzyme assay system (See section 3.2.5).

A)



B)

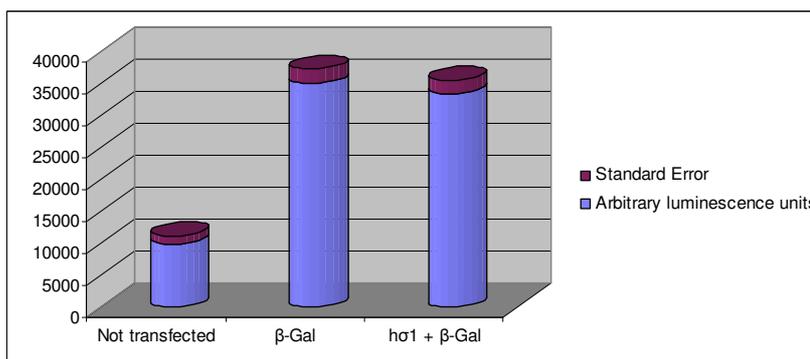


Figure 3.4. β -galactosidase activity in native cells (Not transfected), in cells transfected with pSV- β -Gal vector alone and in cells stably overexpressing $h\sigma_1$ transfected with the pSV- β -Gal vector. **A)** results on HEK-293 cell line; **B)** results on SH-SY5Y cell line.

Results of β -galactosidase assays are reported in figure 3.4. In both HEK-293 and SH-SY5Y cell lines, transiently transfected with pSV- β -Gal, produced luminescence is 3 times higher than in not transfected controls. This result indicates that β -galactosidase is overexpressed only in cells that received the pSV- β -Gal plasmid. Moreover, these data indicate that following transient transfection with pSV- β -Gal vector, expression of β -galactosidase in cells stably transfected with $h\sigma_1$ receptor does not change in comparison to cells transfected with pSV- β -Gal vector alone. Thus, the expression of β -galactosidase is not influenced by the presence of pCMV6 Entry- $h\sigma_1$ vector.

Transfection conditions optimized with pSV- β -Gal were then used in experiments done on cells transfected with pcDNA3.1(-)-hDAT plasmid.

3.3.2 Western blot of stably expressed $h\sigma_1$ -FLAG fusion protein

Overexpression of fusion protein $h\sigma_1$ -FLAG was confirmed with western blot experiments. Cells overexpressing $h\sigma_1$ were then selected and used in experiments.

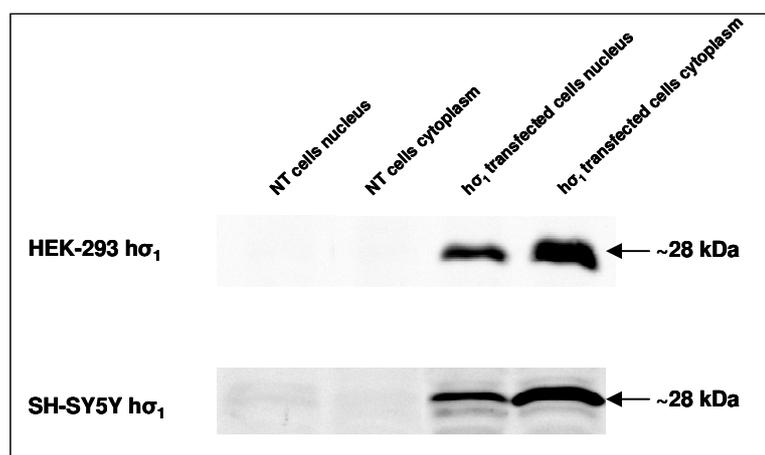


Figure 3.5. Western blot of HEK-293 and SH-SY5Y stably expressing $h\sigma_1$ -FLAG fusion protein compared to not transfected (NT) cells.

3.3.3 *Topology and expression analysis of EGFP-hDAT and hσ₁ in HEK-293 by confocal microscopy*

In the experiments, nuclei were stained with DAPI, which emission falls in blue wavelength range.

Not transfected control are not showed because in these cells any green-shifted fluorescence was detectable.

Since EGFP-hDAT fusion protein is able to produce fluorescence alone while excited at 488 nm, its detection doesn't need conjugation with any secondary fluorescent antibody (Fig.3.6).

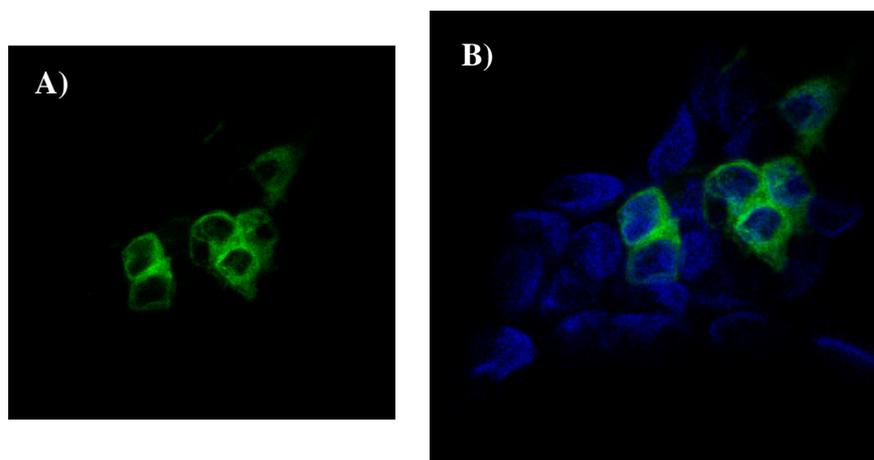


Figure 3.6. Confocal images of HEK-293 transiently transfected with pEGFP-C1-hDAT; **A)** image of EGFP-hDAT alone; **B)** merged image of nucleus marker (blue) and EGFP-hDAT (green); note that EGFP-hDAT fluorescence appears predominantly near the cell surface.

These experiments confirm that HEK-293 cells transiently transfected with the pEGFP-C1-hDAT plasmid express the fluorescent fusion protein. Noteworthy, EGFP-hDAT appears mainly located on the plasma membrane level suggesting that the expressed protein is a functional membrane transporter.

As it concerns the detection of $h\sigma_1$ -FLAG, this fusion protein requires conjugation with a fluorescent probe in order to be detected by confocal microscopy. A red-shifted secondary antibody was chosen as it doesn't interfere with blue DAPI and green EGFP fluorophores.

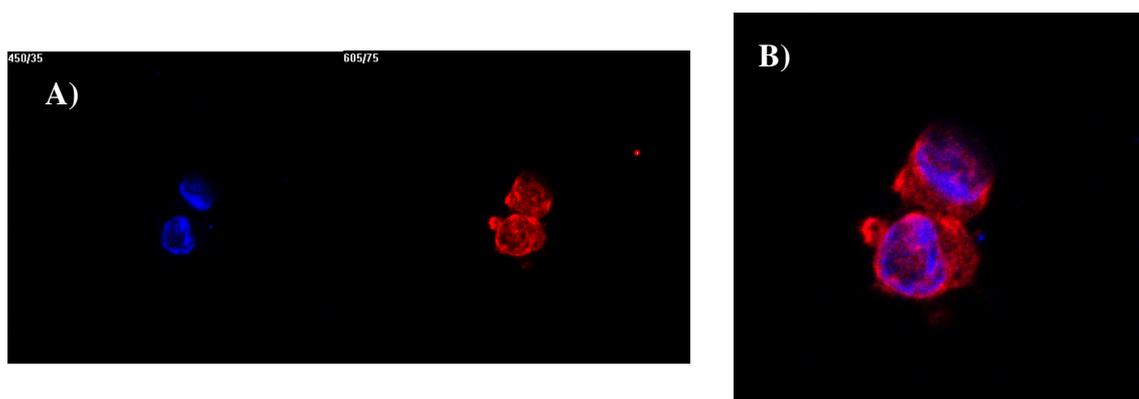


Figure 3.7. Confocal images of HEK-293 stably transfected with pCMV6 Entry-*hSigma1*. **A)** separated images of nuclei stained with DAPI (blue) and $h\sigma_1$ -FLAG receptors labeled with a fluorescent probe (red); **B)** merged image of nucleus and $h\sigma_1$ -FLAG receptor markers; note that $h\sigma_1$ -FLAG receptors fluorescence appears predominantly in the cytoplasm.

These experiments confirm the stable expression of $h\sigma_1$ -FLAG fusion protein. According to previously described subcellular localization of rat σ_1 receptors (see section 1.4.2), overexpressed $h\sigma_1$ receptors are mainly localized in the cytoplasm and less around the cell membrane.

Cotransfection of both EGFP-hDAT and $h\sigma_1$ -FLAG fusion proteins was also confirmed by confocal microscopy techniques. As showed in figure 3.8, both the proteins are expressed in double transfected clones.

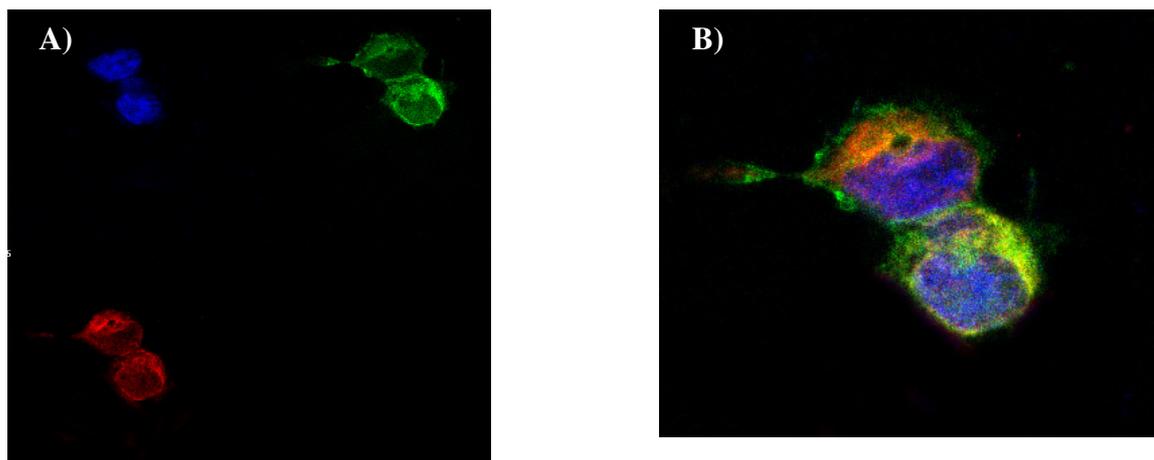


Figure 3.8. Confocal images of HEK-293 cotransfected with pCMV6 Entry-hSigma, and -C1-hDAT; **A)** separated images of nuclei stained with DAPI (blue), hσ₁-FLAG receptors labeled with a fluorescent probe (red) and EGFP-hDAT (green); **B)** merged image of nucleus and hσ₁-FLAG receptor markers and EGFP-hDAT; note that hσ₁-FLAG receptors fluorescence appears predominantly in the cytoplasm whereas EGFP-hDAT appears mainly located on the plasma membrane.

3.3.4 Set up of [³H]DA uptake assay

[³H]DA uptake assays were performed in order to check if native HEK-293 and SH-SY5Y possess a basal uptake activity of the [³H]DA and if, once transfected with pcDNA3.1(-)-hDAT, they are able to express a functional protein capable to elevate [³H]DA uptake. Thus, uptake abilities of not transfected (NT) cells or of cells transfected with pcDNA3.1(-) empty vector and of cells transfected with pcDNA3.1(-)-hDAT were compared counting the recovered radioactivity after 10 minutes of incubation at 37°C in KRH buffer containing the [³H]DA.

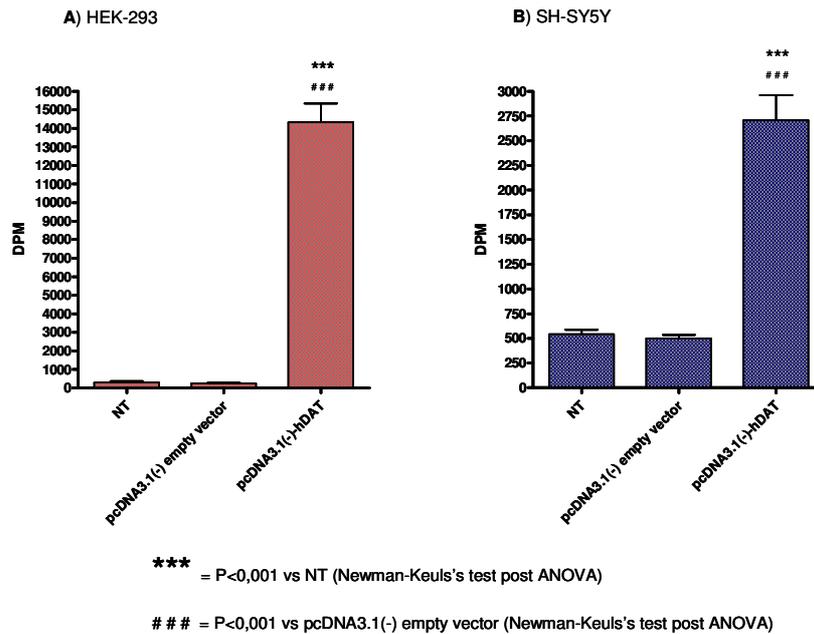
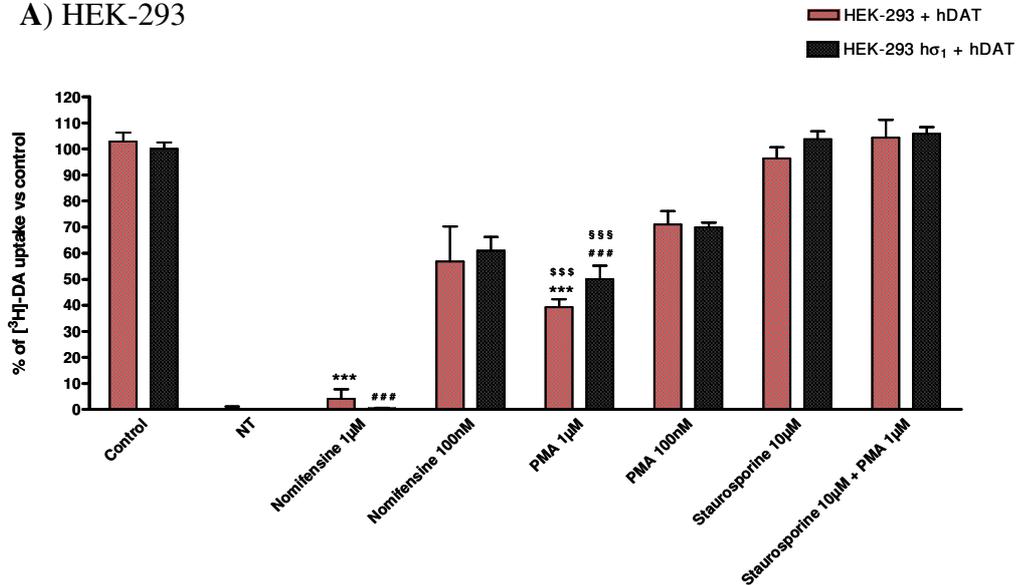


Figure 3.9. DPM values retrieved in three different clones of **A)** results on HEK-293, data are presented as mean \pm SEM of two independent experiments (carried out in triplicate); **B)** results on SH-SY5Y, after 10 minutes of incubation at 37°C with [3 H]DA in KRH buffer data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

Since radioactivity recovered in cells transfected with pcDNA3.1(-)-hDAT is extremely higher compared to controls that did not receive this plasmid, experiments showed in figure 3.6 confirm that uptake of extracellular [3 H]DA depends on hDAT activity. Noteworthy, in transfected HEK-293 (HEK-293-hDAT) the retrieved radioactivity was higher than in transfected SH-SY5Y (SH-SY5Y-hDAT) (15000 DPM vs 2700 DPM) whereas background was almost the same in both cell lines (~600 DPM). This difference between HEK-293-hDAT and SH-SY5Y-hDAT is mainly to ascribe to transfection efficiency, which is known to be pretty much lower in SH-SY5Y. Before to start to perform uptake assays to test the interactions between σ_1 receptors and hDAT, method validation was to examine the

responsiveness of this system to DAT substrates, known to alter its activity and expression.

A) HEK-293



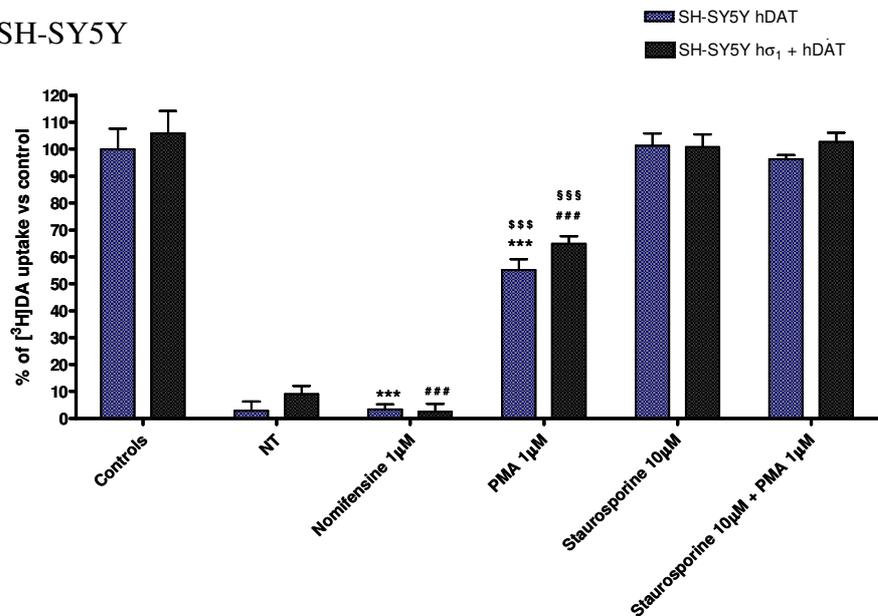
*** = P<0,001 vs HEK-293-hDAT control (Newman-Keuls's test post ANOVA)

= P<0,001 vs HEK-293 h σ_1 + hDAT control (Newman-Keuls's test post ANOVA)

\$\$\$ = P<0,001 vs HEK-293-hDAT treated with staurosporine 10 μ M + PMA 1 μ M (Newman-Keuls's test post ANOVA)

§§§ = P<0,001 vs HEK-293 h σ_1 + hDAT treated with staurosporine 10 μ M + PMA 1 μ M (Newman-Keuls's test post ANOVA)

B) SH-SY5Y



*** = P<0,001 vs SH-SY5Y hDAT control (Newman-Keuls's test post ANOVA)

= P<0,001 vs SH-SY5Y h σ_1 + hDAT control (Newman-Keuls's test post ANOVA)

\$\$\$ = P<0,001 vs SH-SY5Y hDAT treated with staurosporine 10 μ M + PMA 1 μ M (Newman-Keuls's test post ANOVA)

§§§ = P<0,001 vs SH-SY5Y h σ_1 + hDAT treated with staurosporine 10 μ M + PMA 1 μ M (Newman-Keuls's test post ANOVA)

Figure 3.10. *Effect of nomifensine, PMA and staurosporine on [³H]DA uptake in A) results on HEK-293, data are presented as mean ±SEM of four independent experiments (carried out in triplicate); B) results on SH-SY5Y, data are presented as mean ±SEM of two independent experiments (carried out in triplicate).*

Nomifensine is a known DA reuptake inhibitor (Meiergerd and Schenk, 1994); as displayed in figure 3.7 nomifensine at 1 μM completely blocked reuptake of extracellular DA in both HEK-293 and SH-SY5Y and retrieved radioactivity was reduced to not transfected (NT) level.

PMA is known to reduce *in vitro* the V_{max} of [³H]DA uptake by approximately 40% vs control: an effect blocked by the protein kinase inhibitor staurosporine (Pristupa et al., 1998). As it's shown in Fig.3.7, in cells transfected with pcDNA3.1(-)-hDAT, PMA and staurosporine cause the same effects described in literature.

These results demonstrate that the expressed recombinant hDAT is actively working and the conditions optimized for these experiments were used to assay σ receptors ligand and to study their modulation on hDAT activity. Nomifensine, at final concentration of 1 μM, was chosen to estimate background radioactivity in uptake experiments.

3.3.5 Effect of vehicle on [³H]DA uptake

Since most of σ ligands are lipophilic, DMSO was chosen as the vehicle to solubilize these drugs were primarily solubilized at 10⁻² M stock solution. Further dilutions were performed in sterile KRH buffer. Thus, when σ ligands were employed at final concentrations at 100 μM, 10 μM, 1 μM, 0.1 μM and 0.01 μM, concentration of DMSO, expressed in % v/v, was respectively 1%, 0.1%, 0.01%, 0.001% and 0.0001%. Effect of 1h incubation

with these concentration of DMSO on [^3H]DA was also checked before assaying the σ ligands.

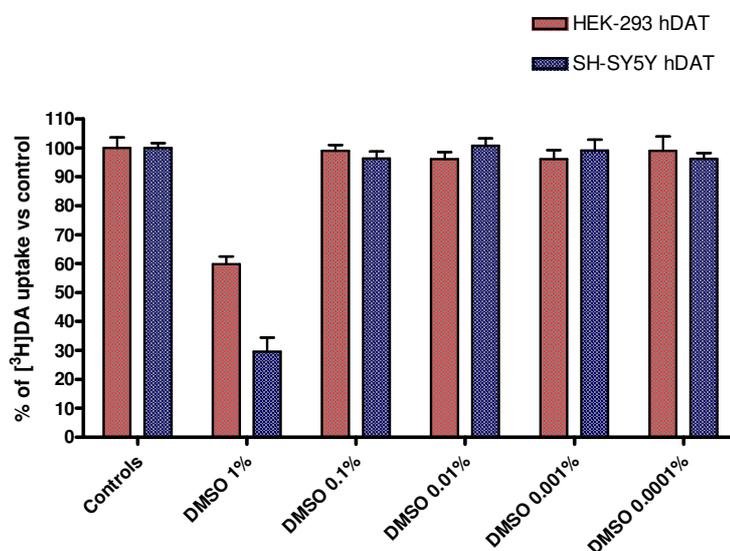


Figure 3.11. Effect of different concentrations of DMSO alone on [^3H]DA uptake.

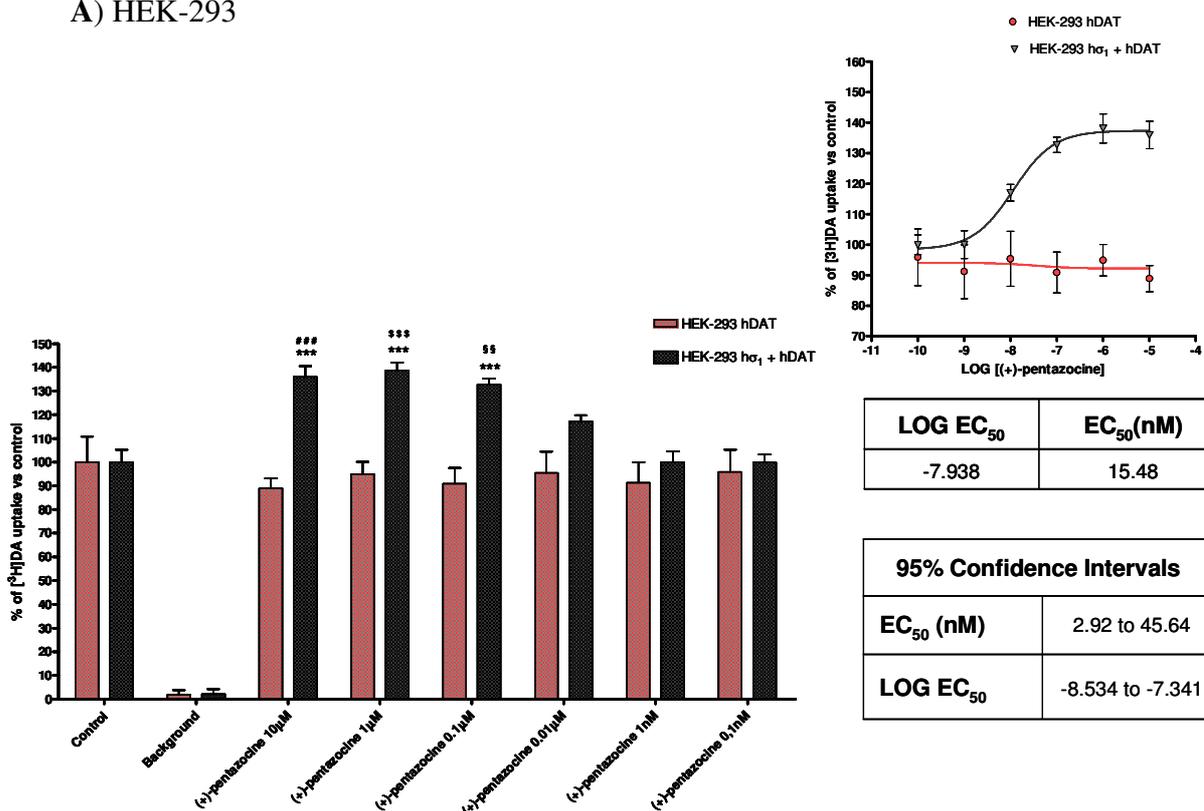
In HEK-293 and SH-SY5Y, DMSO interfered with uptake only when used at 1% v/v. DMSO toxicity is probably the cause of reduction of [^3H]DA uptake observed with 1% (v/v) of DMSO alone; in fact, after 1 h of treatment, cells that received 1% (v/v) of DMSO were detached from the well, floating, then in the medium.

3.4 Results

3.4.1 Effect of (+)-pentazocine on [³H]DA uptake

The first σ ligand employed in [³H]DA uptake assays was (+)-pentazocine, the prototype of σ_1 agonists, which possess high affinity for σ_1 binding sites ($K_i = 16.7$ nM) (Fig.3.8).

A) HEK-293

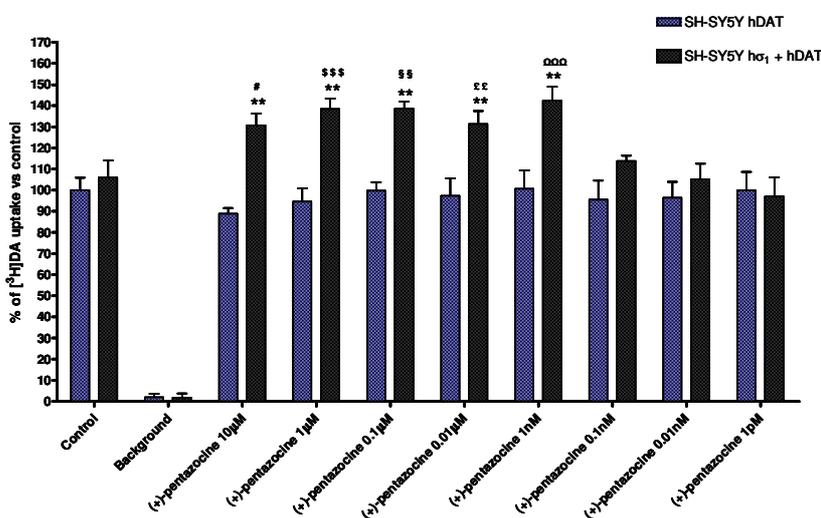


= P < 0,001 vs HEK-293 hDAT treated with 10 μ M (+)-pentazocine (Newman-Keuls's test post ANOVA)

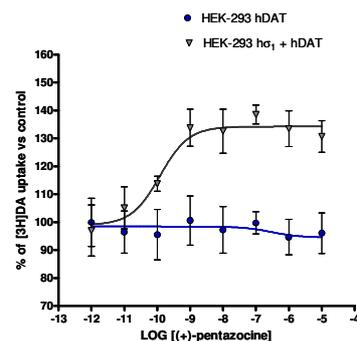
\$\$\$ = P < 0,001 vs HEK-293 hDAT treated with 1 μ M (+)-pentazocine (Newman-Keuls's test post ANOVA)

§§ = P < 0,01 vs HEK-293 hDAT treated with 0.1 μ M (+)-pentazocine (Newman-Keuls's test post ANOVA)

B) SH-SY5Y



****** = P<0,01 vs SH-SY5Y h σ_1 + hDAT control (Newman-Keuls's test post ANOVA)
= P<0,05 vs SH-SY5Y hDAT treated with (+)-pentazocine 10 μ M (Newman-Keuls's test post ANOVA)
\$\$\$ = P<0,001 vs SH-SY5Y hDAT treated with (+)-pentazocine 1 μ M (Newman-Keuls's test post ANOVA)
§§ = P<0,01 vs SH-SY5Y hDAT treated with (+)-pentazocine 0.1 μ M (Newman-Keuls's test post ANOVA)
££ = P<0,01 vs SH-SY5Y hDAT treated with (+)-pentazocine 0.01 μ M (Newman-Keuls's test post ANOVA)
ΩΩΩ = P<0,001 vs SH-SY5Y hDAT treated with (+)-pentazocine 1 nM (Newman-Keuls's test post ANOVA)



LOG EC ₅₀	EC ₅₀ (nM)
-9.89	0.13

95% Confidence Intervals	
EC ₅₀ (nM)	0.04 to 0.44
LOG EC ₅₀	-10.42 to -9.358

Figure 3.12. Effect of different concentrations of (+)-pentazocine on [³H]DA uptake in **A)** results on HEK-293, data are presented as mean \pm SEM of five independent experiments (carried out in triplicate); **B)** results on SH-SY5Y, data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

(+)-pentazocine, concentration-dependently increases the uptake of [³H]DA in cells stably overexpressing h σ_1 , with a maximum increase of about 40% over the basal values. Interestingly, concentration-response studies showed a different trend between HEK-293 and SH-SY5Y cell lines.

In kidney derived cells, the maximum effect of (+)-pentazocine was observed at 0.1 μ M. At 0.01 μ M (+)-pentazocine was less effective and inactive at 1 nM. Considering that K_i of (+)-pentazocine for σ_1 receptor falls in nM range (Tab 1.1), it's interesting to note that the more active

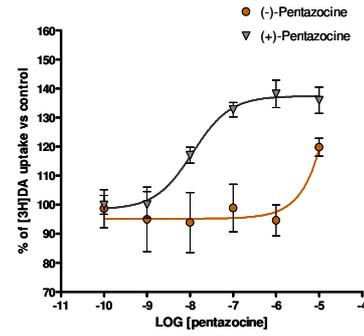
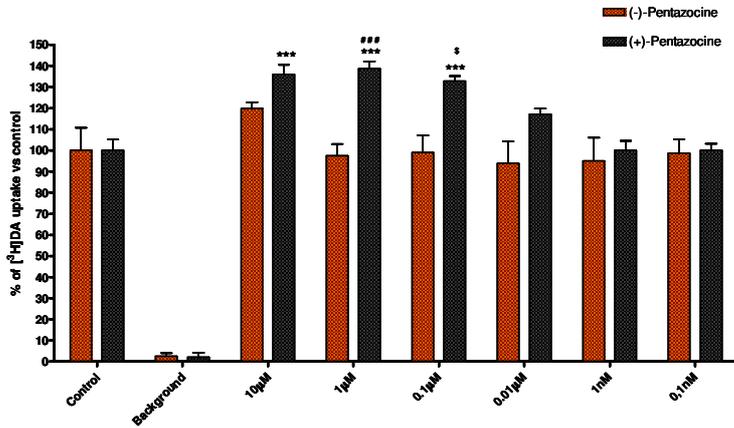
concentrations of the σ_1 agonist (0.1 μM) is higher than expected. To explain this, subcellular localization of σ receptors might be taken in account. At resting state, σ_1 receptors are cytoplasmatic proteins, mainly concentrated in ER lipid rafts. Thus ligands have to cross cells plasmalemma either passively or transported by membrane carriers in order to reach their target. In addition, it has been often reported that high concentrations are required to activate σ_1 receptors (Gudelsky et al., 1995 and 1999; Moison et al., 2003).

In neuroblastoma derived cells, concentration-response curve of (+)-pentazocine better fits with its calculated K_i . In fact, in the SH-SY5Y model, the maximum effect was reached at (+)-pentazocine was used at final concentration of 1 nM. Thus it appears that SH-SY5Y appear to be more sensitive to (+)-pentazocine than HEK-293.

3.4.2 Effects of (-)-pentazocine on [³H]DA uptake

Since the (-) isomer of pentazocine possess a 50 time lower affinity for σ_1 receptors (Tab.1.1), in cells cotransfected with h σ_1 receptor and hDAT concentration-effect curves of (-)-pentazocine were obtained in order to confirm the specific involvement of overexpressed h σ_1 receptors in the reported actions of (+)-pentazocine.

A) HEK-293 hσ₁ + hDAT



	LOG EC ₅₀	EC ₅₀ (nM)
(+)pentazocine	-7.938	15.48
(-)pentazocine	-	-

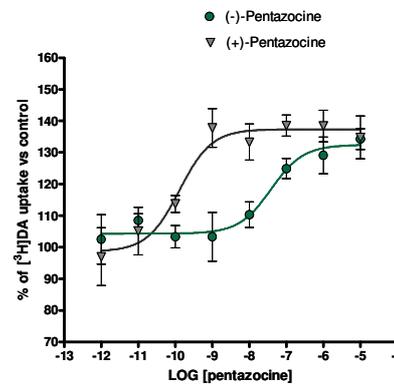
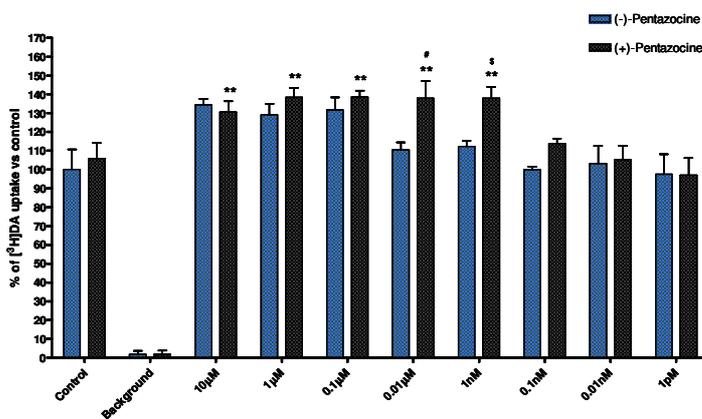
95% Confidence Intervals		
	(-)pentazocine	(+)pentazocine
EC ₅₀ (nM)	-	2.92 to 45.64
LOG EC ₅₀	-	-8.534 to -7.341

*** = P<0,001 vs not treated control (Newman-Keuls's test post ANOVA)

= P<0,001 vs HEK-293 hσ₁ + hDAT treated with 1 µM (-)-pentazocine (Newman-Keuls's test post ANOVA)

\$ = P<0,05 vs HEK-293 hσ₁ + hDAT treated with 0.1 µM (-)-pentazocine (Newman-Keuls's test post ANOVA)

B) SH-SY5Y hσ₁ + hDAT



	LOG EC ₅₀	EC ₅₀ (nM)
(+)pentazocine	-9.92	0.12
(-)pentazocine	-7.413	38.68

95% Confidence Intervals		
	(-)pentazocine	(+)pentazocine
EC ₅₀ (nM)	9.10 to 164.27	2.92 to 45.64
LOG EC ₅₀	-8.041 to -6.784	-8.534 to -7.341

** = P<0,01 vs not treated control (Newman-Keuls's test post ANOVA)

= P<0,05 vs SH-SY5Y hσ₁ + hDAT treated with (-)-pentazocine 0.01 µM (Newman-Keuls's test post ANOVA)

\$ = P<0,05 vs SH-SY5Y hσ₁ + hDAT treated with (-)-pentazocine 1 nM (Newman-Keuls's test post ANOVA)

Figure 3.13. Comparison between effects of (+)- and (-)-pentazocine on [³H]DA uptake in **A)** results on HEK-293, data are presented as mean ±SEM of four independent experiments (carried out in triplicate); **B)** results on SH-SY5Y, data are presented as mean ±SEM of two independent experiments (carried out in triplicate).

(-)-pentazocine was not able to increase [³H]DA uptake in HEK-293 and, in SH-SY5Y, calculated EC₅₀ for (-)-pentazocine is 322-fold higher than EC₅₀ calculated for its (+) isomer. These results are in line with the low affinity of (-)-pentazocine for σ₁ receptors and demonstrate the specific involvement of overexpressed hσ₁ in modulating hDAT [³H]DA uptake.

3.4.3 Effects of σ₁ antagonists

Effects of σ antagonists BD-1047 and NE-100 on [³H]DA uptake were also evaluated in presence and in absence of (+)-pentazocine.

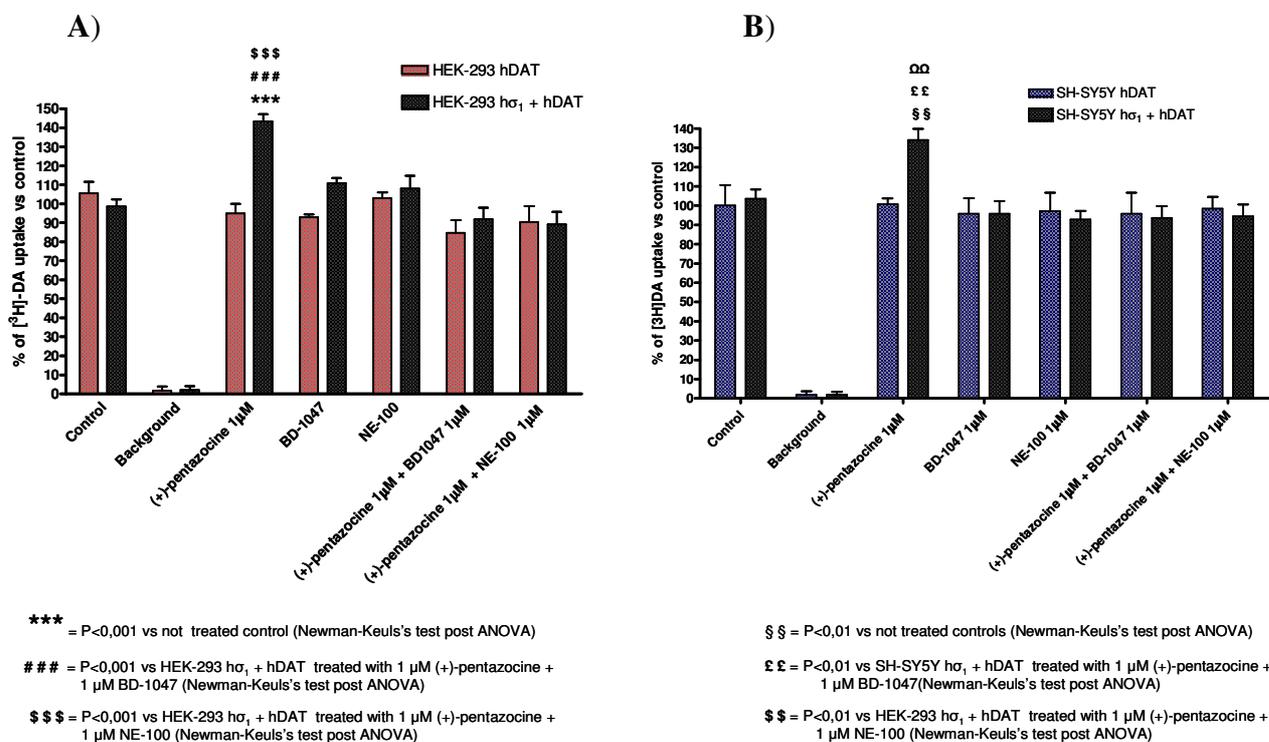


Figure 3.14. Effects of σ antagonists on [^3H]DA uptake; **A)** results on HEK-293, data are presented as mean \pm SEM of three independent experiments (carried out in triplicate); **B)** results on SH-SY5Y, data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

BD-1047 and NE-100 alone did not affect hDAT activity and it is relevant that these classic σ_1 antagonists were able to prevent the activation of h σ_1 by (+)-pentazocine. These results further confirm the specific involvement of transfected h σ_1 as effector of (+)-pentazocine effects.

3.4.4 Effects of SM-21

To evaluate any interference of endogenous σ_2 receptors, the σ_2 selective agonist SM-21 was also tested.

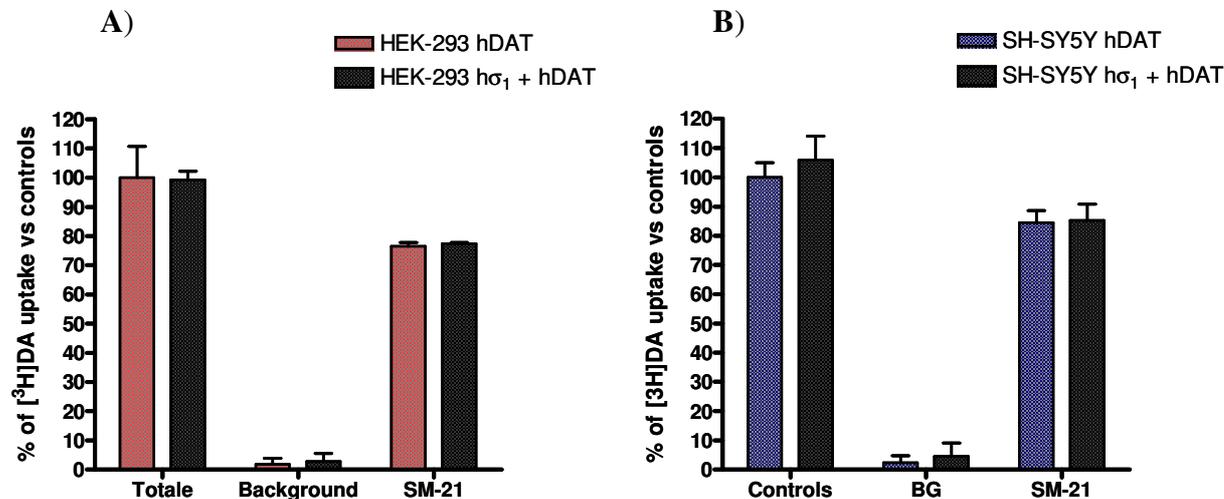


Figure 3.15. Effects of σ_2 selective agonist SM-21 at 1 μM on [^3H]DA uptake in **A)** results on HEK-293; **B)** results on SH-SY5Y.

As showed in figure 3.15, pretreatment with SM-21 slightly reduces [^3H]DA uptake both in HEK-293 and SH-SY5Y native or stably overexpressing h σ_1 receptors. Since in these experiments σ_2 receptors

recruitment could induce the opposite effect observed for the 1 subtype of σ receptors family, it would be interesting in the future to better characterize this feature. However data are not statistically significant and thus, in this thesis, SM-21 effects aren't further discussed.

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CHAPTER 4,

FUNCTIONAL CONSEQUENCES OF σ_1 -MEDIATED INCREASE OF DOPAMINE UPTAKE

4.1 Aim of the study

Uptake assays shown in chapter 3 demonstrate that in HEK-293 and in SH-SY5Y $h\sigma_1$ receptors activation by (+)-pentazocine positively modulates the hDAT uptake.

As described in section 2.7.1, DA is likely to be considered a neurotoxic molecule. In fact, when as consequence of exposure to toxic agents intracellular levels of DA abnormally increase, vesicles can't sequester the DA which is metabolized by MAO (A and B) and COMT with consequent overproduction of oxygen reactive species and toxic catabolites (Hastings, 1995, Hastings et al., 1996). Stress induced by these molecules leads cells to death.

For the second part of this thesis, experiments have been performed in order to investigate functional alterations caused by the (+)-pentazocine-mediated increase of DA uptake; particularly it has been investigated if the increase of intracellular [DA] could affect cells viability.

Basal toxicity of DA was first evaluated in native cells and in cells transiently transfected with pcDNA.31-hDAT. Once obtained control values, a molecular screening has been performed to elucidate relationship between σ_1 agonists and antagonists and uptaken DA toxicity.

Cells viability was estimated using the MTT assay, described in section 4.2.1.

4.2 Methods

Cells culture, transfections conditions and controls are described in chapter 3, section 3.2.

4.2.1 *MTT viability assay*

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a light sensitive yellow salt, highly soluble in water and in phosphate-buffered saline (PBS) which is reduced to insoluble purple formazan in living cells. These reductions take place only when mitochondrial reductase enzymes are active; since these enzymes are active only in undamaged cells, this conversion is used as a measure of cellular viability. Purple formazan is soluble in lipophilic solvents, such as DMSO and isopropyl; the dissolved formazan produces a purple solution and absorbance of this colored solution can be quantified by measuring it between 500 and 600 nm by a spectrophotometer. Thus, when the amount of purple formazan produced by treated cells is compared with the amount of formazan produced by untreated controls, the effectiveness of the treatment in causing death can be deduced through the production of a dose-response curve.

Viability assays were performed in 24-wells plates (Corning) precoated with poly-L-lysine. Five days before viability assays, 70000 cells were seeded in each well of the plate. 24h later, each well was transfected with pcDNA3.1(-)-hDAT. 48h after transfection, HEK-293 and SH-SY5Y were then treated with different concentration of DA and σ ligands, diluted in respective cell medium. Cell toxicity was evaluated 24h after treatments with the colorimetric MTT viability assay. Optimized conditions used in this

research are following reported. Exactly 24h after treatments, cells were washed two times with PBS and, in each well, medium was substituted with 500 μ l of a 200 μ M solution of MTT in sterile PBS and the plate was incubated 1,5h at 37°C. After incubation, plates were centrifuged for 30 minutes at 2600 g in a Beckman Coulter™ Allegra 25 R centrifuge, using a plate rotor to precipitate formazan. Surnatant was rapidly eliminated and formazan was solubilized by adding 500 μ l of DMSO in each well. 24-wells plates were shaken for 1h on a plate shaker at 250 rpm and absorbance was directly measured in a PerkinElmer Victor² luminometer/spectrophotometr (545 nm).

4.2.2 Data analysis

Data were analyzed with GraphPad Prism[®] software (version 4.0; GraphPad Software Inc., San Diego, CA, USA).

Viability data were normalized by setting absorbance values of controls as 100% and of background as 0%. Controls were transfected with pcDNA3.1(-)-hDAT and didn't receive any treatment before being assayed for MTT conversion in formazan. Background data were obtained treating cells with DA at final concentration of 5 mM. In fact, while using such high concentration of DA, spontaneous oxidation of the catecholamine is enough to kill all treated cells.

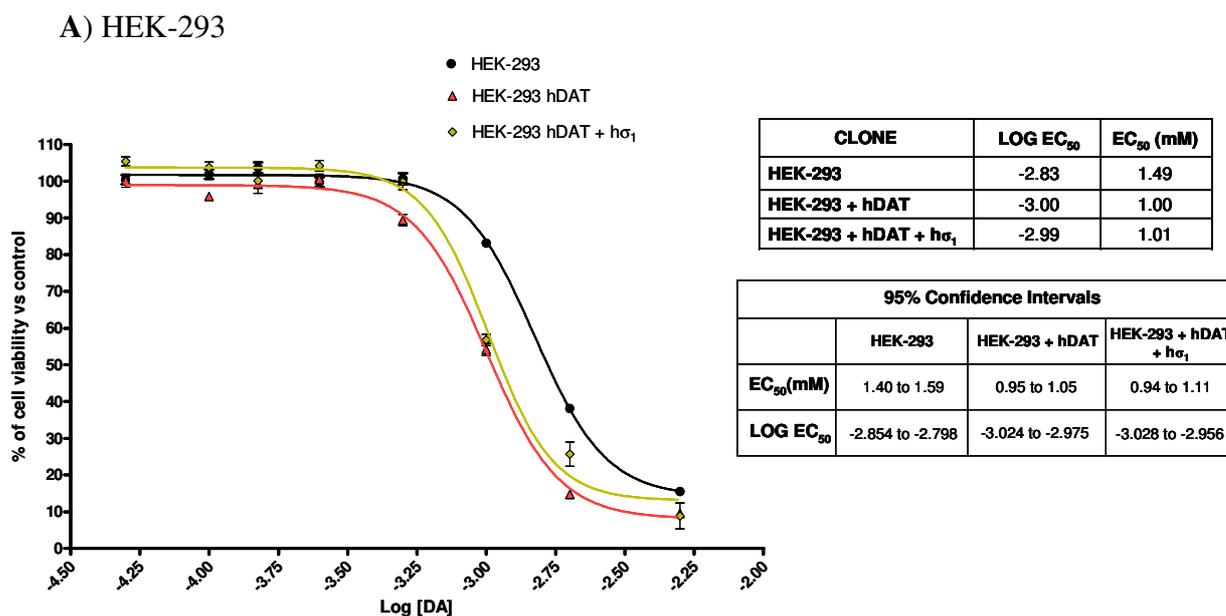
All data are presented as mean \pm standard error of the mean (SEM) for the indicated number of experiments. Statistical significance was determined by Newman-Keuls test post ANOVA using GraphPad Prism Prism[®] software (version 4.0; GraphPad Software Inc., San Diego, CA, USA). Newman-Keuls test post ANOVA was used to compare all data versus controls and among multiple groups. P-values < 0.05 were considered to be significant.

4.2.3 Set up of viability assays

Viability assays were primarily performed in order to check the toxicity of DA at different concentration in native HEK-293 and SH-SY5Y, or in cells transiently transfected with pcDNA3.1(-)-hDAT.

Native cells are not able to uptake extracellular DA and then the catecholamine toxicity should be ascribed mainly to autoxidation of the molecule. Once transfected with hDAT, cells gain the ability to inwardly transport the DA and uptaken DA is metabolized to release toxic species directly in the cytoplasmatic lumen.

A first set of experiments was performed to demonstrate that transfected cells are more sensitive to DA toxicity (Fig.5.1)



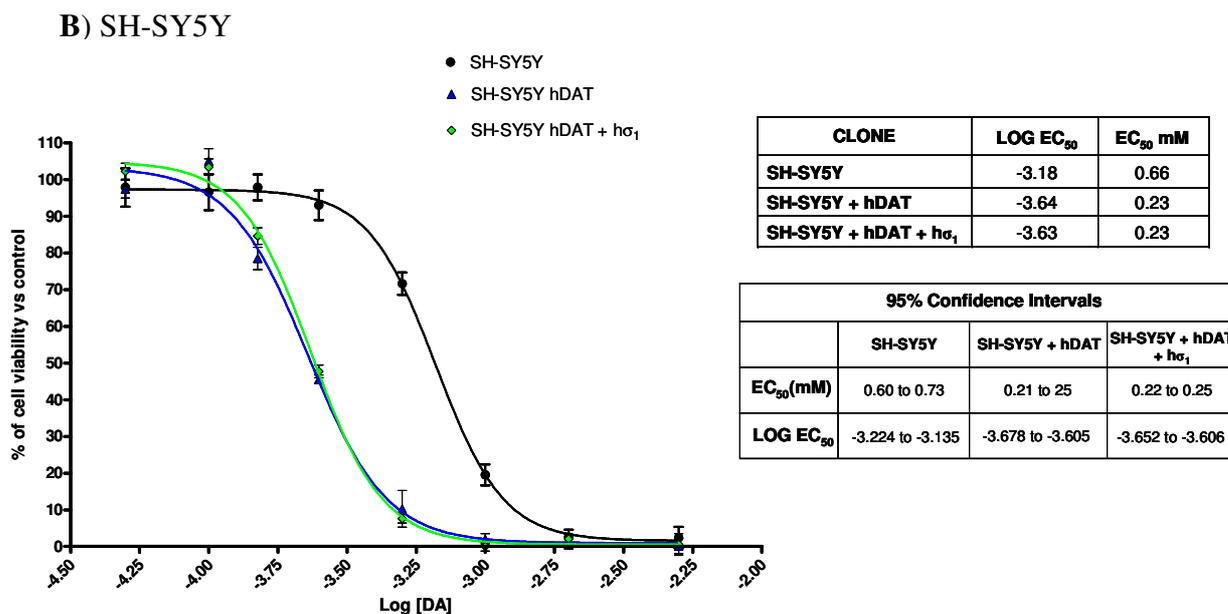


Figure 4.1. Toxicity of different concentration of DA in three different cell clones after 24h of incubation. **A)** HEK-293 the data are presented as mean \pm SEM of four independent experiment (carried out in triplicate); **B)** SH-SY5Y 293 the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

As evinced from EC₅₀ values, DA is more toxic in cells transfected with hDAT. In addition, SH-SY5Y are more sensitive to DA than HEK-293. This is probably due to a different DA metabolism efficiency, which is higher in the neuronal- than in the kidney-derived cell line.

To confirm the role of DA uptake and metabolism in mediating DA toxicity, cells transfected with hDAT were exposed to lethal concentrations of DA (1 mM for HEK-293, 500 μ M for SH-SY5Y) in presence and in absence of nomifensine, a DAT selective inhibitor and of selegiline, a MAO-B inhibitor, at the final concentration of 1 μ M.

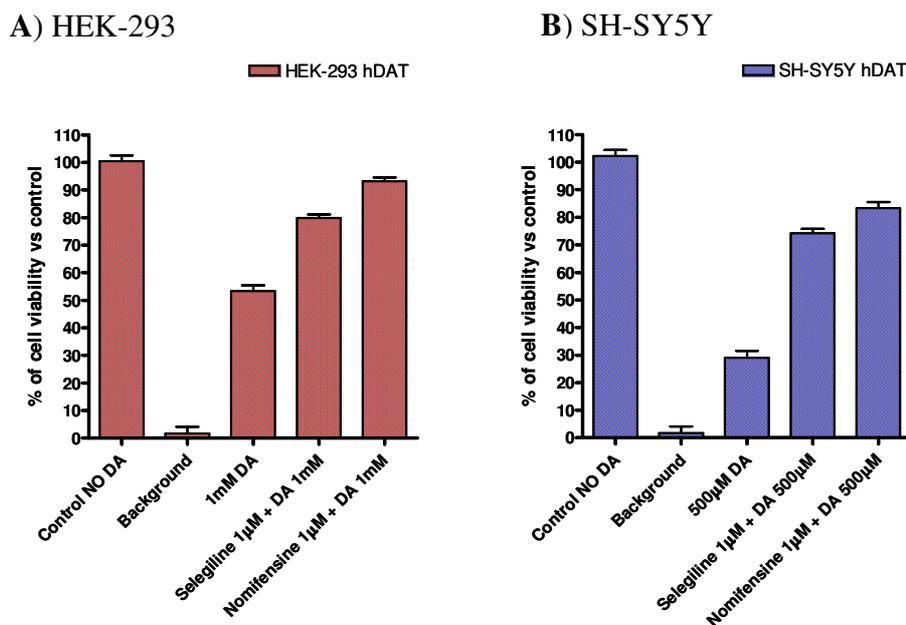


Figure 4.2. Protective effect of nomifensine and selegiline in presence of toxic concentration of DA. **A)** HEK-293 the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate); **B)** SH-SY5Y the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

When cells are exposed to DA in the presence of nomifensine, viability is increased of about 40% in HEK-293 and of about 60% in SH-SY5Y, in comparison to cells exposed to DA alone. Protective activity of nomifensine confirms the role of cell uptake in mediating DA toxicity. Moreover, selegiline is able to decrease the mortality rate; this last finding indicates that, in both the cell lines, oxidative metabolism of DA contribute to the toxicity of this catecholamine.

However, viability never reaches the control values. This is due to the autoxidation of DA which produces toxic species. Thus, the concentrations of DA used to estimate σ_1 contribution to cell toxicity were chosen in order to reduce the DA autoxidation counterpart.

4.2.4 Effect of vehicle on cell viability

As previously reported (see section 3.3.5), most of σ ligands are lipophilic, thus DMSO was chosen as vehicle to solubilize drugs at 10^{-2} M as stock solution. Further dilutions were performed in sterile media. When σ ligands were employed at $100\mu\text{M}$, $10\mu\text{M}$, $1\mu\text{M}$, $0.1\mu\text{M}$ and $0.01\mu\text{M}$, concentration of DMSO, expressed in % v/v, was respectively 1%, 0.1%, 0.01%, 0.001% and 0.0001%. Effect of 1h incubation with these concentration of DMSO alone on cell viability was also checked (Fig.5.3)

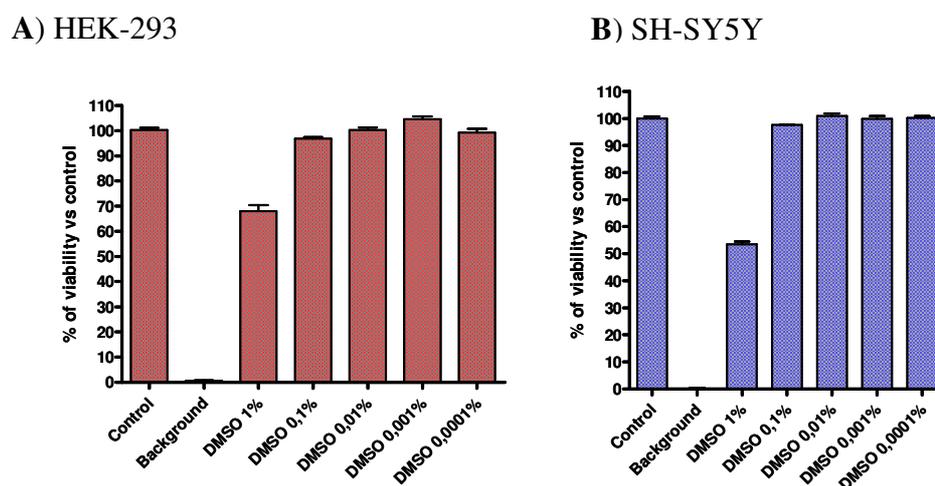


Figure 4.3. Effect of DMSO on cellular viability; **A)** HEK-293 the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate); **B)** SH-SY5Y the data are presented as mean \pm SEM of two independent experiments(carried out in triplicate).

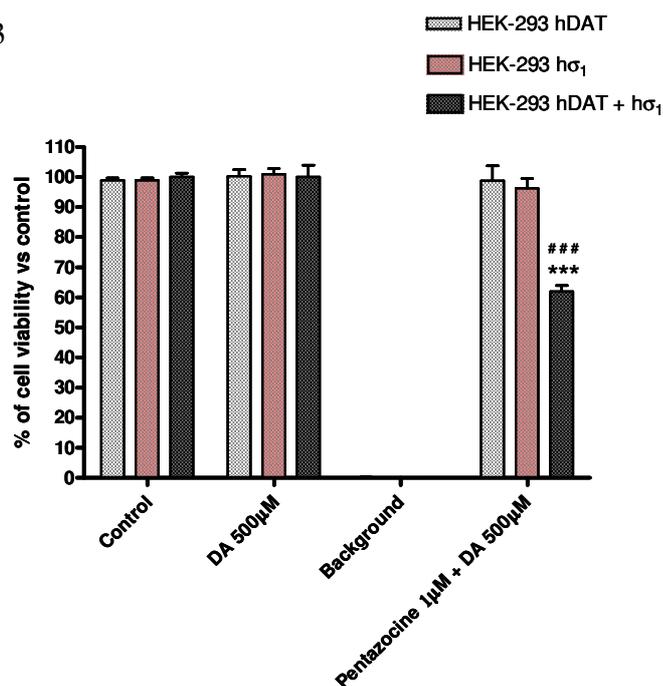
DMSO is toxic when used at the concentration of 1% v/v in both HEK-293 and SH-SY5Y

4.3 Results

4.3.1 Effects of (+)-pentazocine on DA toxicity

Data acquired to set up viability assays were used to choose the concentrations of DA used in first set of experiments, which are respectively 500 μM for HEK-293 and 150 μM for SH-SY5Y. These concentrations are slightly lower than EC_{50} and allow to estimate the σ_1 contribution to cell toxicity avoiding any interference induced by DA autoxidation alone. (+)-pentazocine was used at 1 μM in both cell lines.

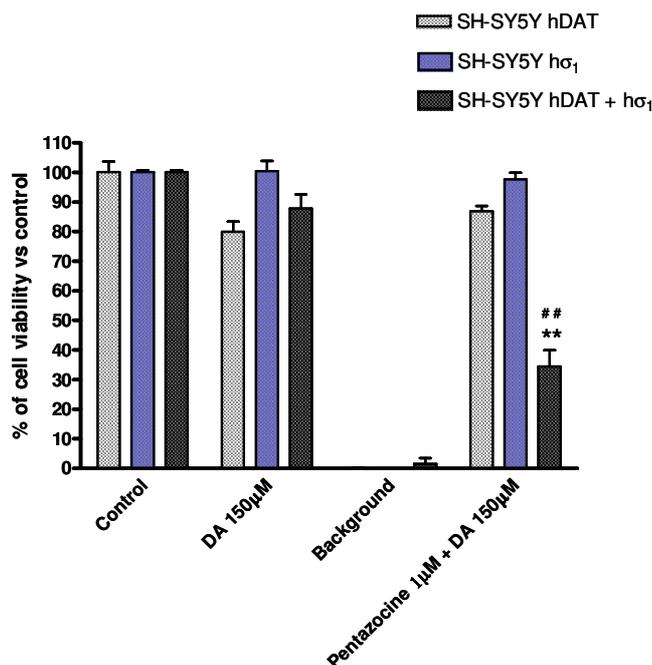
A) HEK-293



*** = $P < 0,001$ vs HEK-293 h σ_1 + (+)-pentazocine 1 μM + DA 500 μM (Newman-Keuls's test post ANOVA)

= $P < 0,001$ vs HEK-293 hDAT + (+)-pentazocine 1 μM + DA 500 μM (Newman-Keuls's test post ANOVA)

B) SH-SY5Y



** = $P < 0,01$ vs SH-SY5Y h σ_1 + (+)-pentazocine 1 μ M + DA 150 μ M (Newman-Keuls's test post ANOVA)

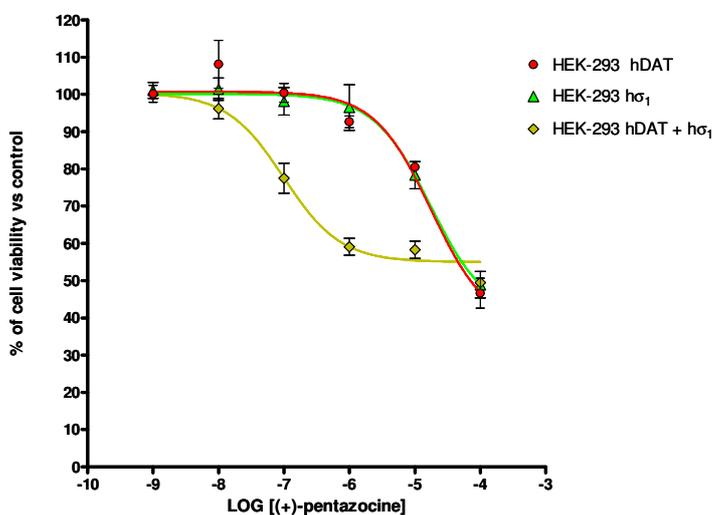
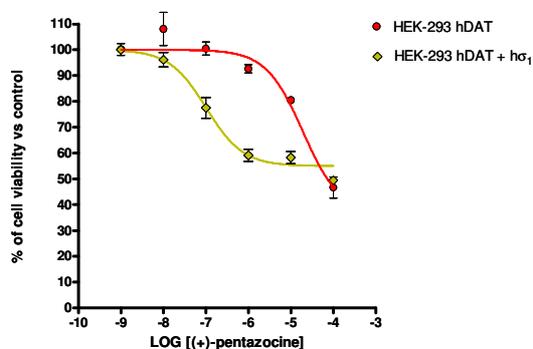
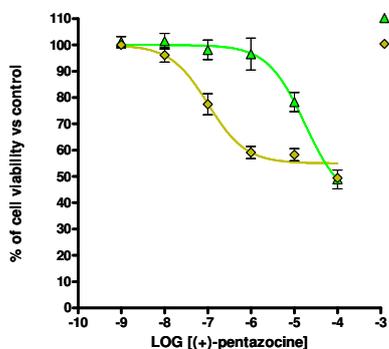
= $P < 0,01$ vs SH-SY5Y hDAT + (+)-pentazocine 1 μ M + DA 150 μ M (Newman-Keuls's test post ANOVA)

Figure 4.4. Effect of (+)-pentazocine at the concentration of 1 μ M on DA toxicity; **A)** HEK-293 the data are presented as mean \pm SEM of five independent (carried out in triplicate); **B)** SH-SY5Y the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

(+)-pentazocine at 1 μ M is able to increase the toxicity of the DA only in cells co-expressing both h σ_1 receptor and hDAT. The increase of DA cell toxicity is to ascribe only to h σ_1 receptor activation.

EC₅₀ curves were also obtained for the σ_1 agonist treating the cells with different concentrations of (+)-pentazocine, in presence of a fixed concentration of DA, respectively 500 μ M for HEK-293 and 150 μ M for SH-SY5Y.

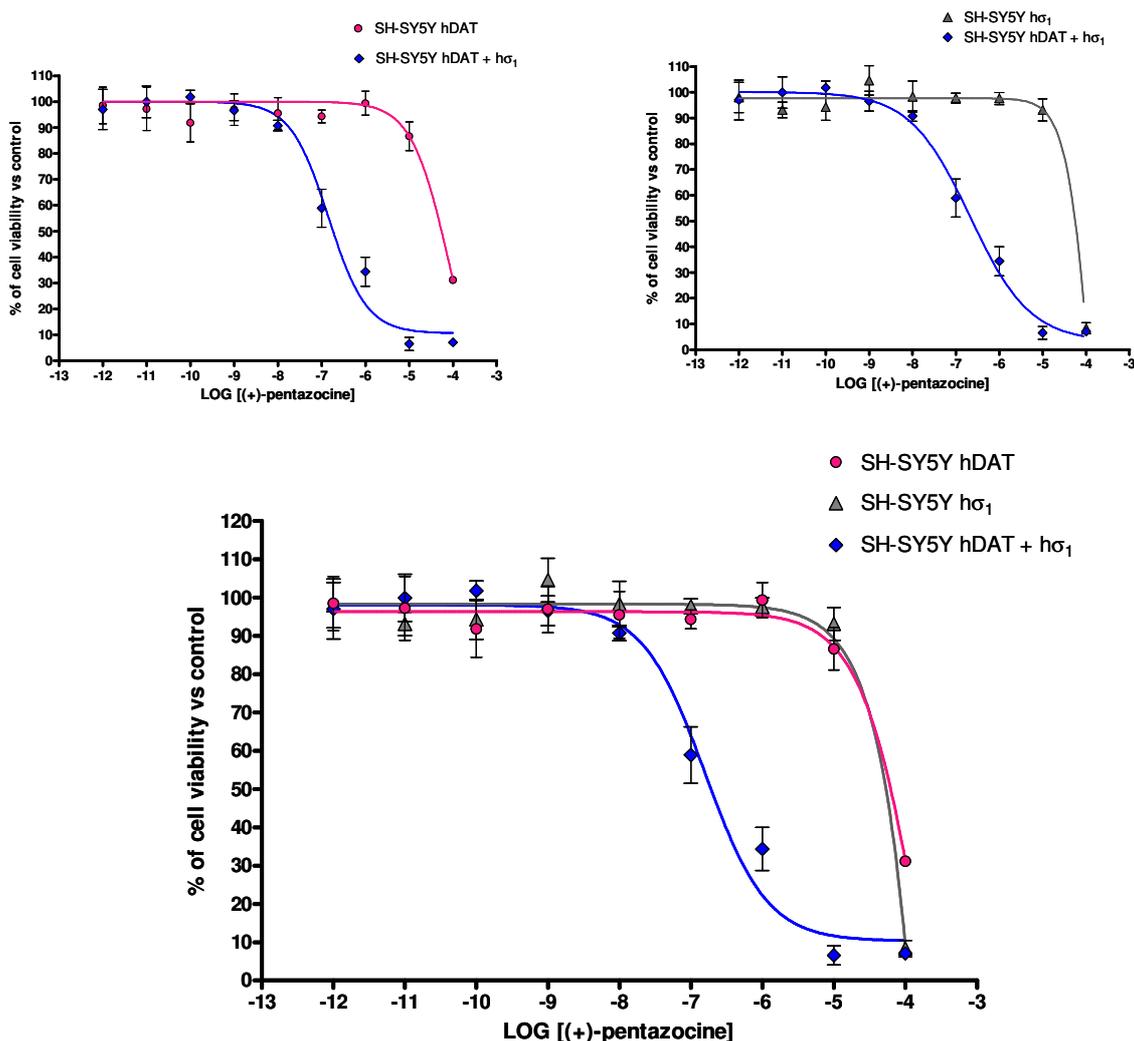
A) HEK-293



CLONE	LOG EC ₅₀	EC ₅₀ (μM)
HEK-293 hDAT	-4.75	17.68
HEK-293 hσ ₁	-4.76	17.36
HEK-293 hDAT + hσ ₁	-7.01	0.01

95% Confidence Intervals			
	HEK-293 hDAT	HEK-293 hσ ₁	HEK-293 + hDAT + hσ ₁
EC ₅₀ (μM)	7.27 to 43.03	8.05 to 37.44	0.06 to 0.17
LOG EC ₅₀	-5.14 to -4.37	-5.10 to -4.43	-7.25 to -6.76

B) SH-SY5Y



CLONE	LOG EC ₅₀	EC ₅₀ (μM)
SH-SY5Y hDAT	-4,62	24.27
SH-SY5Y h σ_1	-4,61	24.78
SH-SY5Y hDAT + h σ_1	-6,80	0.01

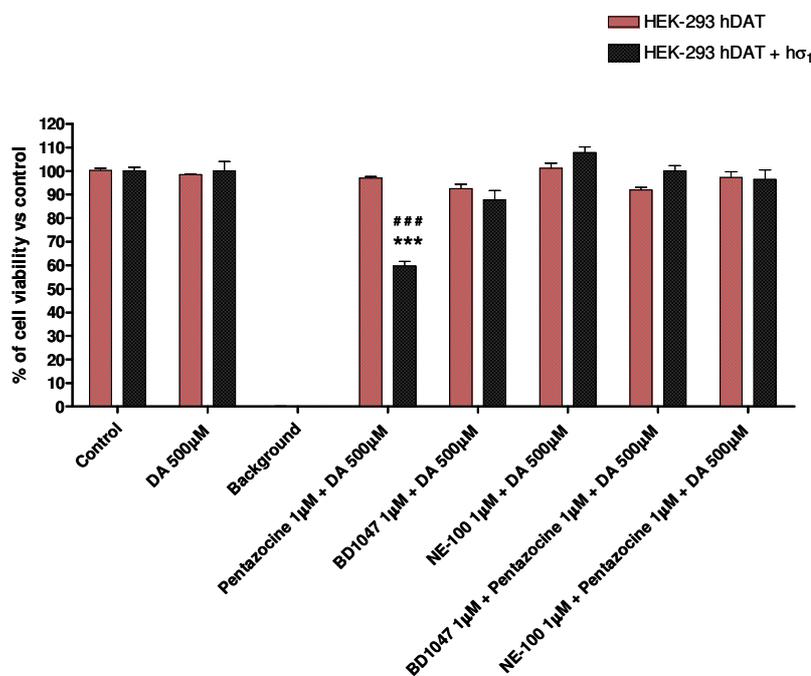
95% Confidence Intervals			
	SH-SY5Y hDAT	SH-SY5Y-293 h σ_1	SH-SY5Y + hDAT + h σ_1
EC ₅₀ (μM)	2.35 to 179.01	2.36 to 180.00	0.09 to 0.27
LOG EC ₅₀	-5.63 to -3.75	-5.63 to -3.75	-7.04 to -6.56

Figure 4.5. EC₅₀ curves of (+)-pentazocine obtained in presence of a fixed concentration of DA; A) HEK-293, concentration of DA was 500 μM, the data are presented as mean

\pm SEM of four independent experiments (carried out in triplicate); **B)** SH-SY5Y, concentration of DA was 150 μ M, the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

In both HEK-293 and SH-SY5Y, EC₅₀ values of cells transfected with hDAT alone are almost superimposable to EC₅₀ of cells transfected with h σ ₁ alone. In these case the toxicity seems to be mediated exclusively by the vehicle (see also Fig. 5.3); therefore these results indicate that (+)-pentazocine alone is not toxic in this model. In cells expressing both h σ ₁ and hDAT, toxicity of DA is increased by (+)-pentazocine in a concentration-dependent manner. These data strongly support the involvement of h σ ₁ receptors. However, to further confirm the role of the stably overexpressed protein, effects of σ ₁ selective antagonists BD-1047 and NE-100 were also evaluated in presence and in absence of (+)-pentazocine.

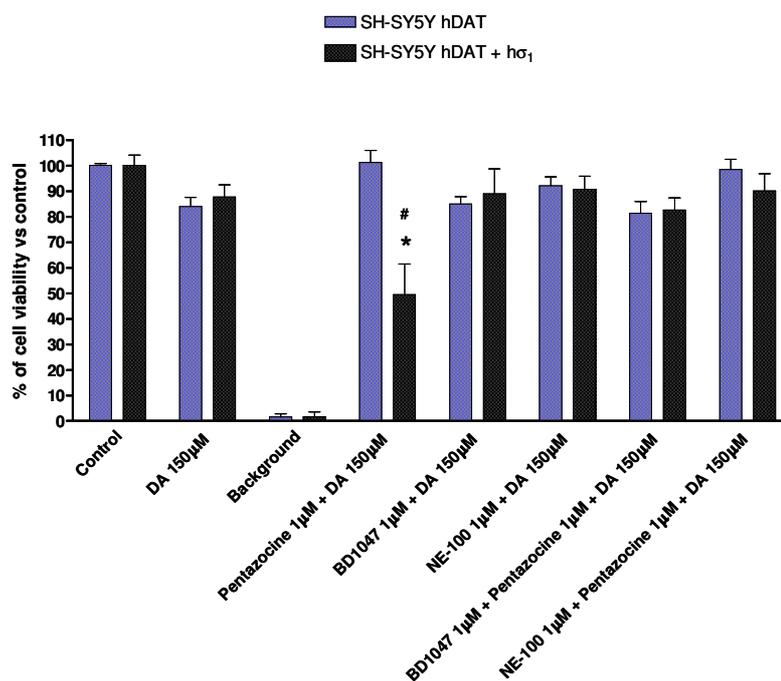
A) HEK-293



*** = P<0,001 vs HEK-293 hDAT + h σ ₁ + BD-1047 + (+)-pentazocine 1 μ M + DA 500 μ M (Newman-Keuls's test post ANOVA)

= P<0,001 vs HEK-293 hDAT + h σ ₁ + NE-100 + (+)-pentazocine 1 μ M + DA 500 μ M (Newman-Keuls's test post ANOVA)

B) SH-SY5Y



* = $P < 0,05$ vs SH-SY5Y hDAT + h σ_1 + BD-1047 + (+)-pentazocine 1 μ M + DA 150 μ M (Newman-Keuls's test post ANOVA)

= $P < 0,05$ vs SH-SY5Y hDAT + h σ_1 + NE-100 + (+)-pentazocine 1 μ M + DA 150 μ M (Newman-Keuls's test post ANOVA)

Figure 4.6. σ_1 selective antagonists BD-1047 and NE-100 block (+)-pentazocine-induced increase of DA toxicity; **A)** HEK-293, the data are presented as mean \pm SEM of four independent experiments (carried out in triplicate); **B)** SH-SY5Y, the data are presented as mean \pm SEM of two independent experiments (carried out in triplicate).

As showed in figure 5.6, both BD-1047 and NE-100 are not toxic when used alone at the concentration of 1 μ M and they are able to revert the (+)-pentazocine-induced increase of DA cell toxicity. These data confirm that when overexpressed h σ_1 receptors, (+)-pentazocine mediates the observed alteration of cell susceptibility to DA toxicity.

In conclusion, it has been shown that (+)-pentazocine increases DA cell toxicity in a concentration-dependent manner only in cells co-expressing h σ_1 and hDAT. σ_1 selective antagonists are able to block the action of the agonist (+)-pentazocine. Since it has been demonstrated in chapter 3 that h σ_1

receptors activation enhances the hDAT uptake activity, the observed (+)-pentazocine-mediated increase of DA cell toxicity is likely to be induced by raised intracellular DA levels.

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CHAPTER 5,

CONFOCAL MICROSCOPY STUDIES

5.1 Aim of the study

The described cross-talking between $h\sigma_1$ receptor and hDAT has been further investigated using confocal microscopy. Methods are described in section 3.2.10 and results are presented in next section (5.2).

5.2 Results

Confocal images were first taken to validate the experimental conditions and to verify if HEK-293 cells transfected with pEGFP-C1-hDAT and pCMV6 Entry-hSigma-1 plasmids were able to express the proteins under investigation. Results of these control experiments are shown in section 3.3.3.

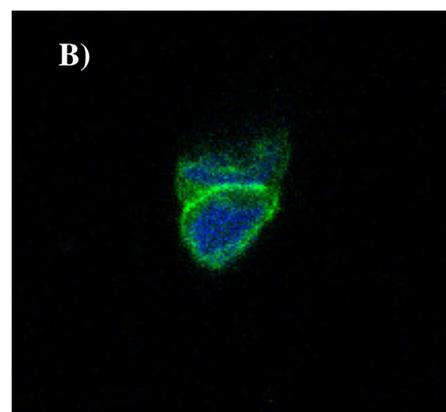
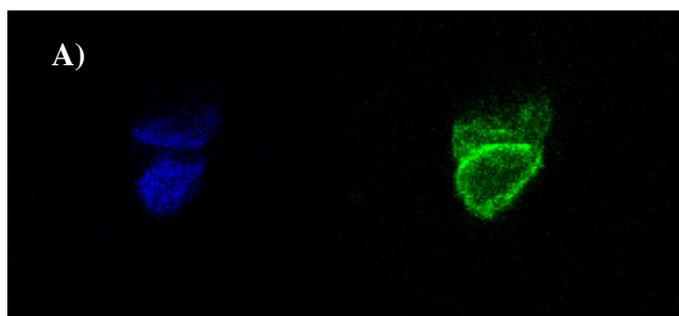
Following studies were performed in order to describe molecular dynamics underlying the σ_1 -mediated increase of DA uptake. Four experimental groups have been then established:

Group #	EGFP-hDAT	$h\sigma_1$-FLAG	(+)-pentazocine 1 μM (1h pretreatment)
1)	+	-	-
2)	+	-	+
3)	+	+	-
4)	+	+	+

5.2.1 Confocal images of groups 1) and 2)

Uptake studies performed in HEK-293 and detailed in chapter 3 demonstrate that the modulation of DA uptake activity induced by (+)-pentazocine is observed only in cells stably expressing the $h\sigma_1$ receptors. For this reason groups 1) and 2) were employed as negative controls, to check if (+)-pentazocine is able to modify the distribution of EGFP-hDAT in absence of overexpressed $h\sigma_1$ receptors. Confocal images of these group are reported in figure 5.1.

Group 1)



Group 2)

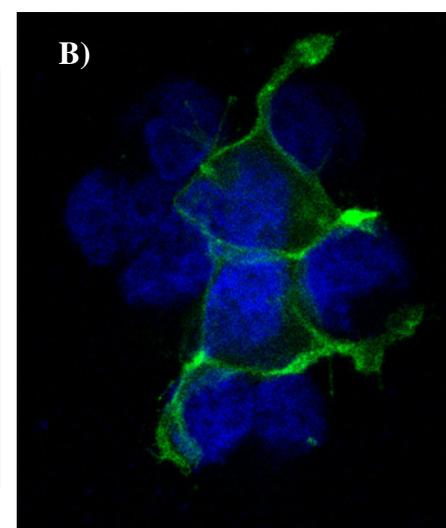
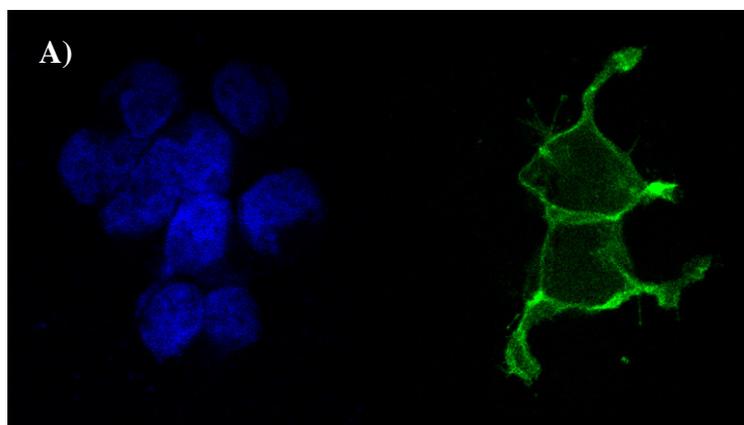


Figure 5.1. Confocal images of group 1) and 2); **A)** separated images of nuclei stained with DAPI (blue) and EGFP-hDAT (green); **B)** merged image of nucleus and $h\sigma_1$ -FLAG receptor markers; note that, in both 1) and 2) groups, EGFP-hDAT fluorescence appears predominantly in near the cell surface.

In both groups, EGFP-hDAT appears mainly distributed on plasmalemma, but a slight green emission is detectable also in the cytoplasm; however it is impossible to distinguish if this cytoplasmatic fluorescence is due to background, or it is given by intracellular recycled transporters. In conclusion, no differences are detectable between group 1) and 2).

5.2.2 Confocal images of groups 3) and 4)

Comparison of images acquired for group 3) and 4) allows to acquire more clear data.

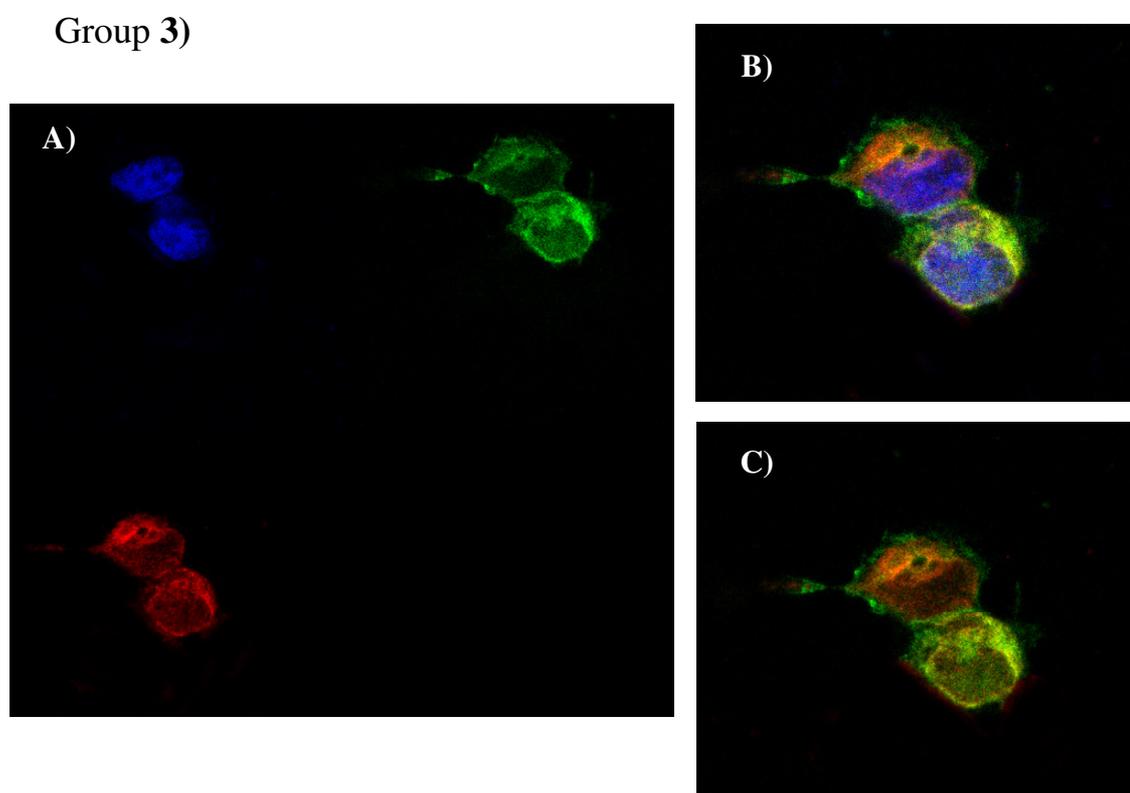
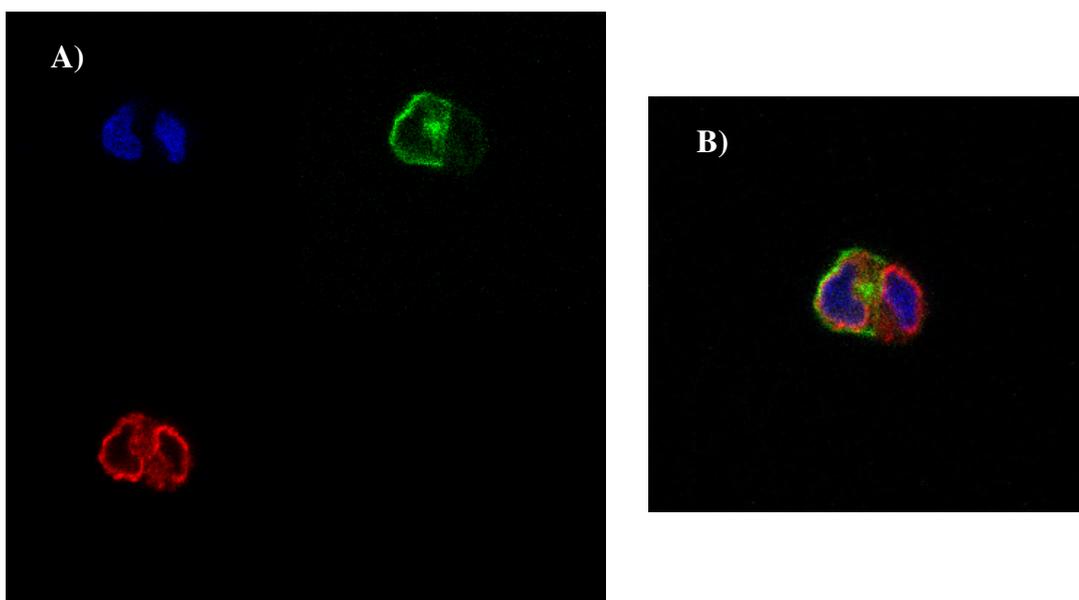


Figure 5.2. Confocal images of group 3); **A)** separated images of nuclei stained with DAPI (blue), $h\sigma_1$ -FLAG receptors labeled with a fluorescent probe (red) and EGFP-hDAT (green); **B)** merged image of nucleus and $h\sigma_1$ -FLAG receptor markers and EGFP-hDAT; **C)** merged image of labeled $h\sigma_1$ -FLAG receptor and EGFP-hDAT, color is due to colocalization of the green and the red fluorescence; note that $h\sigma_1$ -FLAG receptors fluorescence appears predominantly in the cytoplasm whereas EGFP-hDAT appears mainly located on the plasma membrane. However a slight green emission is detectable also in the cytoplasm.

Group 4)



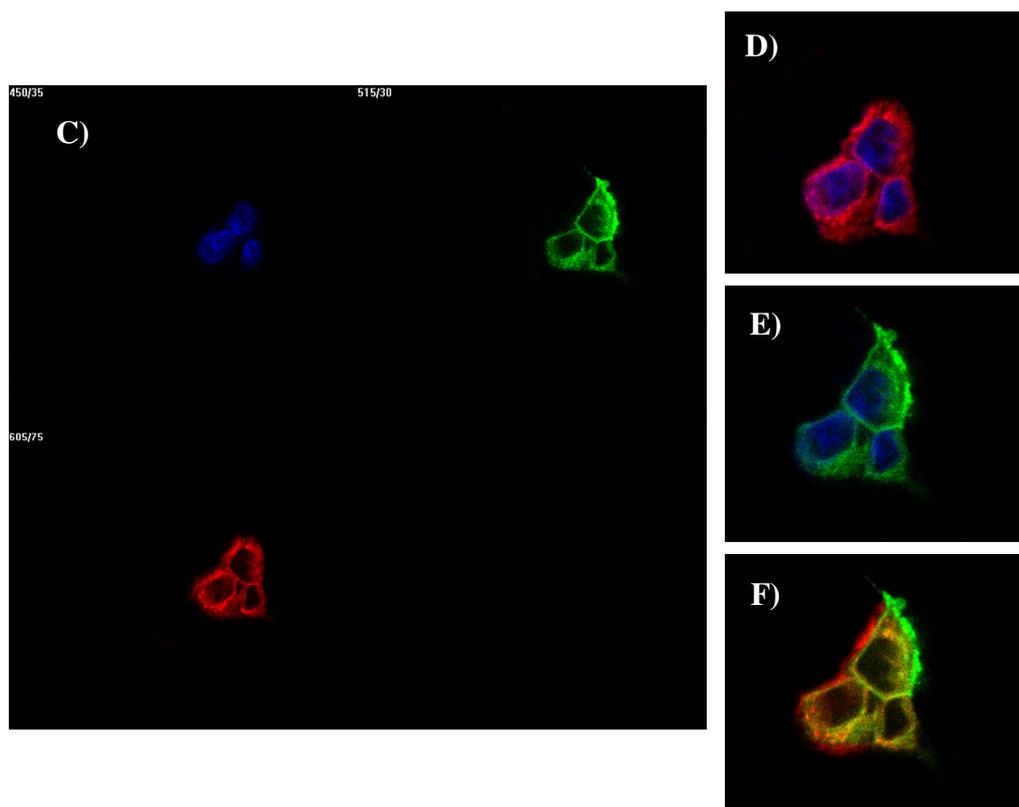


Figure 5.3. Confocal images of group 4); **A)** and **C)** separated images of nuclei stained with DAPI (blue), $h\sigma_1$ -FLAG receptors labeled with a fluorescent probe (red) and EGFP-hDAT (green); **B)** merged image of nucleus and $h\sigma_1$ -FLAG receptor markers and EGFP-hDAT; **D)** merged image of nucleus marker and EGFP-hDAT; **E)** merged image of labeled $h\sigma_1$ -FLAG receptor and nucleus; **F)** merged image of labeled $h\sigma_1$ -FLAG receptor and EGFP-hDAT, yellow color is due to colocalization of the green and the red fluorescence; note that in the group 4) (exposed to (+)-pentazocine for 1 h) $h\sigma_1$ -FLAG receptors fluorescence is more distributed on the plasma membrane, in comparison to the untreated group 3). Also EGFP-hDAT green fluorescence appears more concentrated on plasmalemma in comparison to group 3). In addition, note that green and red fluorescence colocalize at cell surface level.

By comparing group 3) and group 4), redistribution of $h\sigma_1$ receptor (stained in red) is clear. In fact, in non treated cells, $h\sigma_1$ receptors are mainly concentrated in the cytoplasm (see also picture 3.7). Following exposure to

(+)-pentazocine, the red stained protein massively translocates towards the plasma membrane.

As far as it concerns the EGFP-hDAT, if we compare the image of group 3) with the images of group 4), hDAT seems to be more located at plasma membrane level in cells pretreated with (+)-pentazocine.

In addition, $h\sigma_1$ and hDAT appear to colocalize both in treated and in non-treated cells. Thus, in conclusion, these confocal studies show that pretreatment with (+)-pentazocine induce a redistribution of intracellular $h\sigma_1$ receptors and suggest that the observed increase of [^3H]DA uptake could be mediated by a $h\sigma_1$ redistribution from the cytoplasm to the cell membrane where hDAT is localized.

DISCUSSION

CHAPTER 6,

DISCUSSION

6.1 Discussion

The concept of sigma (σ) receptors was first introduced in literature by Martin and colleagues (Martin et al., 1976), up to now, numerous studies have tried to unveil their role in neuronal transmission. This study reports for the first time that in two cells model, HEK-293 and SH-SY5Y cells, $h\sigma_1$ receptor is able to modulate the DA uptake mediated by hDAT.

Results illustrated in this thesis demonstrate that activation of overexpressed $h\sigma_1$ receptors by (+)-pentazocine, the σ_1 agonist prototype, determines an increase of 40% of the extracellular [3 H]DA uptake, in comparison to non-treated controls. The hypothesis that this effect is mediated by the $h\sigma_1$ receptors is confirmed by results obtained in uptake assays, hereafter reported:

- 1) (+)-pentazocine mediates the increase of [3 H]DA uptake only in cells stably transfected with the vector encoding for the fusion protein $h\sigma_1$ -FLAG. EC_{50} value obtained for the σ_1 agonist in HEK-293 cell line is higher than the EC_{50} obtained in SH-SY5Y. This difference is mainly to ascribe to the different nature of these cells.
- 2) (-)-pentazocine, which has a 42 lower affinity for σ_1 than its (+) isomer, is not able to increase the [3 H]DA uptake in HEK-293. In SH-SY5Y, calculated EC_{50} for (-)-pentazocine is 322 fold higher than EC_{50} calculated for the (+)-pentazocine. These results are in line with the different affinity of (+)-and (-) isomers of pentazocine for the σ_1 receptors.

- 3) In both the cell lines, the σ_1 antagonists BD-1047 and NE-100 prevent the positive effect of (+)-pentazocine on DA reuptake; these compounds had no effect on uptake if used alone.

Involvement of σ_1 receptors in the modulation of extracellular levels of DA has been previously described *in vivo* (Gudelsky, 1999; Moison et al., 2003). In these studies, intrastriatal infusion of σ_1 receptors agonists, particularly (+)-pentazocine, produced a biphasic effect on extracellular DA concentration, consisting in a rapid increase, during the first thirty minutes, followed by a more prolonged and marked decrease (Fig.1.6 A and B) (Gudelsky 1999; Moison et al., 2003). The biphasic effect is mainly evident when higher concentrations of the σ_1 ligand are administered. At lower doses, the σ_1 agonists definitely appear to reduce the extracellular [DA] (Gudelsky 1999; Moison et al., 2003). According to these observations, in the rat intravenous administration of σ agonists decreases the firing rate of rats DA neurons (Steinfels et al., 1989; Minabe et al., 1999) and this reduction is prevented by the σ_1 antagonist NE-100. The σ -mediated reduction of extracellular [DA] has been related to inhibition of release of this catecholamine. However, the hypothesis that σ_1 receptors may also increase the DA uptake has never been investigated. Results showed in this Ph.D. dissertation are partially in line with what has been previously reported by Gudelsky and Minabe. In fact, the σ_1 -mediated increase of [3 H]DA uptake could represent another mechanism by which (+)-pentazocine regulates the extracellular [DA] *in vivo*.

In the experimental model adopted in this study, as expected, the major activity of σ_1 selective antagonists BD-1047 and NE-100 was to block the effects of the agonist and, hence, they prevented the (+)-pentazocine-induced increase of the [3 H]DA uptake. σ_1 antagonists have been investigated for the treatment of psychostimulant abuse since they are able to block several

cocaine and amphetamine mediated effects, like sensitization, relapse, hyperlocomotion and overdose death (Maurice et al., 2002). These drugs are also able to directly bind the σ_1 receptors at micromolar concentrations, achievable following their *in vivo* administration (Maurice et al., 2002). As widely described in the literature (see section 2.6.2), both cocaine and amphetamine act increasing the extracellular DA concentrations, which is thought to mediate the rewarding and reinforcing properties of these drugs. Thus, it is not easy to fit our results with these axioms, because in our models the σ_1 agonist actually reduces the extracellular level of DA instead of increase it and σ_1 antagonists would even prevent this decrease.

In the attempting to explain these discrepancies, it is necessary not to consider the dopaminergic system as the only target of σ_1 receptors. In fact, as described in chapter 1, activation of σ_1 receptors may alter several neurotransmitter systems, such as the cholinergic, noradrenergic, GABAergic, serotonergic and glutamatergic systems. This thesis describes a cross talking between the h σ_1 receptor and the hDAT in two cell models; therefore it is not possible to exclude that *in vivo* σ_1 receptors activation by psychostimulants may involve multiple neuronal pathways.

For the second part of this thesis, experiments have been performed in order to investigate functional alterations caused by the (+)-pentazocine-mediated increase of DA uptake; particularly it has been investigated if the increase of intracellular [DA] could affect cells viability. In fact, when intracellular levels of DA abnormally increase, vesicles can't sequester the DA which is metabolized by MAO (A and B) and COMT with consequent overproduction of oxygen reactive species and toxic catabolites (Hastings, 1995, Hastings et al., 1996). Stress induced by these molecules may leads cells to death.

As is possible to evince from data showed in chapter 4, DA is more toxic in cells that transiently express the hDAT, underlying the role of cell uptake in mediating DA toxicity. SH-SY5Y are more sensitive to DA than the HEK-293; this difference is probably due to DA metabolism efficiency, which is higher in the neuronal- than in the kidney-derived cell line.

For what it concerns the role of the transfected $h\sigma_1$ in modulating the DA cell toxicity, this study demonstrates that (+)-pentazocine increases DA cell toxicity in a concentration-dependent manner only in cells co-expressing $h\sigma_1$ and hDAT. Such effects are evident exclusively when DA is present at a concentration slightly lower than EC_{50} that allow to estimate the σ_1 contribution to cell toxicity avoiding any interference induced by DA autoxidation alone (respectively μM for HEK-293 and $150 \mu\text{M}$ for SH-SY5Y). EC_{50} curves of (+)-pentazocine were thus obtained.

Published studies concerning *in vivo* DA metabolism modulation by σ_1 receptors demonstrate that some σ_1 ligands are able to increase extracellular levels of two DA metabolites, dihydroxy phenylacetic acid (DOPAC) and homovanillic acid (HVA), in the striatum and medial prefrontal cortex (Matsuno et al., 1995; Skuza et al., 1998; Kanzaki et al., 1992). Our results are in line with these findings. In fact the increase of DA metabolism could be induced through the σ_1 -mediate raise of intracellular DA levels; moreover, the increase of extracellular DA metabolism markers help to explain the σ_1 -mediated increase of DA cell toxicity described.

Relevant is the protective action of σ_1 antagonists versus the (+)-pentazocine-stimulated decrease of viability in presence of DA. In fact, toxic effects of psychostimulants are mainly mediated by alterations of extracellular DA concentrations (Gluck et al., 2001). In this context, the σ_1 antagonists, which were proposed as antipsychostimulant therapy, may also have a role in protecting neurons from psychostimulant-induced toxicity.

A mapping study of σ_1 receptors in Parkinson's disease (PD) using the σ ligand [^{11}C]SA4503 and positron emission tomography (PET) has been performed (Mishina et al., 2005). In PD patients, binding of the radiotracer was significantly lower on the more affected than the less affected side of the anterior putamen, but there was no significant difference with respect to the binding between patients and controls. However, in this study changes of basal activity of σ_1 have not been described. Further studies are necessary to better explore any functional relationship between the disappearance of DA and of the σ_1 binding sites in the same neurons.

The data of this thesis support the idea that activation of σ_1 receptors, by any endogenous or exogenous ligand, may facilitate DA uptake and thus, by increasing its cellular concentration, may contribute to cause cell death. It could be suggested the σ_1 binding sites, located inside the dopaminergic neurons, may contribute to the vulnerability of these cells by elevating intracellular levels of free DA. Numerous compounds, including several drugs of abuse, bind to σ_1 receptors and activating them could facilitate the damage of dopaminergic neurons exposed to higher intracellular DA levels. If this hypothesis will be confirmed by future investigations, it could be appropriated to test σ_1 receptor antagonists in PD experimental models as adjuvant to conventional therapies.

In the last part of this thesis, functional cross-talking between h σ_1 receptor and hDAT has been further investigated using confocal microscopy. From the acquired data it could be suggested that, following exposure to (+)-pentazocine, the h σ_1 receptors massively translocate towards the plasma membrane. Translocation of rat σ_1 receptors are known to occur after 1 h of exposure to (+)-pentazocine (Hayashi and Su, 2003). However this is the first time that a translocation of the human subtype σ_1 receptor in HEK-293 is described. In addition, colocalization of hDAT and h σ_1 appears to occur both

in treated and in non-treated cells, suggesting that the observed increase of [³H]DA uptake could be mediated by the hσ₁ redistribution from the cytoplasm to the cell membrane where hDAT is localized. Any physical interaction between the two proteins remains to be proved.

6.2 Concluding remarks

In this thesis, modulatory effects of hDAT by hσ₁ receptors has been described in cell models.

It has been demonstrated that the σ₁ receptor agonist (+)-pentazocine induces a potentiation of the hDAT [³H]DA uptake activity only in cells stably expressing the hσ₁ receptor in a concentration-dependent manner. This effect is abolished by pretreatment with σ₁ antagonists BD-1047 and NE-100, which are not able to modify the [³H]DA uptake if tested alone.

It has been demonstrated that viability of cells is strongly affected by the level of the uptaken DA; in viability assays, (+)-pentazocine increases cells susceptibility to DA toxicity in a concentration-dependent manner only in groups of cells that co-express the hσ₁ and the hDAT. This effect is prevented by pretreatment with σ₁ antagonists BD-1047 and NE-100. The significant increase of toxicity induced by (+)-pentazocine could be explained by virtue of the raised intracellular [DA], which has been proved in DA uptake assays. Confocal microscopy studies showed that hσ₁ receptors are able to translocate toward the membrane following exposure to (+)-pentazocine; in addition colocalization of hDAT and hσ₁ appear to occur both in treated and in non-treated cells. Thus, hσ₁ translocation could represent a key step in mediating the observed increase of [³H]DA uptake.

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