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# **G Protein-Coupled Receptor Heteromers Are Key Players in Substance Use Disorder.**

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## **Highlights**

- Heteromers are functional entities with behavioral impact
- Heteromers are increasingly recognized as key players in substance use disorder
- Heteromers may be part of larger functional multiprotein complexes
- Heteromers represent emerging innovative therapeutic targets

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## **Abstract**

G protein–coupled receptors (GPCR) represent the largest family of membrane proteins in the human genome. Physical association between two different GPCRs is linked to functional interactions which generates a novel entity, called heteromer, with specific ligand binding and signaling properties. Heteromerization is increasingly recognized to take place in the mesocorticolimbic pathway and to contribute to various aspects related to substance use disorder. This review focuses on heteromers identified in brain areas relevant to drug addiction. We report changes at the molecular and cellular levels that establish specific functional impact and highlight behavioral outcome in preclinical models. Finally, we briefly discuss selective targeting of native heteromers as an innovative therapeutic option.

## **Keywords**

addiction, substance use disorder, G protein-coupled receptors, heteromers, rodents, opioid, cannabinoid, dopamine, cocaine, adenosine, psychostimulant, alcohol

## 1. Introduction

Drug addiction or Substance Use Disorder (SUD) is a multifactorial disease that emerges from the combination of genetic predisposition, socio-economic environment and the nature of the drug itself. Addiction **develops from initial recreational drug use but also emerges following misuse and/or abuse of prescription drugs after an initial therapeutic use or in patients that self-medicate. It progresses towards compulsive drug seeking and consumption until this excessive behavior** cannot be controlled despite adverse consequences to an individual's social and professional life. In addition, negative emotional states, such as anxiety and irritability, characterize abstinence. Therefore, maintaining long-term abstinence is the major challenge to be faced by addicts and SUD is now recognized as a chronic relapsing neuropsychiatric disorder (Koob and Volkow, 2010).

All drugs alter dopaminergic tone by promoting activation of the mesocorticolimbic pathway. This neurocircuit consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (Nacc), amygdala and prefrontal cortex (PFC). Glutamatergic projections from the PFC also contribute to the core regulation process of this pathway. Additional brain areas then modulate the activity of this circuit in response to external stimuli such as spatial (hippocampus) and non-spatial (amygdala) cues, or internal states (amygdala, hypothalamus, raphe nuclei) (Figure 1). Importantly, addiction is now acknowledged to also develop in response to behavioral patterns **such as gambling or excessive food consumption**. In these pathologies, endogenous opioid and/or endocannabinoid peptides are released that activate their cognate receptor to stimulate the dopaminergic mesocorticolimbic pathway as observed for exogenous drugs of abuse (Noori et al., 2016).

Despite common modulation of the mesocorticolimbic pathway, the molecular targets of drugs of abuse differ significantly. Opiates, tetrahydrocannabinol (THC) or the psychostimulants caffeine or hallucinogen lysergic acid diethylamide (LSD) bind to G protein-

coupled receptors (GPCR), namely the opioid, cannabinoid, adenosine or serotonin receptors respectively. Alcohol mainly targets ligand-gated channels whereas psychostimulants such as amphetamines or cocaine modulate the dopamine transporter. Accordingly, pharmacological approaches have revealed that SUD is a complex process and that drug induced neuroplasticity covers multiple aspects including interactions triggered between different neuromodulatory systems. Cross-talk can take place at the molecular level through direct interactions between two GPCRs. This gives rise to a novel entity, a heteromer, which is characterized by specific ligand binding, signaling and/or trafficking properties. Cross-talk can also occur at the cellular level through simultaneous activation of several signaling cascades and at the integrated level through concomitant involvement of distinct neuronal circuits. Moreover, interactions can simultaneously occur at the three levels adding complexity to the ways in which drugs of abuse exert their effects.

Here, we review native heteromers formed by associations of two GPCRs or by association of a GPCR with an ion channel, in brain areas related to SUD. We focus on *in vivo* functional alterations and highlight behavioral consequences. We also include data collected in co-transfected cells that provide insight into the heteromerization process or add to our comprehension of heteromer signaling. Finally, we briefly address the potential of these heteromers as novel therapeutic targets.

## **2. The concept of heteromerization in GPCRs**

All GPCRs have a common architecture made of seven  $\alpha$ -helical transmembrane domains (TM) and possess an extracellular N-terminus and intracellular C-terminus. Specific recognition of the ligands takes place in the extracellular N-terminus and loops, but ligand binding also requires docking in a pocket within the helical bundle. Binding of an agonist leads

to receptor activation which in turn affects interaction with intracellular partners and initiates multiple downstream signaling events.

Based on specific structural determinants, GPCRs are usually sorted into five different classes with classes A, B, C and F being expressed in mammals (Fredriksson and Schiöth, 2005). GPCRs exhibit a high propensity to aggregate (Salim et al., 2002), which raised the question of criteria for a minimal functional unit. About 20 years ago, functional GABA<sub>B</sub> receptors from class C GPCRs were shown to require mandatory association of GABA<sub>B1</sub> and GABA<sub>B2</sub> receptors to be functional (Jones et al., 1998). Obligatory dimerization has subsequently been shown for other class C GPCRs such as the metabotropic glutamate receptors or the taste receptors (Rondard et al., 2011). In contrast, several class A GPCRs including rhodopsin,  $\beta_2$  adrenergic, neurotensin 1, leukotriene B<sub>2</sub> or mu opioid receptors are able to bind ligands, activate G proteins and recruit  $\beta$ -arrestin or G protein kinases in a monomeric form when reconstituted in a lipidic environment (reviewed in (Vischer et al., 2015)). The ability of a receptor to initiate signaling cascades as a monomeric entity does not however preclude interaction with other monomers from the same or a different receptor type to form a larger functional complex. For example, two monomers were found associated with one heterotrimeric G protein in the case of the serotonin 5HT<sub>4</sub> receptor (Pellissier et al., 2011). Activation of one monomer was enough to stimulate the G protein but co-activation led to a positive cross-talk indicating that this receptor rather functions as a homodimer. Similarly, the minimal signaling unit of the dopamine D<sub>2</sub> receptor was defined as homodimer. Here again, activity was maximal upon agonist binding to one monomer but was modulated by the constitutive activity of the second monomer indicating asymmetric functional interaction (Han et al., 2009). Whether the minimal functional unit involves a monomer or a homodimer thus appears receptor dependent (reviewed in (Ferre et al., 2014)).

In native tissue, class A GPCRs usually form clusters where they functionally interact with the surrounding receptors forming the so-called homomers if only one receptor type is present or heteromers if associated with a different receptor/protein (Khelashvili et al., 2010).

Heteromers have been extensively studied in co-transfected cells using biochemical, biophysical, or pharmacological approaches often combined with generation of mutant receptors (for reviews see (Fernandez-Duenas et al., 2012; Maggio et al., 2013; Vischer et al., 2015). **Early on, major efforts were invested to overcome technical limitations and validate their existence in native tissue.** In 2007, the International Union of Basic and Clinical Pharmacology defined criteria for validation of heteromer formation in class A GPCRs (Pin et al., 2007) comprising three main criteria:

1) There should be evidence for physical association in native tissues or primary cells. This can be obtained by co-immunoprecipitation, co-localization with antibody at electron microscopic level or energy transfer technologies using labeled ligands or knock-in animals. The use of the proximal ligation assay (PLA), developed more recently, is also widely accepted as appropriate to detect *in situ* receptor physical proximity.

2) In native tissues, heteromerization must induce a specific functional property, either a new pharmacological profile and/or the activation of a specific transduction cascade, distinct from those induced by each receptor expressed alone.

3) Disruption of the physical interaction (knock-out animals, RNA<sub>i</sub> technology, interfering peptide) must drastically alter the specific functional properties assigned to heteromers.

In the past years, our understanding of the functional impact of *in vivo* heteromerization has significantly improved and the concept has gained acceptance. Next to heteromer formation involving two class A GPCRs, heteromer formation between class A and class C GPCRs or between GPCRs and other proteins such as ion channels have also been reported.

This, in turn, has introduced an additional level of complexity in our interpretation of the behavioral responses observed upon receptor activation but has also expanded the repertoire of tools enabling modulation of cellular activity and physiological pathways.

### **3. Modulation of striatal dopaminergic transmission by heteromers**

Dopamine transmission in the striatum is central to SUD though modulated in different ways by drugs of abuse. Opiates, cannabis and alcohol, impact the core VTA-Nacc dopaminergic axis by reducing GABAergic tonic inhibition on dopamine release whereas psychostimulants modulate the activity of dopaminergic receptors in the basal ganglia both contributing to the reinforcing effect of a drug. In the striatum, medium spiny neurons (MSN) play a crucial role in this process by integrating information and propagating it through two main output pathways (Figure 2A). The direct striato-nigral pathway projects to the internal globus pallidus and substantia nigra (SN) and expresses dopamine D<sub>1</sub> receptors together with dynorphin and substance P. The indirect striato-pallidal pathway projects to the external globus pallidus and expresses dopamine D<sub>2</sub> receptors together with enkephalins (Tepper et al., 2007). More recently, a third striatal output has been postulated which is composed of a discrete neuronal population of the ventral striatum, mostly in the Nacc shell. These neurons co-express D<sub>1</sub> and D<sub>2</sub> receptors, dynorphin, enkephalin, GABA, and glutamate and may project to the SN and VTA (Perreault et al., 2012a). Importantly, each pathway expresses a combination of postsynaptic GPCRs coupled to inhibitory G $\alpha$ i/o or excitatory G $\alpha$ s subunits, providing the opportunity to further modulate neuronal activity through receptor heteromerization (Figure 2B).

### **4. Class A-class A heteromers with established modulatory role in SUD**

Here, we review functional properties of heteromers showing altered expression levels upon drug exposure and/or specific impact on behaviors related to SUD (Figure 3).

#### **4.1 Adenosine $A_{2a}$ -dopamine $D_2$ heteromers**

Heteromerization of adenosine  $A_{2a}$  and dopamine  $D_2$  receptors has been extensively studied in native tissue. Close physical proximity of  $A_{2a}$  and  $D_2$  receptors was detected by *in situ* PLA in the striatum of C57BL6/J mice (He et al., 2016; Trifilieff et al., 2011) and confirmed in rats by electron microscopy and time-resolved fluorescence resonance energy transfer (FRET) (Fernandez-Duenas et al., 2015). Co-internalization was detected upon activation by  $D_2$  or  $A_2$  agonists in SHSY5Y neuroblastoma and in primary striatal neurons (Hillion et al., 2002; Huang et al., 2013). Bivalent ligands bound to  $D_2$  or  $A_{2a}$  receptors with higher affinity than the corresponding monovalent control ligands in striatal membranes but not in membranes expressing one receptor only suggesting synergistic binding at  $A_{2a}$ - $D_2$  heteromers (Soriano et al., 2009), which supports close physical proximity of  $A_{2a}$  and  $D_2$  receptors.

Remarkably, changes in the extent of physical association between  $A_{2a}$  and  $D_2$  receptors were reported in several behavioral paradigms relevant to SUD. Psychostimulants induce sensitization of the dopaminergic system and progressively disrupt goal directed behaviors in favor of goal insensitive habitual responding (Furlong et al., 2017). This transition is facilitated by  $D_1$  receptor activation and inhibited by  $D_2$  receptors. In the striatum of C57BL6/J mice,  $A_{2a}$ - $D_2$  heteromers were more abundant in the dorsolateral than in the dorsomedial striatum in basal conditions (He et al., 2016; Trifilieff et al., 2011). However, *in situ* PLA revealed higher signal in the dorsomedial and dorsolateral striatum in a paradigm of habit formation (random interval training) whereas it remained unchanged in a paradigm of goal directed behavior (random ratio training) (He et al., 2016). This suggests that  $A_{2a}$ - $D_2$

heteromers participate in the negative regulation of D<sub>2</sub> signaling in the striatum and contribute to the control of abnormal habit formation taking place during drug addiction especially during relapse. This view is in line with accelerated habit formation following methamphetamine sensitization observed when D<sub>2</sub> receptors are pharmacologically blocked before instrumental learning (Nelson and Killcross, 2013). Moreover, these animals were unable to inhibit responding in extinction and reacquisition phases even when they acquired an aversion to the reinforcer (Nelson and Killcross, 2013). A<sub>2a</sub>-D<sub>2</sub> heteromers may therefore also contribute to the development of compulsive behaviors.

In a model of cocaine self-administration, *in situ* PLA revealed increased A<sub>2a</sub>-D<sub>2</sub> heteromers in the Nacc shell but not in the dorsal striatum of rats (Borrito-Escuela et al., 2017). Injection of the A<sub>2a</sub> TM V peptide in the Nacc disrupted the A<sub>2a</sub>-D<sub>2</sub> heteromers as shown by decreased *in situ* PLA signal (Borrito-Escuela et al., 2018). It also suppressed the inhibitory effect of the A<sub>2a</sub> agonist CGS21680 on cocaine self-administration (Borrito-Escuela et al., 2018), in agreement with reports demonstrating that reinstatement of cocaine self-administration is decreased or favored by A<sub>2a</sub> agonists or antagonists respectively (O'Neill et al., 2012).

Dynamical changes have also been described in a model of voluntary alcohol intake (intermittent access to 20% alcohol for at least 12 weeks), which led to higher detection of A<sub>2a</sub>-D<sub>2</sub> heteromers in the Nacc shell and dorsal striatum by *in situ* PLA (Feltmann et al., 2018). This is in line with the regulation of ethanol's rewarding effect in mice by A<sub>2a</sub> receptors expressed in striatopallidal neurons (Arolfo et al., 2004). Ethanol interacts directly with the adenosine system by blocking nucleoside transporters in the cell membrane. More specifically, ethanol selectively inhibits the type 1 equilibrative nucleoside transporter (ENT1) leading to decreased levels of striatal adenosine (Dunwiddie and Masino, 2001). Using ENT1 knock-out mice to mimic reduced adenosine availability, Nam *et al.* (2013) showed that dampening A<sub>2a</sub>

signaling in the dorsomedial striatum promoted ethanol seeking behaviors through decreased CREB activity and enhanced goal-oriented behaviors, hence contributing to excessive ethanol drinking (Nam et al., 2013). Ethanol also decreased ENT1 expression in the Nacc where reduced levels of adenosine could contribute to ethanol dependence and tolerance (Nam et al., 2013). In line with these observations, mice lacking  $A_{2a}$  showed reduced sensitivity to the hypnotic effects of alcohol, increased alcohol consumption and reduced alcohol-withdrawal seizures (El Yacoubi et al., 2001). At the level of  $A_{2a}$ - $D_2$  heteromers, reduced  $A_{2a}$  activity would decrease  $D_2$  signaling thereby contributing to reduce alcohol withdrawal symptoms but, at the same time, increasing alcohol consumption. Interestingly, the  $A_{2a}$  antagonist caffeine is often present as adulterant in the preparation of illicit drugs and in energy drinks consumed in combination with alcohol or other psychostimulants.  $A_{2a}$ - $D_2$  heteromers could thus mediate caffeine stimulant effects that would mask some of the acute intoxicating effects of ethanol and promote ethanol seeking behavior (reviewed in (Ferre, 2016)).

At the molecular level, the domains at the interface of the two receptors have been widely explored *in vitro* using a variety of approaches including mutagenesis, receptor chimeras, glutathione S transferase pull-down coupled to mass spectrometry and molecular modeling. The transmembrane domain TM V and third intracellular loop IL3 of the  $D_2$  receptor were identified as part of the contact area and were required to observe antagonistic pharmacological interactions (Torvinen et al., 2004) (Borrito-Escuela et al., 2010). More precisely, the arginine cluster 217RRRRKR222 present in the third intracellular loop IL3 of both short  $D_{2S}$  and long  $D_{2L}$  receptor isoforms form electrostatic interactions with two aspartate residues ( $D401A$ ,  $D40_{2a}$ ) and the phosphorylated S374 residue in the  $A_{2a}$  C-terminus (Borrito-Escuela et al., 2010; Ciruela et al., 2004).

Recently, a functional heterotetramer composed of  $D_2$ - $D_2$  and  $A_{2a}$ - $A_{2a}$  homomers has been proposed to integrate the experimental data collected over the years (Borrito-Escuela et

al., 2016; Fuxe et al., 2010; Navarro et al., 2014; Taura et al., 2017) and, in particular, the psychostimulant effects of the A<sub>2a</sub> antagonist caffeine (Ferre et al., 2018) (Figure 4). In the heterotetramer, the transmembrane domain TM VI is present at the interface between A<sub>2a</sub>-A<sub>2a</sub> or D<sub>2</sub>-D<sub>2</sub> receptor homomers and transmembrane domains IV and V contribute to the A<sub>2a</sub>-D<sub>2</sub> heteromeric interface (Navarro et al., 2018b). This macromolecular complex is pre-coupled to the partner G proteins and two adenylate cyclase AC5 molecules and undergoes dynamic reorganization upon agonist binding (Navarro et al., 2018b).

#### **4.2 Dopamine D<sub>2</sub>-sigma1 receptor heteromers**

The sigma1 receptor was originally wrongly classified within the opioid receptor family. The recently solved 3D crystal structure revealed a trimeric protein with one transmembrane domain in each protomer. (Schmidt et al., 2016). This ubiquitous protein intervenes in various cellular functions, is predominantly located in the endoplasmic reticulum (ER) but has also been detected at the plasmalemma and in synaptosomal fractions (recently reviewed in (Sambo et al., 2018)). In addition, the sigma1 receptor has an affinity of about 2µM for cocaine and metamphetamine in rodent brain tissue (Matsumoto et al., 2002; Nguyen et al., 2005).

Physical proximity between the postsynaptic long isoform of the D<sub>2</sub> receptor (D2L) and the sigma1 receptor was identified by *in situ* PLA and co-immunoprecipitation in the mouse striatum (Navarro et al., 2013). In a protocol of cocaine self-administration, dopamine D<sub>2</sub>-sigma1 proximity was increased in the rat Nacc shell and decreased in the dorsal striatum as shown by *in situ* PLA (Borrito-Escuela et al., 2017). Since cocaine similarly affected A<sub>2a</sub>-D<sub>2</sub> and D<sub>2</sub>-sigma1 heteromers in the two regions, one can postulate that the three proteins associate into larger A<sub>2a</sub>-D<sub>2</sub>-sigma1 heterocomplexes. Interestingly, direct action of 150 µM cocaine in CHO cells devoid of dopamine transporter but endogenously expressing the sigma1 receptor

(Mei and Pasternak, 2001) led to decreased BRET signal between co-expressed A<sub>2</sub> and D<sub>2</sub> receptors (Marcellino et al., 2010). This strongly suggests that cocaine concentrations compatible with those measured in cocaine abusers could affect the conformation of the A<sub>2a</sub>-D<sub>2</sub>-sigma1 heterocomplex.

From a functional point of view, co-administration of cocaine and the D<sub>2</sub>/D<sub>3</sub> agonist quinpirole decreased ERK1/2 phosphorylation in the mouse striatum. In contrast, cocaine did not induce ERK1/2 phosphorylation and could not modify the level of ERK1/2 phosphorylation induced by quinpirole in sigma1 knock-out mice (Navarro et al., 2013). Moreover, in the presence of sigma1 antagonists (PD144418 or BD1063), cocaine effects were reduced, and cocaine failed to amplify the effects of the agonist quinpirole on K<sup>+</sup> evoked dopamine and glutamate release from striatal synaptosomes (Beggiato et al., 2017; Navarro et al., 2013). Altogether, data therefore support a functional role for D<sub>2</sub>-sigma1 heteromers in the effects of cocaine.

Expression of D<sub>2</sub>-sigma1 heteromers is also modulated by chronic alcohol suggesting a more general implication in SUD. In a model of voluntary alcohol consumption with intermittent access to 20% ethanol, a decrease in PLA signal for D<sub>2</sub>-sigma1 heteromers was observed in the dorsal striatum where it was paralleled by a decrease in D<sub>2</sub>-D<sub>2</sub> homomers and an increase in A<sub>2a</sub>-D<sub>2</sub> heteromers (Feltmann et al., 2018).

#### ***4.3 Dopamine D<sub>1</sub> - dopamine D<sub>2</sub> heteromers***

In the basal ganglia, the strict separation in D<sub>1</sub> and D<sub>2</sub> receptor expression patterns has been challenged by the description of a subset of striatal MSNs co-expressing D<sub>1</sub> and D<sub>2</sub> receptors, dynorphin, enkephalin, GABA and glutamate (Hasbi et al., 2011) (Perreault et al., 2012b).

Heteromerization of D<sub>1</sub> and D<sub>2</sub> dopamine receptors was then hypothesized. To support this view, S. George and collaborators accumulated evidence of D<sub>1</sub>-D<sub>2</sub> physical proximity. First, FRET signal was detected on primary striatal cultures between secondary antibodies coupled to the AlexaFluor 350 to localize D<sub>2</sub> receptors and secondary antibodies coupled to the AlexaFluor 488 to localize D<sub>1</sub> receptors suggesting co-localization within the same membrane domains (Verma et al., 2010). Using FRET efficiency, the proximity of D<sub>1</sub> and D<sub>2</sub> receptors was estimated to 5-7 nm in clusters located at the surface of MSNs in the Nacc core (Hasbi et al., 2009; Perreault et al., 2016). *In vivo* D<sub>1</sub>-D<sub>2</sub> physical proximity was also confirmed by *in situ* FRET approaches in the rat striatum (Hasbi et al., 2018). Co-immunoprecipitation from striatal rat brain (Hasbi et al., 2009; Lee et al., 2004) and human striatum (Pei et al., 2010) further supported close receptor proximity. More recently, endogenous D<sub>1</sub>-D<sub>2</sub> heteromers were visualized using *in situ* PLA in primary striatal cultures and Nacc from rat (Perreault et al., 2016) as well as in the Nacc shell from the long-tail macaque (Perreault et al., 2016; Rico et al., 2017). More specifically, about 30% of the MSNs in the rat Nacc shell and more than 20% in the rat Nacc core expressed D<sub>1</sub>-D<sub>2</sub> heteromers at the cell surface versus only 5% of the MSNs in the dorsal striatum as revealed by *in situ* PLA with labeled primary antibodies (Hasbi et al., 2018). Using the same approach, no D<sub>1</sub>-D<sub>2</sub> heteromers were detected in the mouse striatum of mice deficient for either receptor (Hasbi et al., 2018). At the molecular level, heteromerization would take place between two adjacent arginine residues located on the D<sub>2</sub> third intracellular loop IL3 and two adjacent glutamic acid residues located on the D<sub>1</sub> C-terminal tail (O'Dowd et al., 2012). Accordingly, a TAT-D<sub>1</sub> peptide encoding the critical portion of the D<sub>1</sub> C-terminus disrupted D<sub>1</sub>-D<sub>2</sub> co-immunoprecipitation (Perreault et al., 2016) and abolished the PLA signal (Hasbi et al., 2018) in the rat striatum.

Functionally, D<sub>1</sub>-D<sub>2</sub> heteromers would specifically signal through Gαq/11 leading to CAMKII activation, intracellular calcium release and increased BDNF expression when co-

activated by the D<sub>1</sub> agonist SKF81297 and the D<sub>2</sub> agonist quinpirole or when activated by the selective D<sub>1</sub>-D<sub>2</sub> agonist SKF83959 (Hasbi et al., 2009; Lee et al., 2004; Perreault et al., 2016; Rashid et al., 2007). Activation of this signaling cascade was disrupted by the TAT-D<sub>1</sub> peptide encoding the critical portion of the D<sub>1</sub> C-terminus thereby establishing dependence on D<sub>1</sub>-D<sub>2</sub> receptor interaction (Perreault et al., 2016). Increased BDNF expression suggests an impact on maturation and differentiation of striatal neurons which could account for drug-induced plasticity.

Recently, the role of D<sub>1</sub>-D<sub>2</sub> heteromerization on several behavioral outputs related to cocaine consumption was uncovered using disruption of D<sub>1</sub>-D<sub>2</sub> heteromers by the TAT-D<sub>1</sub> peptide in the rat striatum (Hasbi et al., 2018; Perreault et al., 2016). This approach provided convincing evidence that activation of D<sub>1</sub>-D<sub>2</sub> heteromers by the D<sub>1</sub>-D<sub>2</sub> agonist SKF83959 blocked acquisition and expression of cocaine induced CPP whereas disruption of striatal D<sub>1</sub>-D<sub>2</sub> heteromers potentiated conditioned place preference to cocaine (Hasbi et al., 2018; Perreault et al., 2016). Similarly, **behavioral sensitization induced by repeated exposure to cocaine, which is characterized by an augmented locomotor response to a subsequent psychostimulant challenge injection, was abolished by the D<sub>1</sub>-D<sub>2</sub> agonist SKF83959 and potentiated by the disruption of striatal D<sub>1</sub>-D<sub>2</sub> heteromers** (Hasbi et al., 2018; Perreault et al., 2016). **Moreover,** activation of D<sub>1</sub>-D<sub>2</sub> heteromers by the D<sub>1</sub>-D<sub>2</sub> agonist SKF83959 abolished cocaine self-administration and reinstatement to cocaine seeking whether promoted by drug priming or cue association (Hasbi et al., 2018). Again, disrupting D<sub>1</sub>-D<sub>2</sub> physical interactions led to an opposite phenotype with potentiation of both drug self-administration and reinstatement (Hasbi et al., 2018). Western blot and immunohistochemical analyses in the NAcc revealed that activation of the D<sub>1</sub>-D<sub>2</sub> heteromer likely counteracted cocaine-induced ERK phosphorylation and ΔFos B accumulation and reduced cocaine evoked field potentials signals by increasing the phosphorylation of DARPP32 at the Thr75 residue (Hasbi et al., 2018).

Interestingly, D<sub>1</sub>-D<sub>2</sub> heteromer expression increases with age as it is less abundant in the caudate putamen and in the Nacc shell, but not in the Nacc core, of juvenile rats compared to adults (Perreault et al., 2010; Perreault et al., 2012b; Perreault et al., 2016). This increase in expression was paralleled by increased G $\alpha$ q coupling in 8-month-old rats compared to 3-month-old animals despite no change in individual receptor expression (Rashid et al., 2007). Data therefore suggest a shift towards D<sub>1</sub>-D<sub>2</sub> G $\alpha$ q coupling with age and lower D<sub>1</sub>-D<sub>2</sub> tonic inhibition in juvenile animals. This may account for the increased susceptibility to cocaine's behavioral effects observed in adolescents (Spear, 2016) and is in line with the observation that behavioral sensitization to psychostimulants such as cocaine requires activation of both D<sub>1</sub> and D<sub>2</sub> receptors (Capper-Loup et al., 2002) and CAMKII (Pierce et al., 1998) in the Nacc.

Importantly, the implication of D<sub>1</sub>-D<sub>2</sub> heteromers is not restricted to cocaine but extends to natural reinforcers. Indeed, activation of D<sub>1</sub>-D<sub>2</sub> heteromers by the D<sub>1</sub>-D<sub>2</sub> agonist SKF83959 reduced sucrose preference in a two-bottle free choice paradigm whereas heteromer disruption with the TAT-D<sub>1</sub> peptide had the opposite effect (Hasbi et al., 2018). In addition, in a paradigm of restricted food access, activation of D<sub>1</sub>-D<sub>2</sub> heteromers reduced the motivation to seek and consume palatable food (Hasbi et al., 2018).

#### ***4.4 Mu opioid-dopamine D<sub>1</sub> heteromers***

Co-immunoprecipitation of mu opioid and dopamine D<sub>1</sub> receptors in striatal membranes from wild type, but not from D<sub>1</sub> or mu receptor knock-out mice, established the physical proximity of the two receptors (Tao et al., 2017). The D<sub>1</sub> antagonist SCH23390 reduced mu opioid receptor signaling as revealed by reduced G protein activation, decreased ERK1/2 phosphorylation and less c-Fos induction in wild type but not D<sub>1</sub> knock-out mice (Tao et al., 2017). These data are indicative of the cross-antagonism exerted by D<sub>1</sub> antagonists on mu opioid signaling. Accordingly, chronic systemic co-administration of the D<sub>1</sub> antagonist

SCH23390 and the mu agonist morphine reduced morphine induced hyperlocomotion, sensitization acquisition and expression (Tao et al., 2017). Systemic administration or local infusion in the Nacc of the D<sub>1</sub> antagonist SCH23390 also decreased expression of the locomotor sensitization to the mu agonists morphine and DAMGO (Tao et al., 2017). Similarly, morphine or DAMGO induced locomotor sensitization was markedly decreased upon intra Nacc injection of an ERK inhibitor (Tao et al., 2017). D<sub>1</sub> impact was independent of dopaminergic transmission from the VTA as it was also observed in rats with 6-OHDA lesion in the NAcc (Tao et al., 2017). Finally, no locomotor sensitization to morphine was observed in knock-out mice deficient for the D<sub>1</sub> receptor (Tao et al., 2017). Altogether, data establish that mu-D<sub>1</sub> heteromers play a critical role in opiate hyperlocomotion and locomotor sensitization.

#### ***4.5 Mu-delta opioid heteromers***

Mapping mu and delta opioid receptors in the brain using double fluorescent knock-in mice revealed co-expression in discrete neuronal populations located in subcortical networks essential for survival, including the perception and processing of aversive stimuli (Erbs et al., 2015). Evidence of close physical proximity at the supraspinal level included detection with mu-delta selective antibodies (Gupta et al., 2010), mu-delta co-immunoprecipitation in the hippocampus (Erbs et al., 2015) and antinociceptive activity upon intracerebroventricular (i.c.v.) injection of MDAN-21, a bivalent ligand bridging the mu agonist oxymorphone and the delta antagonist naltrindole (Daniels et al., 2005). Direct physical interaction was shown in the Nacc through disruption by a sequence corresponding to the delta carboxy tail (Kabli et al., 2013), and was also established in the spinal cord through disruption by a peptide corresponding to the mu transmembrane domain TM I (He et al., 2011), and in DRGs through

disruption with a peptide corresponding to the delta second intracellular loop IL2 (Xie et al., 2009).

Immunohistochemical detection with mu-delta specific antibodies revealed that chronic morphine treatment increased mu-delta heteromer expression in regions associated with the mesocorticolimbic pathway such as the VTA, Nacc, striatum, PFC, hippocampus and, hypothalamus (Gupta et al., 2010), pointing to a modulatory role in SUD. Of note, chronic morphine treatment enhanced delta opioid receptor expression at the cell surface through a mu opioid receptor dependent pathway (reviewed (Gendron et al., 2015)) compatible with mu-delta heteromer formation in the ER (Décaillot et al., 2008; Xie et al., 2009). Enhanced delta opioid receptor expression restricted to the circuit of thermal nociception was also reported in the spinal cord in a model of voluntary alcohol consumption (van Rijn et al., 2012) indicating that increase in functional delta receptors could be achieved upon consumption of drugs of abuse other than morphine. Whether the underlying mechanism relies on mu-delta heteromerization however requires further confirmation.

Pharmacological evidence also points to a link between mu-delta heteromers and SUD-related behaviors. Targeting mu-delta heteromers with the bivalent ligand MDAN-21 (Daniels et al., 2005) or with the biased agonist CYM51010 (Gomes et al., 2013) induced less tolerance and physical dependence compared to morphine. This is in line with high mu-delta co-expression in brainstem nuclei tightly connected to the autonomous system (Erbs et al., 2015) that suggests mu-delta modulation of somatic and autonomic symptoms during drug withdrawal. Moreover, TAN-67, identified as a mu-delta receptor selective agonist in co-transfected HEK 293 cells, decreased ethanol but not sucrose consumption in wild type but not delta receptor knock-out mice (van Rijn and Whistler, 2009).

From a functional perspective, occupancy of the binding site of one receptor increased ligand binding and signaling to the other receptor in SK-N-SH neuroblastoma cells that

endogenously co-express the two receptors (Gomes et al., 2004; Gomes et al., 2011; Gomes et al., 2000). Interestingly, the nature of the first ligand did not seem important since an agonist, antagonist or inverse agonist induced similar effects. Data therefore suggest that mu-delta heteromerization induced cross-allosteric modulation with a positive cooperativity promoted upon binding of the first ligand. Heterotetramers formed by association of mu homomers with delta homomers have been postulated (Golebiewska et al., 2011), which could accommodate bidirectional cross-allosteric modulation as for A<sub>2a</sub>-D<sub>2</sub> heterotetramers (Bonaventura et al., 2015) (Navarro et al., 2018b).

Co-activation of mu and delta opioid receptors in native tissue also resulted in a positive cross-talk. Indeed, increased hyperpolarization was observed in a subset of GABAergic neurons in VTA slices upon co-application of the delta antagonist TIPP $\psi$  together with mu agonist DAMGO or upon co-application of the mu antagonist CTAP together with the delta agonists deltorphin II or DPDPE (Margolis et al., 2017). Given their enhanced expression in the VTA of morphine dependent animals ((Gupta et al., 2010), Massotte unpublished), mu-delta heteromers could thus contribute to increase dopamine release in the Nacc.

Mu-delta heteromerization also alters G protein coupling. In mu-delta co-transfected CHO cells, inhibition of forskolin induced cAMP production was maintained after pertussis toxin treatment. This led a postulate that mu-delta heteromers would no longer signal through pertussis toxin sensitive inhibitory G $\alpha$ i/o proteins but would recruit the inhibitory pertussis toxin insensitive G $\alpha$ z subunit (George et al., 2000). The G $\alpha$ z subunit is expressed in the striatum and also abundant in the hippocampus (Friberg et al., 1998) where extensive mu-delta heteromerization is present. G $\alpha$ z is recruited by heroin, morphine, buprenorphine, and methadone (Sanchez-Blazquez et al., 2001). Moreover, mu-delta preferential coupling to G $\alpha$ z would not be desensitized by chronic morphine administration in the rat striatum and hippocampus (George et al., 2000; Kabli et al., 2014) and G $\alpha$ z deficient mice show increased

morphine tolerance (Hendry et al., 2000). These data suggest that  $G\alpha_z$  dependent mu-delta signaling could also play a role in drug addiction.

Heteromerization not only affects G protein-dependent signaling but also alters  $\beta$  arrestin-dependent pathways. In neuroblastoma SKNSH endogenously co-expressing mu and delta opioid receptors, activation by the mu agonist DAMGO led to a spatio-temporal change in the ERK1/2 phosphorylation profile with prolonged activation in the cytoplasmic compartment (Rozenfeld and Devi, 2007). In the presence of the delta selective antagonist TIPP $\psi$ , the phosphorylation profile reversed to the one observed for the mu opioid receptor expressed alone (Rozenfeld and Devi, 2007) as expected if dependent on mu-delta heteromerization. The change in ERK1/2 phosphorylation profile was also abolished in the presence of  $\beta$ -arrestin 2 siRNA (Rozenfeld and Devi, 2007). Efficient recruitment of the  $\beta$ -arrestin 2 dependent signaling pathway was also observed upon activation by the biased mu-delta agonist CYM51010 (Gomes et al., 2013) that promotes co-internalization of mu and delta opioid receptors in primary hippocampal neurons (Derouiche et al., 2018). These results suggest increased cytoplasmic signaling by internalized mu-delta heteromers in line with recent reports on GPCR signaling from the endosomal compartment (Tsvetanova et al., 2015) and indicate changes in the spatiotemporal control of neuronal activity.

#### ***4.6 Mu opioid-Galanin1 heteromers***

Mu opioid receptors are widely distributed throughout the brain (Erbs et al., 2015). Galanin1 (Gal1) receptors are mostly present in the VTA, Nacc, and SN, suggesting that they may contribute to the galanin-mediated regulation of dopamine neurotransmission (Moreno et al., 2017). Gal1 receptors are also highly expressed in the locus coeruleus, where they may also modulate noradrenergic transmission during opiate withdrawal (Hawes and Picciotto, 2004). Physical proximity between the mu opioid and Gal1 receptors was established in co-transfected

cell by BRET and bimolecular fluorescent complementation (Moreno et al., 2017). In the VTA, the mu antagonist CTOP was able to counteract galanin induced ERK1/2, AKT and CREB phosphorylation, a cross-antagonism attributed to mu-Gal1 heteromers (Moreno et al., 2017). In addition, galanin could not prevent dopamine release promoted by local infusion of the mu agonist endomorphin 1 when mu-Gal1 heteromers were disrupted by injection of a peptide corresponding to the mu transmembrane domain TM V (Moreno et al., 2017). This suggests a negative cross-talk by which galanin dampens mu opioid receptor signaling. Moreover, enhanced ERK activation was observed in the VTA, Nacc and amygdala in galanin knock-out mice following acute morphine administration compared to wild type mice (Hawes et al., 2008). Importantly, conditioned place preference to morphine was abolished in galanin knock-out mice (Hawes et al., 2008). These results therefore substantiate galanin-dependent modulation of the reinforcing properties of opiates. In humans, the single nucleotide polymorphism rs948854 localized in the promoter region of the galanin gene is known to be associated with anxiety related disorders. It may also indicate increased vulnerability to opiate addiction since an increased number of homozygous carriers for the minor allele (G) was detected among opioid addicts (Beer et al., 2013).

#### ***4.7 Cannabinoid CB<sub>1</sub>- serotonin 5HT<sub>2a</sub> heteromers***

THC binds to CB<sub>1</sub> receptors which mediate both its beneficial effects such as analgesia, anxiolytic action and neuroprotection and detrimental effects such as dependence, anxiogenic action or memory impairment (Zou and Kumar, 2018). In addition, cannabis consumption in adolescents is well known to facilitate the appearance of psychosis involving the serotonergic system (Karila et al., 2014). CB<sub>1</sub> receptors are highly expressed in the rat striatum on presynaptic cortical inputs and postsynaptic dendrites of cells located in the patch and matrix compartments (Rodriguez et al., 2001). Since serotonergic 5HT<sub>2a</sub> receptors are strongly

expressed in the PFC and are also present in the striatum (Pazos et al., 1985; Weber and Andrade, 2010), the existence of CB<sub>1</sub>-5HT<sub>2a</sub> heteromers has been postulated to underlie the functional interaction between the endocannabinoid and serotonergic systems in this context.

CB<sub>1</sub>-5HT<sub>2a</sub> heteromers were identified in the cortex (somatomotor layers 1,2,3), hippocampus (CA3) and, dorsal striatum by *in situ* PLA in wild type mice but not in mice deficient for either receptor (Vinals et al., 2015). Infusion (i.c.v.) of the CB<sub>1</sub> receptor transmembrane domains TM V or TM VI but not TM VII domain disrupted the PLA signal, indicating that physical association involve TM V and TMVI (Vinals et al., 2015). Importantly, no CB<sub>1</sub>-5HT<sub>2a</sub> heteromers could be detected in the Nacc (Vinals et al., 2015).

*In vivo* disruption of the CB<sub>1</sub>-5HT<sub>2a</sub> physical contact as well as use of 5HT<sub>2a</sub> knock-out mice revealed that some THC effects such as hypolocomotion, hypothermia, analgesia, anxiogenic effect were exclusively mediated by the CB<sub>1</sub> receptor (Vinals et al., 2015). In a protocol of self-administration, the reinforcing properties of the cannabinoid agonist WIN55,212-2 also remained unaffected in 5HT<sub>2a</sub> knock-out mice. This suggested no involvement of CB<sub>1</sub>-5HT<sub>2a</sub> heteromers in agreement with the absence of PLA signal in the Nacc (Vinals et al., 2015). However, following chronic THC administration, symptoms of withdrawal precipitated with the cannabinoid antagonist rimonabant were significantly attenuated in 5HT<sub>2a</sub> knock-out mice compared to wild type animals pointing to a possible involvement of the CB<sub>1</sub>-5HT<sub>2a</sub> heteromers in physical dependence (Vinals et al., 2015). In addition, memory impairment (novel object recognition task), anxiolytic effect (elevated plus maze) and increased social interaction induced by acute THC administration were reduced in 5HT<sub>2a</sub> knock-out mice and/or upon disruption of the physical contact between the two receptors (Vinals et al., 2015). This indicates that CB<sub>1</sub>-5HT<sub>2a</sub> heteromers modulate these THC dependent effects, in particular, aspects of the THC associated cognitive deficits.

At the cellular level, CB<sub>1</sub>-5HT<sub>2a</sub> heteromerization switched the 5HT<sub>2a</sub> receptor coupling from the G $\alpha$ <sub>q</sub> to the G $\alpha$ <sub>i</sub> pathway in co-transfected HEK293 cells (Vinals et al., 2015). Co-activation of the receptors in co-transfected HEK293 cells reduced cell signaling by increasing cAMP inhibition and reducing  $\beta$ -arrestin recruitment without altering ERK1/2 and Akt phosphorylation. (Vinals et al., 2015). Bidirectional cross-antagonism was also observed upon simultaneous administration of an antagonist for one receptor and an agonist for the other on cAMP production,  $\beta$ -arrestin recruitment, ERK1/2 and Akt phosphorylation (Vinals et al., 2015). Both negative crosstalk and cross-antagonism were reversed upon physical disruption of the CB<sub>1</sub>-5HT<sub>2a</sub> interaction in co-transfected HEK293 cells (Vinals et al., 2015). Importantly, both were observed in brain regions where CB<sub>1</sub>-5HT<sub>2a</sub> physical association was observed and were absent in 5HT<sub>2a</sub> knock-out mice (Vinals et al., 2015).

#### **4.8 Cannabinoid CB<sub>1</sub>-adenosine A<sub>2a</sub> heteromers**

Adenosine A<sub>2a</sub> and CB<sub>1</sub> receptors are expressed in dendritic spines of striatopallidal GABAergic neurons and their close physical proximity was established by co-immunoprecipitation from rat striatal membranes (Carriba et al., 2007; Ferreira et al., 2015). Further data in favor of heteromerization were collected in co-transfected cells by BRET experiments (Carriba et al., 2007). In SHSY5Y neuroblastoma that endogenously co-express the two receptors, efficient CB<sub>1</sub> receptor coupling to inhibitory G protein requires simultaneous activation of A<sub>2a</sub> receptors and is abolished in the presence of an A<sub>2a</sub> selective antagonist indicative of cross-antagonism (Carriba et al., 2007). Accordingly, blocking A<sub>2a</sub> receptors with the A<sub>2a</sub> antagonist MSX-3 counteracted the decrease in locomotor activity promoted by intrastriatal bilateral administration of the CB<sub>1</sub> agonist WIN55212-2 (Carriba et al., 2007). In addition, decreased conditioned place preference to THC was observed in A<sub>2a</sub> receptor knock-out mice supporting a role for CB<sub>1</sub>-A<sub>2a</sub> heteromers (Soria et al., 2004).

Functional interaction of A<sub>2a</sub> and CB<sub>1</sub> receptors in presynaptic corticostriatal synapses was also postulated using radioligand binding in nerve terminal membranes, glutamate release assay in acutely isolated nerve terminals and paired-pulse ratio measurements in isolated monosynaptic contacts in corticostriatal slices (Ferreira et al., 2015). However, in this case, A<sub>2a</sub> receptor activation dampened CB<sub>1</sub> receptor function and CB<sub>1</sub>-mediated inhibition of corticostriatal glutamatergic inputs (Ferreira et al., 2015). These results suggest that activation of the A<sub>2a</sub> receptor would have opposite effects on CB<sub>1</sub> receptor signaling depending on the pre- or post-synaptic localization of the CB<sub>1</sub>-A<sub>2a</sub> heteromer.

## **5. Class A-Class A Heteromers with potential role in SUD**

Here, we review heteromers for which impact on cellular mechanisms relevant to SUD have been evidenced, although no direct behavioral outcome has been reported yet.

### ***5.1 Mu opioid-cannabinoid CB<sub>1</sub> heteromers***

Both mu opioid and cannabinoid CB<sub>1</sub> receptors are primary targets of drugs of abuse and widely expressed throughout the brain (Erbs et al., 2015; Nguyen et al., 2010). Heteromerization between these two receptors is thus bound to strongly alter drug effects. BRET measurements in co-transfected HEK293 cells established the ability of the two receptors to physically interact and ultrastructural analysis in the rat caudate putamen revealed discrete co-localization of the two receptors in MSNs (Rios et al., 2006). The presence of CB<sub>1</sub> receptors in mu enriched patches suggested that the two receptors could modulate the output of these GABAergic neurons (Rodriguez et al., 2001). This may account for the similar impact of the two receptors on motor activity.

Interestingly, THC was reported to allosterically decrease opiate binding at the mu opioid receptor in rat brain membranes (Vaysse et al., 1987). In addition, simultaneous co-activation of the receptors in SK-N-SH neuroblastoma cell line or rat striatal membranes decreased G protein-dependent signaling (Rios et al., 2006). This bidirectional negative

crosstalk was associated with decreased activation of the G protein-Src-STAT3 pathway in mu-transfected Neuro<sub>2a</sub> cells and a negative impact on neuriteogenesis (Rios et al., 2006) which may contribute to drug related neuroplasticity.

Social play has a strong rewarding value and is modulated by neurotransmitters involved in reward and motivation. Accordingly, increased activity of the opioid, endocannabinoid or dopamine receptors in the Nacc enhanced social play in rodents ((Manduca et al., 2016) and refs therein). Systemic injection of the endocannabinoid 2-arachidonoyl (2-AG) hydrolysis inhibitor JZL184 increased social play behavior in adolescent rodents (Manduca et al., 2016). This effect was blocked by infusion in the NAcc of the mu antagonist CTAP and was absent in mu knock-out mice (Manduca et al., 2016). Reciprocally, systemic injection of the mu opioid agonist morphine increased social play and was abolished by the CB<sub>1</sub> antagonist SR1417-16 or in CB<sub>1</sub> knock-out mice (Manduca et al., 2016). Whole cell recordings of miniature excitatory postsynaptic currents revealed functional presynaptic interaction between CB<sub>1</sub> and mu opioid receptors. Indeed, CB<sub>1</sub> antagonists reduced the inhibition exerted by the mu opioid receptor on excitatory transmission influencing Nacc neurons and *vice versa* (Manduca et al., 2016). Altogether, these data revealed bidirectional cross-antagonism taking place at mu-CB<sub>1</sub> heteromers and strongly suggest that functional mu-CB<sub>1</sub> heteromers in the Nacc can modulate the rewarding properties of drugs of abuse.

## **5.2 Cannabinoid CB<sub>1</sub>-CB<sub>2</sub> heteromers**

Cannabinoid CB<sub>1</sub> receptors are the primary molecular target for THC. CB<sub>1</sub> receptors are widely distributed throughout the brain (Nguyen et al., 2010). In the striatum, they are often located presynaptically on GABAergic terminals of interneurons or collaterals from MSNs (Matyas et al., 2006) but they are also present in glutamatergic terminals (Huang et al., 2001). Striatal postsynaptic CB<sub>1</sub> receptors are located in the somatodendritic compartment of

dynorphin and enkephalin positive MSNs (Callen et al., 2012; Martin et al., 2008) and references therein). CB<sub>2</sub> receptors are mostly expressed outside the CNS but evidence is accumulating for their presence in the brain where they are mostly postsynaptic ((Callen et al., 2012) and references therein). Physical proximity indicative of heteromer formation with the CB<sub>1</sub> receptor was reported using *in situ* PLA in the rat striatum (Callen et al., 2012).

As described for mu opioid-CB<sub>1</sub> heteromers, CB<sub>1</sub>-CB<sub>2</sub> co-activation resulted in a negative cross-talk on the Akt/PKB signaling and CB<sub>1</sub>-CB<sub>2</sub> cross antagonism decreased ERK1/2 and Akt/PKB phosphorylation, thereby reducing neurite outgrowth (Callen et al., 2012). Activation of the striatal microglia increased CB<sub>1</sub>-CB<sub>2</sub> heteromerization. Moreover, microglial activation changed the negative crosstalk on CB<sub>1</sub>-CB<sub>2</sub> signaling observed in resting cells upon co-stimulation by the CB<sub>1</sub> agonist ACEA and the CB<sub>2</sub> agonist JWH133 into a positive cross-talk in activated cells (Navarro et al., 2018a). This is in line with a study indicating that activation of the endocannabinoid system, and more specifically of the CB<sub>2</sub> receptor, before methamphetamine administration reduced the neurotoxicity of the drug (Nader et al., 2014). Also, cocaine increased CB<sub>1</sub> and decreased CB<sub>2</sub> expression in the PFC and hippocampus of adolescent rats (Garcia-Cabrerizo and Garcia-Fuster, 2016). Although CB<sub>1</sub>-CB<sub>2</sub> heteromers have not been evidenced in the cortex so far, one can postulate that cocaine could prevent their neuroprotective effect by decreasing their expression, which may contribute to the addiction vulnerability observed in adolescents (Bagot et al., 2015). CB<sub>1</sub>-CB<sub>2</sub> heteromers may therefore represent a therapeutic target to reduce neuroinflammation that develops in SUD (Karoly et al., 2015).

### ***5.3 Dopamine D<sub>3</sub>/D<sub>3nf</sub> heteromers***

Several pieces of evidence link the dopamine D<sub>3</sub> receptor to SUD. First, expression of dopamine D<sub>3</sub> receptors is increased in several brain areas from cocaine abusers,

metamphetamine users and alcohol dependent patients (for reviews (Prieto, 2017; Sokoloff and Le Foll, 2017). Second, the Ser9Gly polymorphism known to enhance ERK signaling and to be responsible for essential tremor has also been linked to early onset of heroin dependence in Han Chinese subjects (Kuo et al., 2014). Third, D<sub>3</sub> receptors are involved in locomotor responses to spatial novelty and psychostimulants (Pritchard et al., 2006). Indeed, D<sub>3</sub> receptors modulate glutamatergic pathways from the PFC to subcortical areas, either directly by interacting with N-methyl-D-aspartate (NMDA) receptors in the Nacc, or indirectly by controlling dopamine release from VTA neurons. Because D<sub>3</sub> receptors are associated with the ventral forebrain mesolimbic system, they influence reward, emotions and motivation but also affects drug seeking and relapse, particularly related with drug associated cues (for a recent review (Sokoloff and Le Foll, 2017).

Co-immunoprecipitation in rat brain revealed physical proximity between the D<sub>3</sub> and truncated D<sub>3nf</sub> receptors (Nimchinsky et al., 1997). In baculovirus infected Sf9 cells, D<sub>3</sub>-D<sub>3nf</sub> heteromerization abolished D<sub>3</sub> activity by inhibiting ligand binding to the receptor (Elmhurst et al., 2000). In co-transfected HEK 293 cells, D<sub>3</sub>-D<sub>3nf</sub> association led to predominant cytoplasmic mislocalization of the D<sub>3</sub> receptor (Karpa et al., 2000). This inactive variant seems thus to play a regulatory role and to reduce functional D<sub>3</sub> receptor availability. In the high responder/low responder model, individual differences in the locomotor responses to an inescapable, novel spatial environment predict individual differences in the locomotor and reinforcing effects of psychostimulants (Pritchard et al., 2006). In this model, the D<sub>3</sub>/D<sub>3nf</sub> ratio was decreased in the PFC, SN and VTA of rat high responder to novelty compared to low responder but remained unaffected in the Nacc (Pritchard et al., 2006). Accordingly, the D<sub>3</sub>/D<sub>3nf</sub> ratio has been proposed to play a role in cocaine sensitization and addiction (Richtand, 2006). Surprisingly, in a protocol of sensitization to nicotine involving three exposures to the

drug, the  $D_3/D_{3nf}$  ratio was increased in the Nacc of adolescent and adult rats suggesting a potential increase in receptor  $D_3$  receptor availability (Smith et al., 2015).

#### ***5.4 Dopamine $D_1$ - $D_3$ heteromers***

$D_1$  and  $D_3$  receptors were co-immunoprecipitated from rat brain membranes (Fiorentini et al., 2008). Co-activation of the receptors potentiated the stimulatory effect of dopamine on cAMP and led to co-internalization of the two receptors in co-transfected HEK 293 cells (Fiorentini et al., 2008). Since agonist stimulation of  $D_3$  receptors did not promote  $D_3$  receptor internalization when expressed alone, this suggested a switch towards  $D_1$  receptor specific desensitization mechanisms (Fiorentini et al., 2008). In striatal membranes, enhanced agonist affinity was observed at the  $D_1$  receptor upon  $D_3$  receptor co-activation (Marcellino et al., 2008). In reserpinized mice, a model to study the function of postsynaptic striatal dopamine receptors without the influence of endogenous dopamine,  $D_3$  receptor activation potentiated  $D_1$  dependent locomotor activity (Marcellino et al., 2008). This positive cross-talk was counteracted by a  $D_3$  selective antagonist and abolished in  $D_3$  knock-out mice (Marcellino et al., 2008).  $D_1$ - $D_3$  heteromers by modulating the direct striatonigral pathway may therefore contribute to psychostimulant locomotor sensitization.

#### ***5.5 Dopamine $D_1$ -histamine $H_3$ -sigma1 heteromers***

The histamine  $H_3$  receptor is abundantly expressed in the striatum and mostly located post-synaptically in both  $D_1$  and  $D_2$  expressing MSNs. The interaction of the histamine  $H_3$  receptor with the dopaminergic system and its implication in alcohol or psychostimulant abuse is well established (Bakker et al., 2006; Ellenbroek, 2013; Panula and Nuutinen, 2011). Physical proximity of histamine  $H_3$  and dopamine  $D_1$  receptors was verified by co-immunoprecipitation experiments in rat (Moreno et al., 2011a) and mouse (Moreno et al.,

2014) striatal membranes. It was further confirmed in the mouse striatum using PLA (Moreno et al., 2014).

At the cellular level, the D<sub>1</sub> receptor couples to the G $\alpha$ s subunit and increases cAMP production. However, in neuroblastoma cells co-transfected with D<sub>1</sub> and H<sub>3</sub> receptors, D<sub>1</sub> agonists led to reduced forskolin induced cAMP suggesting that the D<sub>1</sub> receptor now activated the inhibitory G $\alpha$ i/o pathway through its association with the H<sub>3</sub> receptor (Ferrada et al., 2009). Co-transfection of D<sub>1</sub> and H<sub>3</sub> receptors led to ERK1/2 phosphorylation upon activation by H<sub>3</sub> agonists which was not observed when the H<sub>3</sub> receptor was expressed alone (Ferrada et al., 2009). Accordingly, the ability of H<sub>3</sub> receptors to stimulate ERK1/2 phosphorylation was not observed in D<sub>1</sub> receptor knock-out mice (Moreno et al., 2011a) supporting a role for D<sub>1</sub>-H<sub>3</sub> heteromerization. Moreover, D<sub>1</sub> receptor expression was decreased and no ERK1/2 phosphorylation following D<sub>1</sub> agonist activation was observed in the striatum of H<sub>3</sub> receptor knock-out mice suggesting that the presence of the H<sub>3</sub> receptor is required for the D<sub>1</sub> receptor to be fully functional (Kononoff Vanhanen et al., 2016). However, H<sub>3</sub> receptor activation inhibited D<sub>1</sub> dependent ERK1/2 phosphorylation in striatal membrane revealing a negative crosstalk on D<sub>1</sub> signaling when the two receptors were simultaneously activated (Moreno et al., 2014).

Antagonists for either receptor blocked ERK1/2 phosphorylation in striatal membranes or neuroblastoma cells co-transfected with D<sub>1</sub> and H<sub>3</sub> receptors indicating that D<sub>1</sub>-H<sub>3</sub> heteromerization was also associated with bidirectional cross-antagonism (Ferrada et al., 2009; Moreno et al., 2014).

Acute cocaine application on organotypic cultures from mouse striatum inhibited D<sub>1</sub>-H<sub>3</sub> bidirectional cross antagonism and reduced the negative cross-talk exerted by H<sub>3</sub> receptor activation on D<sub>1</sub> receptor signaling (Moreno et al., 2014). A similar impact on D<sub>1</sub>-H<sub>3</sub> heteromer signaling was observed following chronic exposure to cocaine using striatal slices from rats

that self-administered the drug for 6-11 weeks (Moreno et al., 2014). Since none of the receptors has affinity for cocaine, association with the sigma1 receptor that efficiently binds this psychostimulant was postulated (Matsumoto et al., 2002). Co-immunoprecipitation and *in situ* PLA experiments both confirmed the physical proximity between sigma1 and D<sub>1</sub> or H<sub>3</sub> receptors (Moreno et al., 2014). Accordingly, all tested effects of cocaine on D<sub>1</sub>-H<sub>3</sub> heteromer signaling were abolished in striatal organotypic cultures from mice deficient for the sigma1 receptor (Moreno et al., 2014). Since drugs of abuse all increase extracellular dopamine level which initiates oxidative stress and neurotoxicity (Cunha-Oliveira et al., 2008), the authors explored the functional role of D<sub>1</sub>-H<sub>3</sub> heteromers on striatal neuron survival. Activation of D<sub>1</sub> receptors induced cell death that was counteracted by activation of the H<sub>3</sub> receptor or by co-administration of D<sub>1</sub> or H<sub>3</sub> selective antagonists (Moreno et al., 2014). Both effects were absent following exposure of the organotypic slices to cocaine (Moreno et al., 2014). Involvement of the sigma1 receptor in the cocaine effects on neuronal cell death was then investigated. Pretreatment with a sigma1 agonist mimicked cocaine effects whereas pretreatment with a sigma1 antagonist abolished blockade of both D<sub>1</sub>-H<sub>3</sub> cross-antagonism and negative crosstalk on D<sub>1</sub> signaling by cocaine (Moreno et al., 2014). Altogether, these data point to D<sub>1</sub>-H<sub>3</sub>-sigma1 heterocomplexes as potential target for the treatment of cocaine abuse.

### ***5.6 Dopamine D<sub>2</sub>-histamine H<sub>3</sub> heteromers***

The histamine H<sub>3</sub> receptor was co-immunoprecipitated with the dopamine D<sub>2</sub> receptor in rat striatal membranes establishing physical proximity between them (Moreno et al., 2011a). In mice deficient for the H<sub>3</sub> receptor, D<sub>2</sub> agonist induced locomotion was altered and D<sub>2</sub> agonists did not promote ERK1/2 phosphorylation in the striatum (Kononoff Vanhanen et al., 2016) supporting functional interaction in D<sub>2</sub>-H<sub>3</sub> heteromers.

Since D<sub>2</sub> receptors can also form heteromers with sigma1 (see section 4.2) or D<sub>1</sub> (see section 4.3) receptors, larger complexes associating H<sub>3</sub>-D<sub>2</sub>-sigma1 receptors or H<sub>3</sub>-D<sub>2</sub>-D<sub>1</sub>-sigma1 receptors may exist whose modulatory role of the striatal GABAergic signaling remains to be elucidated.

### **5.7 Adenosine A<sub>1</sub>-adenosine A<sub>2a</sub> heteromers**

The G $\alpha$ i/o coupled adenosine A<sub>1</sub> and G $\alpha$ s coupled A<sub>2a</sub> receptors co-localize in glutamatergic terminals in the rat striatum as indicated by co-immunoprecipitation and electron microscopy (Ciruela et al., 2006). Upon A<sub>1</sub>-A<sub>2a</sub> heteromerization, activation of the A<sub>2a</sub> receptor reduces the affinity and efficacy of A<sub>1</sub> agonists consistent with a negative cross-talk on A<sub>1</sub> signaling (Ciruela et al., 2006). A<sub>1</sub> receptors have higher affinity for adenosine than A<sub>2a</sub> receptors. Accordingly, at low adenosine concentrations, preferred activation of A<sub>1</sub> receptors will inhibit glutamate release whereas, at higher concentrations, adenosine will also bind to A<sub>2a</sub> receptors and facilitate glutamate release by inhibiting A<sub>1</sub> signaling through A<sub>2a</sub>-A<sub>1</sub> heteromers. Caffeine is a non-selective antagonist of A<sub>1</sub> and A<sub>2a</sub> receptors with arousing and psychomotor effects similar to prototypical psychostimulants such as cocaine or amphetamines. Caffeine has higher affinity for A<sub>2a</sub> receptors than for A<sub>1</sub> receptors in transfected cells expressing only one receptor type but A<sub>2a</sub> affinity for caffeine decreases upon A<sub>2a</sub>-A<sub>1</sub> heteromerization (Ciruela et al., 2006). Acute caffeine that results in low adenosine concentration would block A<sub>1</sub> receptors and enhance glutamatergic release. On the contrary, chronic caffeine would result in higher adenosine concentrations through inhibition of the ENT1 transporter and would inhibit glutamatergic release through A<sub>1</sub>-A<sub>2a</sub> heteromers (Ciruela et al., 2006). A<sub>1</sub>-A<sub>2a</sub> heteromers can therefore contribute to modulate striatal activity and may also associate with D<sub>2</sub> and sigma1 receptors to form larger heterocomplexes.

### **5.8 Class A- class A heteromers with unexplored role in SUD**

*In vivo* physical proximity has been reported for several additional class A receptor pairs involving at least one of the receptors playing a role in SUD. This includes cannabinoid CB<sub>1</sub>-delta opioid receptors (cortex, striatum) (Bushlin et al., 2012), dopamine D<sub>2</sub>-somatostatin sst<sub>2</sub> receptors (primary striatal cultures) (Baragli et al., 2007), adenosine A<sub>2a</sub>-purinergic P<sub>2</sub>Y<sub>1</sub> receptors (cortex) (Yoshioka et al., 2002), mu opioid-adrenergic A<sub>2a</sub> receptors (primary hippocampal cultures, nucleus of the solitary tract) (Jordan et al., 2003; Sun et al., 2015), histamine H<sub>3</sub> receptor- non-functional histamine H<sub>3</sub> truncated splice variant (cortex, caudate putamen, hippocampus) (Bakker et al., 2006). The functional relevance of these heteromers in the context of SUD remains to be established but they could enrich the actual possibilities to modulate the various aspects of drug addiction.

Also, additional heteromers have been described that do not involve receptors for drugs of abuse but modulate neuronal and/or glial functions such as those involving association of opioid and chemokine receptors (reviewed in (Melik Parsadaniantz et al., 2015) (Kramp et al., 2011)) or heteromerization of serotonin receptors (reviewed in (Gaitonde and Gonzalez-Maeso, 2017)). They are key players in neuroinflammation, nociception or depression, and may therefore contribute to the expression of symptoms associated with SUD.

### **6. Class A-class C heteromers and SUD**

Much less is known to date on the functional consequences of heteromerization between these class A and class C GPCRs in the context of drug addiction, However, the wide distribution of GABA<sub>B</sub> receptors (Charles et al., 2001), the presence of mGlu<sub>5</sub> receptors in the cortex, striatum and Nacc and the high cortical expression of mGlu<sub>2</sub> subunits (Ferraguti and Shigemoto, 2006) designate them as potential modulators of neuronal activity in the context of SUD and associated psychosis.

### **6.1 Serotonin 5HT<sub>2a</sub>-metabotropic glutamate mGlu<sub>2</sub> heteromers**

The implication of the serotonergic system in mood, anxiodepressive disorders and other psychosis such as schizophrenia is well established. This neuromodulatory system is also the target of hallucinogenic substances isolated from mushrooms (psilocybin, mescaline) or of synthetic compounds such as LSD, which can also be abused leading to tolerance and psychological dependence (Das et al., 2016). These hallucinogenic drugs bind to 5HT<sub>2a</sub> receptors but not only recruit the canonical 5HT<sub>2a</sub> dependent Gαq/11 pathway but also stimulate Gαi/o signaling (Gonzalez-Maeso et al., 2008). Therefore, they modify the Gαi/Gαq coupling and signaling balance in favor of the inhibitory signaling cascade.

Serotonin 5HT<sub>2a</sub> and metabotropic glutamate mGlu<sub>2</sub> receptors were co-immunoprecipitated in mouse (Fribourg et al., 2011) and human cortical membranes (Gonzalez-Maeso et al., 2008). The two receptors were also co-localized near synaptic junctions in mouse cortical neurons (Moreno et al., 2012). Studies using chimeras containing various ratios of mGlu<sub>2</sub> receptor showed that the mGlu<sub>2</sub> transmembrane domains TM IV and V are mandatory for association with 5HT<sub>2a</sub> receptors with three alanine residues (A677, A681, A685) at the TM IV intracellular end essential for heteromerization (Moreno et al., 2012).

In oocytes from *Xenopus laevis* co-expressing 5HT<sub>2a</sub> and mGlu<sub>2</sub> receptors, 5HT<sub>2a</sub> mediated Gαq/11 activation in response to serotonin was decreased by 50% and mGlu<sub>2</sub> mediated Gαi/o signaling in response to glutamate was enhanced by 200%. Activation of 5HT<sub>2a</sub>-mGlu<sub>2</sub> heteromers by endogenous ligands therefore favored Gαi over Gαq signaling. This shift was specific to the 5HT<sub>2a</sub>-mGlu<sub>2</sub> receptor pair since it was not observed upon co-expression of the 5HT<sub>2a</sub> receptor with the non-interacting mGlu<sub>3</sub> receptor (Fribourg et al., 2011). In addition, occupancy of one of the receptors by an agonist, an antagonist or an inverse agonist differently affected Gαq/Gαi coupling. In oocytes co-expressing the two receptors, agonists favored signaling through their cognate receptor and decreased signaling through the

associated receptor indicative of bidirectional negative cross-talk. In contrast, positive allosteric modulation was observed with inverse agonists. Indeed, inverse agonists inhibited signaling through their cognate receptor and enhanced signaling through the associated receptor (Fribourg et al., 2011). Similar increases in signaling of the partner receptor by inverse agonist was also observed in cortical neurons *in vivo* (Fribourg et al., 2011). Accordingly, activation of the mGlu<sub>2</sub> receptor by the agonist LY379268 suppressed the G $\alpha$ i dependent hallucinogenic effect of the 5HT<sub>2a</sub> agonist DOI in membranes from the mouse PFC (Gonzalez-Maeso et al., 2008) and the effects of the inverse agonist LY341495 were significantly attenuated in 5HT<sub>2a</sub> knock-out mice (Fribourg et al., 2011; Gonzalez-Maeso et al., 2008). The causal link between 5HT<sub>2a</sub>-mGlu<sub>2</sub> physical association and the head twitch behavioral response to hallucinogens was then conclusively established. In mice deficient for the mGlu<sub>2</sub> receptor, administration of the 5HT<sub>2a</sub> agonist DOI did not induce head twitching revealing the essential role of this G $\alpha$ i coupled receptor (Moreno et al., 2011b). Rescuing mGlu<sub>2</sub> receptor expression in the mouse frontal cortex upon herpes viral infection restored the behavioral response (Moreno et al., 2012). However, no behavioral response was observed following re-expression of a mGlu<sub>2</sub> receptor missing the three alanine residues in the transmembrane domain TM IV that are essential for physical association with the 5HT<sub>2a</sub> (Moreno et al., 2012), confirming that 5HT<sub>2a</sub>-mGlu<sub>2</sub> heteromers mediate the specific response to hallucinogens.

## **6.2 Adenosine A<sub>2a</sub>- metabotropic glutamatergic mGlu<sub>5</sub> heteromers**

Physical proximity of adenosine A<sub>2a</sub> and metabotropic glutamate mGlu<sub>5</sub> receptors was first determined by co-immunoprecipitation using rat striatal membranes (Ferre et al., 2002). Detection of A<sub>2a</sub>-mGlu<sub>5</sub> heteromers by *in situ* PLA in mice revealed homogenous distribution throughout the striatum that was not affected in a protocol of habit formation (random interval training) or goal directed behavior (random ratio training) (He et al., 2016). The same approach

uncovered increased  $A_{2a}$ -mGlu<sub>5</sub> heteromers in the Nacc shell following cocaine self-administration (Borrito-Escuela et al., 2017). Co-injection (i.c.v.) of the  $A_{2a}$  agonist CGS21680 and the mGlu<sub>5</sub> agonist CHPG led to increased c-Fos expression in the Nacc but not in the caudate putamen or in the cingulate cortex (Ferre et al., 2002). Dual-probe microdialysis in freely moving rats revealed that co-administration of the  $A_{2a}$  agonist CGS21680 and the mGlu<sub>5</sub> agonist CHPG led to synergistic effect and negatively modulated dopamine D<sub>2</sub> signaling thereby enhancing glutamate and GABAergic extracellular levels in the globus pallidus (Beggiato et al., 2016). This suggests that  $A_{2a}$ -mGlu<sub>5</sub> heteromers may serve to overcome the strong tonic inhibitory effect of dopamine on striatal  $A_{2a}$  function and may contribute to modulate locomotor activation and sensitization by psychostimulants. In addition, increased levels of heteromerization between  $A_{2a}$ -D<sub>2</sub>, D<sub>2</sub>-sigma1,  $A_{2a}$ -mGlu<sub>5</sub> receptors detected by *in situ* PLA in the NAcc shell (Borrito-Escuela et al., 2017) suggest that these four receptors may associate in larger signaling platforms to modulate the striatopallidal activity.

### **6.3 Muscarinic $M_2$ -metabotropic $GABA_{B2}$ heteromers**

Co-expression of the  $GABA_{B2}$  but not the  $GABA_{B1}$  subunit in neuronal PC12 cells rescued surface expression of the muscarinic  $M_2$  receptor and functional coupling to GIRK channels upon chronic stimulation with acetylcholine (Boyer et al., 2009). Mutagenesis combined with FRET established that the interaction took place between the proximal C-terminal domain of  $GABA_{B2}$  (P741 to V776) and the last 23 amino acids of the C-terminal domain of the muscarinic  $M_2$  receptor. Importantly, the interaction site on the  $GABA_{B2}$  receptor was distinct from the coiled-coil domain responsible for the obligatory  $GABA_{B1}/GABA_{B2}$  dimerization (Boyer et al., 2009). Therefore, it is unknown whether muscarinic  $M_2$  receptors interact with the  $GABA_{B2}$  receptor only or rather with the functional dimeric  $GABA_{B1/2}$  receptor. Co-immunoprecipitation from rat cortical membrane of  $GABA_{B2}$

subunit together with the muscarinic M<sub>2</sub> receptor confirmed *in vivo* proximity (Boyer et al., 2009) and suggested that the heteromer may play a role in drug addiction by modulating the endogenous acetylcholine tone at presynaptic level. A possible outcome would be a contribution of these heteromers to the discriminative stimulus properties of cocaine (Joseph and Thomsen, 2017).

## **7. Class A GPCR-ion channels heteromers with potential role in SUD**

Physical association involving class A GPCRs is not restricted to interactions with another GPCR but can also involve ion channels, which would account for the growing complexity of interactions already identified. This emphasizes the need to adapt our current view of a functional unit to encompass broader associations taking place within a cluster of proteins of more than two types.

### **7.1 Dopamine receptor- Voltage-gated Ca<sup>2+</sup> channel heteromers**

Physical proximity between dopamine D<sub>2</sub> receptors and voltage gated N-type Ca<sup>2+</sup> channels was identified by co-immunoprecipitation in the rat striatum (Kisilevsky and Zamponi, 2008) where the G $\alpha$ i/o coupled D<sub>2</sub> receptors exert robust voltage dependent and independent inhibition of N-type channels. Importantly, voltage independent D<sub>2</sub> inhibition appeared to be mediated through D<sub>2</sub>-N-type channel heteromerization since obliterated upon disruption of physical association. In addition, D<sub>2</sub>-N-type channel association increased cell surface expression of the channel and promoted D<sub>2</sub> agonist-induced internalization in co-transfected HEK 293 cells (Kisilevsky and Zamponi, 2008).

Dopamine D<sub>1</sub> receptors couple to G $\alpha$ s and enhance L-type Ca<sup>2+</sup> channels through voltage independent pathways involving PKA activation. In the rat PFC, however, D<sub>1</sub> receptors co-immunoprecipitated with N-type Ca<sup>2+</sup> channels and inhibited their activity by a combination of voltage-dependent (G $\beta$  $\gamma$ ) and independent (PKA) pathways (Kisilevsky et al., 2008). In co-

transfected HEK 293 cells, D<sub>1</sub>-N-type channel heteromer formation increased surface expression of the channel and promoted co-internalization following D<sub>1</sub> activation (Kisilevsky et al., 2008). In primary cortical neurons, D<sub>1</sub> receptors regulated channel distribution in dendritic compartments. Importantly, D<sub>1</sub> regulation of N-type channel activity appeared selective to the PFC and was not observed in the striatum where no D<sub>1</sub>-channel physical proximity could be detected. The reason for this region specificity, however, remains unknown. Given the neuroplasticity induced by drugs of abuse that involve increased D<sub>1</sub> signaling (Volkow and Morales, 2015), this type of interaction provides a mechanism by which D<sub>1</sub> could contribute to dopaminergic inhibition of N-type channel activity during back propagating action potentials and hence could participate in the control exerted by the PFC on the striatum. The functional contribution of these heteromers in the context of SUD remains however to be established.

### ***7.2 Opioid like receptor ORL1- Voltage-gated Ca<sup>2+</sup> channel heteromers***

Physical proximity between the G $\alpha$ i/o coupled opioid ORL1 receptor and the voltage gated N-type Ca<sup>2+</sup> channels was established in the rat brain by co-immunoprecipitation (Beedle et al., 2004). Similar to the inhibitory control exerted by D<sub>1</sub> or D<sub>2</sub> receptors in the brain, N-type channel association with ORL1 resulted in a tonic inhibition of the channels in DRGs. The latter was attributed to ORL1 constitutive activity. The agonist nociceptin also led to G protein dependent inhibition of N-type channels and promoted receptor-channel co-internalization in the DRG, both mechanisms contributing to reduce Ca<sup>2+</sup> entry (Altier et al., 2006). Regulation of N-type channel surface expression was also observed upon co-expression with D<sub>1</sub> or D<sub>2</sub> dopamine receptors (Kisilevsky et al., 2008; Kisilevsky and Zamponi, 2008), and may reflect a general mechanism by which N-type channel expression and activity are regulated. ORL1 and its endogenous ligand nociceptin/orphanin-FQ (N/OFQ) are widely distributed throughout

the brain and are present in areas that critically mediate motivated and addictive behaviors such as the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the medial PFC, the VTA, or the Nacc (Ozawa et al., 2015). Activation of ORL1 regulates drug reward and drug-seeking behaviors associated with opiates, psychostimulants or ethanol (reviewed in (Witkin et al., 2014)). With respect to ethanol-mediated behaviors, ORL1 agonists attenuate conditioned place preference, ethanol self-administration, and ethanol-seeking behaviors in rat lines genetically-selected for high alcohol preference presumably through decreased dopamine release (reviewed in (Witkin et al., 2014)). In addition, mu opioid receptors can associate with ORL1 as shown by co-immunoprecipitation in the DRGs where activation of the mu opioid receptor led to co-internalization of the N-type channel. This was not observed when the ORL1 receptor was absent (Evans et al., 2010) and therefore indicates formation of larger heterocomplexes. Because of the broad overlap in the distributions of mu and ORL1 receptors (Ozawa et al., 2015) and the broad expression of N-type channels in the brain (Kamp et al., 2012), such complexes are likely to form at supraspinal levels and may therefore participate to the response to drugs of abuse, especially when considering that the mu opioid receptor acts as a gateway for many of these (Contet et al., 2004).

### ***7.3 Class A receptor-NMDA channel heteromers***

Co-immunoprecipitation experiments uncovered physical proximity between the dopamine D<sub>1</sub> and the NR<sub>1</sub> subunit of NMDA channels in the rat hippocampus (Pei et al., 2004) and striatal post-synaptic membrane preparations (Fiorentini et al., 2003). Interaction between the two proteins takes place in the ER and increases D<sub>1</sub> receptor surface expression (Fiorentini et al., 2003) through contacts involving their C-termini (Pei et al., 2004). In addition, D<sub>1</sub> heteromerization with NR<sub>1</sub> enhanced D<sub>1</sub> signaling in primary hippocampal neurons (Pei et al.,

2004) and abolished its internalization upon agonist activation in co-transfected HEK293 cells (Fiorentini et al., 2003)

Physical proximity was also identified by co-immunoprecipitation in the mouse periaqueductal gray between mu opioid receptors and the NR<sub>1</sub> subunit of NMDA channels (Rodriguez-Munoz et al., 2012). Disruption of the complex by morphine contributed to the development of tolerance (Rodriguez-Munoz et al., 2012). Considering the ubiquitous distribution of the NR<sub>1</sub> subunit, mu opioid-NMDA heteromers are likely to form in other brain regions. In addition, mu-D<sub>1</sub> and D<sub>1</sub>-NMDA heteromers have been identified in the striatum suggesting the possibility of larger heterocomplexes that may represent a yet unrecognized modulator of neuronal activity contributing to SUD.

## **8. Heteromers as novel therapeutic targets**

Very few novel pharmacological targets have proven effective in the clinic over the past three to four decades. Only minimal advances have been made, which emphasizes the need to address SUD treatment from a different perspective. Heteromerization as a mean for different GPCRs to alter behaviour by functioning together may therefore represents an alternative way to think about biological underpinnings of addiction. Indeed, heteromers have been detected in the human brain as for dopamine D<sub>1</sub>-D<sub>2</sub> and 5HT<sub>2a</sub>-mGlu<sub>2</sub> pairs (Gonzalez-Maeso et al., 2008; Pei et al., 2010). In preclinical models, unambiguous implication in SUD has now been established for several receptor pairs. Although this identifies heteromers as novel therapeutic targets, the design of selective ligands is still in its infancy.

Bivalent ligands have been developed to simultaneously bind two receptors in physical contact. Administration (i.c.v.) of the bivalent ligand MDAN-21 composed of the mu agonist oxymorphone and the delta antagonist naltrindole induced analgesia with low tolerance and low physical dependence (Daniels et al., 2005). This provided a proof of concept that the

strategy is of therapeutic interest. Selective targeting of mu-delta heteromers may indeed represent a valuable option, in particular for patients on opiate maintenance treatment.

Integrated dual acting drugs combining heteromer selective targeting with a mixed agonist/ antagonist profile were also explored as a therapeutic approach. The strategy has been validated with eluxadoline, a mixed mu opioid agonist delta opioid antagonist recently approved by the FDA (Viberzi) for the treatment of irritable bowel syndrome. Arguments in favor of binding to mu-delta heteromers include lower efficacy in mice deficient for the delta opioid receptor and reduced signaling in the presence of mu-delta selective antibodies (Fujita et al., 2014). In the context of SUD, targeting  $A_{2a}$ - $D_2$  heteromers with a mixed  $A_{2a}$ -antagonist- $D_2$  agonist (Jorg et al., 2015) may have therapeutic application by reducing habit formation developing upon chronic usage of psychostimulants.

Attempts were also made to develop biased ligands with preferential affinity for heteromers. SKF83959 specifically targets  $D_1$ - $D_2$  heteromers and attenuates cocaine-induced locomotor sensitization and reinforcing properties as well as reinstatement of cocaine seeking (Hasbi et al., 2018). CYM51010, a monovalent ligand with preferential affinity for mu-delta heteromers, induced potent thermal analgesia comparable to morphine but lower tolerance and physical dependence (Gomes et al., 2013). Since CYM51010 showed no rewarding properties, this ligand is currently evaluated in preclinical models as an alternative to current opiate maintenance strategies such as methadone or buprenorphine (Massotte unpublished results).

Finally, the concept of signalosome is also gaining importance in the context of SUD. Based on established physical proximity, complexes involving adenosine  $A_{2a}$ , dopamine  $D_2$ , sigma1 receptors and N-type channels, composed of histamine  $H_3$ , dopamine  $D_1$ , sigma1 receptors and N-types channel or associating mu opioid, dopamine  $D_1$  and NMDA channels can now be reasonably postulated as plausible SUD-relevant functional units that fine-tune intracellular cascades. These large protein aggregates form signaling platforms and represent

an extended view of the current concept of heteromerization. Better characterization of these large functional ensembles should therefore pave the way to novel therapeutic options.

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## **Figure legends**

### **Figure 1: The mesocorticolimbic pathway in rodents.**

The mesocorticolimbic pathway is involved in the reinforcing properties of drugs of abuse. Dopaminergic projections (DA) originating from the ventral tegmental area (VTA) are shown. Nacc, nucleus accumbens ; SN, substantia nigra; CeA, central amygdala; BNST, bed nucleus of the stria terminalis; LH, lateral hypothalamus.

### **Figure 2: Heteromers modulate dopamine transmission**

(A) Native heteromers can modulate the direct striato-nigral pathway expressing the dopamine D<sub>1</sub> receptor and the indirect striato-pallidal pathway expressing the dopamine D<sub>2</sub> receptor. A third striatal output is composed of a discrete neuronal population of the ventral striatum expressing D<sub>1</sub>-D<sub>2</sub> heteromers. Cross-talks within the heteromers and impact on neuronal activity are indicated. MSN, medium spiny neuron; Glu, glutamate.

(B) Neuroanatomical distribution of native heteromers that can modulate the mesocorticolimbic pathway and contribute to substance use disorder.

### **Figure 3: Altered expression of heteromers in the context of substance use disorder.**

Expression of heteromers is dynamic and affected by the physiological state and/or by drugs of abuse. Changes in heteromer expression identified in the mesocorticolimbic pathway by proximity ligation assay or selective antibodies are indicated.

### **Figure 4: Proposed model for adenosine A<sub>2a</sub> receptor and dopamine D<sub>2</sub> receptor interactions in A<sub>2a</sub>-D<sub>2</sub> heterotetramers.**

The association of two A<sub>2a</sub>-D<sub>2</sub> heteromers generates a heterotetramer in which multiple interactions can simultaneously take place in the presence of ligands. Activation of the

adenosine  $A_{2a}$  receptor by adenosine or an exogenous agonist negatively modulates  $D_2$  agonist affinity and efficacy through physical interactions within the  $A_{2a}$ - $D_2$  heteromer. This allosteric modulation therefore dampens dopamine  $D_2$  receptor G-protein-dependent and independent signaling. Reciprocally, activation of the dopamine  $D_2$  receptor by dopamine or an exogenous agonist can negatively cross-modulate  $A_{2a}$  receptor affinity and signaling. Additional cross-talks take place at the level of the signaling cascades. They correspond to canonical interactions between stimulatory  $G_{\alpha s}$  dependent and inhibitory  $G_{\alpha i/o}$  dependent pathways. They modulate cAMP production in opposite directions. The heterotetrameric organization enables additional modulations by  $A_{2a}$  antagonists such as caffeine that would decrease the negative crosstalk on  $D_2$  receptor and reduce  $G_{\alpha s}$  dependent signaling (adapted from (Taura et al., 2017) (Navarro et al., 2018b) .

**Table 1**  
**Heteromers with identified and potential implication in SUD**

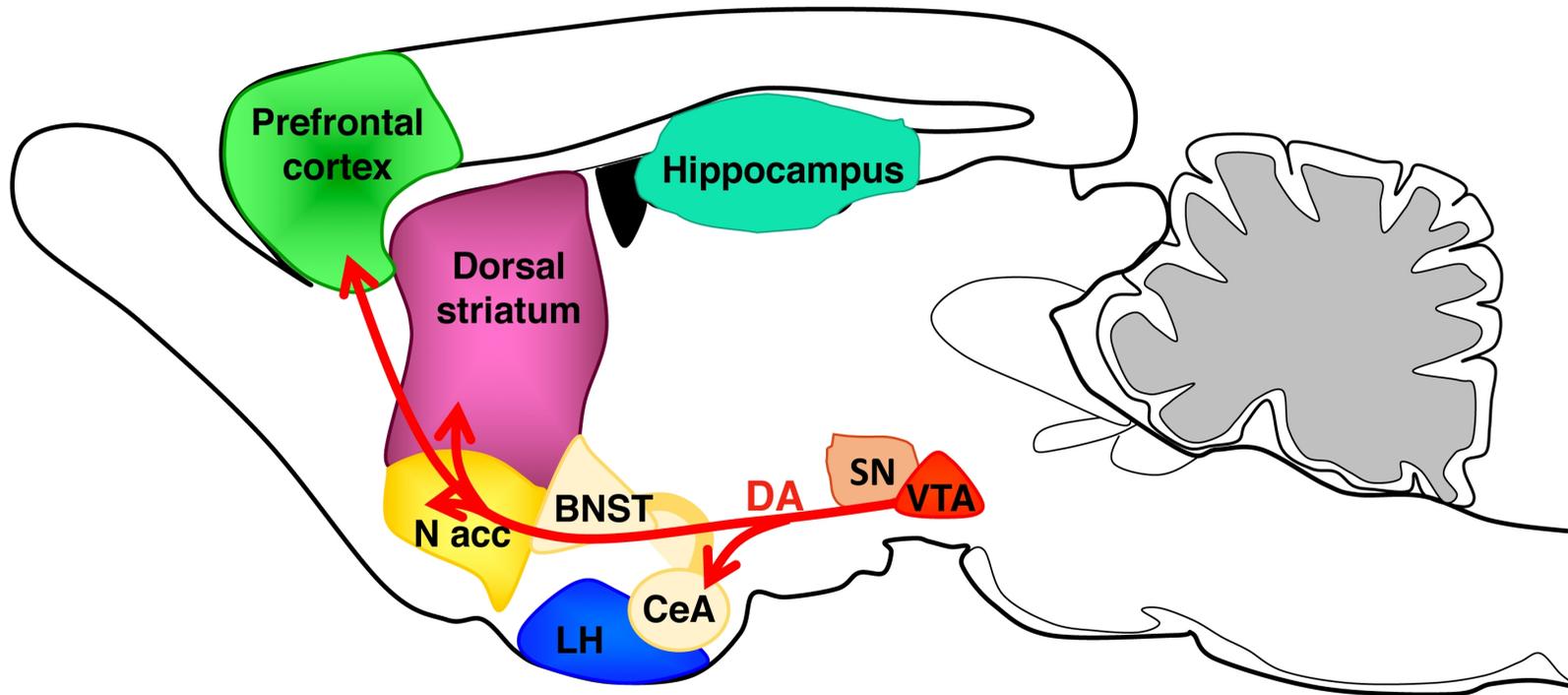
Abbreviations: Co-IP: co-immunoprecipitation, FRET: fluorescence resonance energy transfer, nd: not determined, PLA: proximity ligation assay

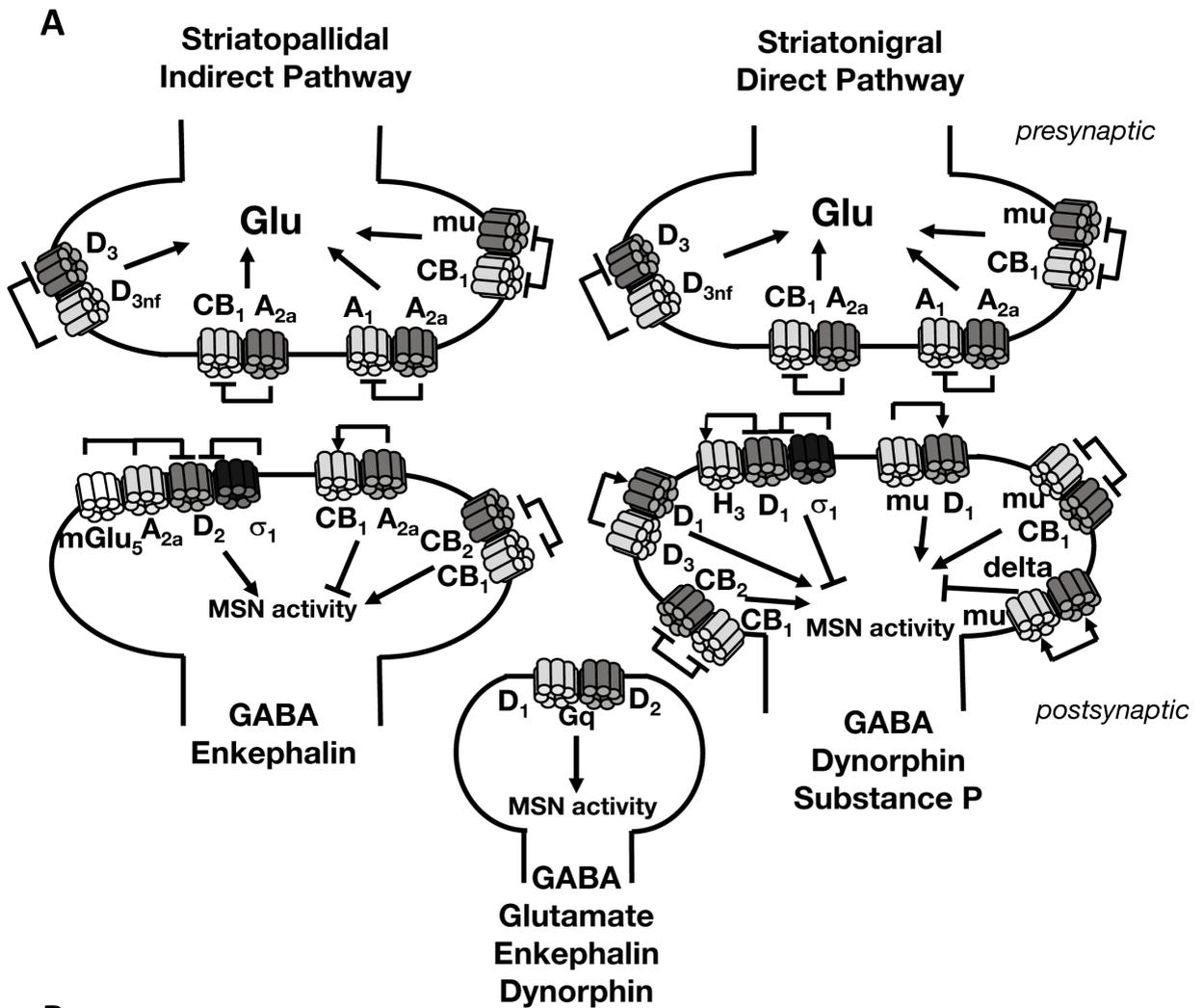
Heteromer	In vivo physical proximity		Impact on signaling properties	Postulated role in SUD	References
	Tissue	Techniques			
<b>Class A-Class A heteromers with established role in SUD</b>					
A <sub>2a</sub> -D <sub>2</sub>	Rat and mouse striatum  Expression modulated by cocaine, ethanol or habit formation	Co-IP  PLA  Bivalent ligands  Electron microscopy  Time resolved-FRET  Disruptive peptide	Bidirectional negative cross-talk  Co-internalization	Habit formation  Drug relapse (cocaine reinstatement)  ↑ Ethanol consumption  ↓ Ethanol withdrawal  Caffeine stimulatory effect	(Borrito-Escuela et al., 2017; Borrito-Escuela et al., 2018; El Yacoubi et al., 2001; Feltmann et al., 2018; Fernandez-Duenas et al., 2015; He et al., 2016; Hillion et al., 2002; Huang et al., 2013; Marcellino et al., 2007; Soriano et al., 2009; Trifilieff et al., 2011)
D <sub>2</sub> -sigma1	Rat and mouse striatum  Expression modulated by cocaine or ethanol	Co-IP  PLA	Negative cross-talk on D2 signaling	Binds and mediates cocaine negative cross-talk on D <sub>2</sub> signaling	(Beggiato et al., 2017; Borrito-Escuela et al., 2017; Feltmann et al., 2018; Navarro et al., 2013)
D <sub>1</sub> -D <sub>2</sub>	Rat, mouse, long-tail macaque, human striatum	Co-IP  FRET	Gαq/11 signaling (BDNF pathway)  ↑BDNF	↓Cocaine locomotor sensitization  ↓ Cocaine reinforcing properties (CPP)	(Hasbi et al., 2009; Hasbi et al., 2018; Lee et al., 2004; Pei et al., 2010; Perreault et al., 2016; Rashid et al., 2007; Rico et al., 2017)

	Expression increased with age	PLA Disruptive peptide		<p>↓Cocaine self-administration</p> <p>↓Cocaine seeking (reinstatement of self-administration by drug or cue priming)</p> <p>↓Susceptibility to cocaine in adulthood</p> <p>↓Sucrose preference</p> <p>↓motivation (palatable food)</p> <p>Drug induced neuroplasticity</p>	
Mu-D <sub>1</sub>	Mouse striatum	Co-IP	Cross-antagonism on mu signaling	Locomotor sensitization to opiates	(Tao et al., 2017)
Mu-delta	Mouse hippocampus, VTA  Expression modulated by chronic morphine	Co-IP  Heteromer specific antibody  Bivalent ligand  Disruptive peptide	<p>Bidirectional positive cross-talk on ligand binding and signaling</p> <p>Gαz coupling</p> <p>Co-internalization</p> <p>↑ β-arrestin signaling</p>	<p>↓tolerance</p> <p>↓physical dependence</p> <p>↓ethanol consumption</p>	(Daniels et al., 2005; Derouiche et al., 2018; Erbs et al., 2015; George et al., 2000; Gomes et al., 2013; Gomes et al., 2004; Gomes et al., 2011; Gomes et al., 2000; Gupta et al., 2010; He et al., 2011; Kabli et al., 2010; Kabli et al., 2013; Margolis et al., 2017; Rozenfeld and Devi, 2007; van Rijn and Whistler, 2009)
Mu-Gal1	Mouse VTA	Disruptive peptide	<p>Cross-antagonism on Gal1 signaling</p> <p>Negative cross-talk on mu signaling</p>	↓Opiates reinforcement and withdrawal	(Hawes et al., 2008; Moreno et al., 2017)
CB <sub>1</sub> -5HT <sub>2a</sub>	Mouse cortex, hippocampus, dorsal striatum	PLA  Disruptive peptide	<p>Bidirectional cross-antagonism</p> <p>Negative cross-talk</p> <p>5HT<sub>2a</sub> coupling to Gαi/o</p>	<p>↑THC physical dependence</p> <p>↑cognitive deficits associated to THC (memory impairment, anxiety, reduced social interaction)</p>	(Vinals et al., 2015)

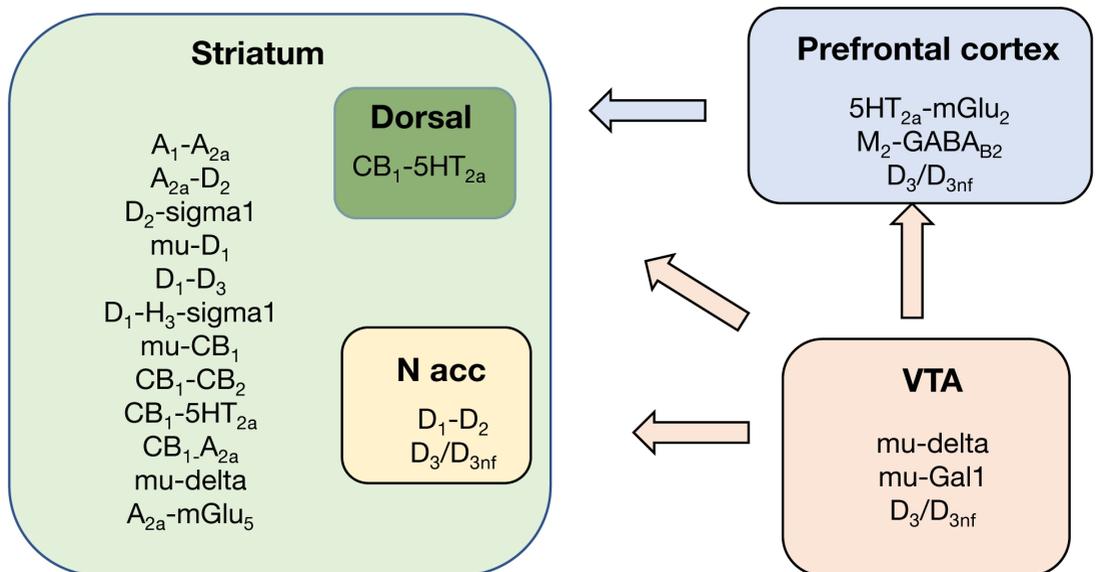
			activation of mTOR pathway		
CB <sub>1</sub> -A <sub>2a</sub>	Rat striatum	Co-IP	Cross-antagonism on CB <sub>1</sub> signaling	THC locomotor sensitization THC reinforcement (CPP)	(Carriba et al., 2007; Ferreira et al., 2015; Soria et al., 2004)
<b>Class A-Class A heteromers with potential role in SUD</b>					
Mu-CB1	Rat striatum	Electron microscopy	Bidirectional negative cross-talk Bidirectional cross-antagonism	Social play ↓ induced neuroplasticity	(Manduca et al., 2016; Rios et al., 2006 ; Rodriguez et al., 2001)
CB <sub>1</sub> -CB <sub>2</sub>	Rat striatum  Activated microglia	PLA	Bidirectional cross-antagonism Negative cross-talk  Negative cross-talk in resting microglia Positive cross-talk in activated microglia Bidirectional cross-antagonism	↓ psychostimulant-induced neurotoxicity  neuroinflammation	(Callen et al., 2012)  (Navarro et al., 2018a)
D <sub>3</sub> /D <sub>3nf</sub>	Rat brain	Co-IP	↓ D <sub>3</sub> cell surface expression, ligand binding	Impulsivity  Sensitization to cocaine or nicotine	(Karpa et al., 2000; Nimchinsky et al., 1997; Pritchard et al., 2006; Smith et al., 2015)
D <sub>1</sub> /D <sub>3</sub>	Rat brain	Co-IP	co-internalization  positive cross talk on D1 signaling	↑ Locomotor sensitization to psychostimulants	(Fiorentini et al., 2008; Marcellino et al., 2008)
D <sub>1</sub> -H <sub>3</sub> -sigma1	Rat and mouse striatum	Co-IP  PLA	Bidirectional cross-antagonism  Negative cross-talk on D <sub>1</sub> signaling (D <sub>1</sub> coupling to Gαi/o, H <sub>3</sub> activation of ERK1/2)	<i>Ex vivo</i> regulation of striatal GABAergic activity by acute and chronic cocaine  neuroprotective	(Ferrada et al., 2009; Kononoff Vanhanen et al., 2016; Moreno et al., 2011a; Moreno et al., 2014)

			sigma1 mediates cocaine disruption of bidirectional cross-antagonism and negative cross-talk on D <sub>1</sub> signaling		
A <sub>1</sub> -A <sub>2a</sub>	Rat striatum	Co-IP  Electron microscopy	↓A <sub>2a</sub> affinity for caffeine	Caffeine stimulant effects	(Ciruela et al., 2006)
<b>Class A-class C heteromers with a role in SUD</b>					
5HT <sub>2a</sub> -mGlu2	Mouse, human cortex	Co-IP  Electron microscopy Interfering peptide	Modulation of Gα <sub>i/o</sub> -Gα <sub>q/11</sub> coupling in favor of Gα <sub>i/o</sub>	Effects of hallucinogens (LSD)	(Fribourg et al., 2011 ; Gonzalez-Maeso et al., 2008; Moreno et al., 2011b; Moreno et al., 2012)
A <sub>2a</sub> -mGlu5	Rat, mouse striatum  Expression modulated by cocaine	Co-IP  PLA	Positive cross-talk	↓ Sensitization to psychostimulants	(Beggiato et al., 2016; Borroto-Escuela et al., 2017; Ferre et al., 2002 ; He et al., 2016)
M <sub>2</sub> -GABA <sub>b2</sub>	Rat cortex	Co-IP	↑ M <sub>2</sub> signaling	Cocaine discrimination	(Boyer et al., 2009; Joseph and Thomsen, 2017)
<b>GPCR-ion channel heteromers with potential role in SUD</b>					
D <sub>2</sub> - N-type Ca <sup>2+</sup> channel	Rat striatum	Co-IP	↓Channel activity	nd	(Kisilevsky and Zamponi, 2008)
D <sub>1</sub> - N-type Ca <sup>2+</sup> channel	Rat PFC	Co-IP	↓Channel activity	nd	(Kisilevsky et al., 2008)
ORL1-N-type Ca <sup>2+</sup> channel	Rat brain	Co-IP	↓Channel activity	nd	(Beedle et al., 2004; Evans et al., 2010)
Mu –NMDA NR1	Mouse PAG	Co-IP	Modulation NMDA-CAMKII pathway	nd	(Rodriguez-Munoz et al., 2012)
D <sub>1</sub> -NMDA	Rat striatum, hippocampus	Co-IP	↑ D1 surface expression  positive cross-talk on D1 signaling	nd	(Fiorentini et al., 2003; Pei et al., 2004)





**B**



### Dorsal striatum

<i>Cocaine</i>	<i>Ageing</i>
↔ A <sub>2a</sub> -D <sub>2</sub>	↑ D <sub>1</sub> -D <sub>2</sub>
↓ D <sub>2</sub> -sigma1	
<i>Alcohol</i>	<i>Habit formation</i>
↑ A <sub>2a</sub> -D <sub>2</sub>	↑ A <sub>2a</sub> -D <sub>2</sub>
↓ D <sub>2</sub> -sigma1	
<i>Chronic morphine</i>	
↑ mu-delta	

### Nucleus accumbens

<i>Cocaine</i>	<i>Ageing</i>
↑ A <sub>2a</sub> -D <sub>2</sub> (shell)	↑ D <sub>1</sub> -D <sub>2</sub> (shell)
↓ D <sub>2</sub> -sigma1 (shell)	
<i>Alcohol</i>	<i>Chronic morphine</i>
↑ A <sub>2a</sub> -D <sub>2</sub> (shell)	↑ mu-delta

### VTA

*Chronic morphine*  
↑ mu-delta

